HAL
open science

# Mapping binding sites for the PDE4D5 cAMP-specific phosphodiesterase to the N- and C-domains of beta-arrestin using spot-immobilized peptide arrays

George S Baillie, David R Adams, Narinder Bahri, Thomas M Houslay, Suryakiran Vadrevu, Dong Meng, Xiang Li, Allan Dunlop, Graeme Milligan, Graeme B Bolger, et al.

## To cite this version:

George S Baillie, David R Adams, Narinder Bahri, Thomas M Houslay, Suryakiran Vadrevu, et al.. Mapping binding sites for the PDE4D5 cAMP-specific phosphodiesterase to the N- and C-domains of beta-arrestin using spot-immobilized peptide arrays. Biochemical Journal, 2007, 404 (1), pp.71-80. 10.1042/BJ20070005 . hal-00478731

**HAL Id: hal-00478731**

**https://hal.science/hal-00478731v1**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Biochemical Journal Immediate Publication. Published on 8 Feb 2007 as manuscript BJ20070005

# Mapping binding sites for the PDE4D5 cAMP-specific phosphodiesterase to the N- and C-domains of βarrestin using spot-immobilised peptide arrays.

George S. Baillie[#∞], David R. Adams[†], Narinder Bhari[#], Thomas M. Houslay[¥], Suryakiran Vadrevu[#], Dong Meng[#], Xiang Li, Allan Dunlop, Graeme Milligan, Graeme B. Bolger[‡], Enno Klussmann[¶] and Miles D. Houslay[#§]

[‡]From the [#]Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK; [¥] Bioinformatics Research Centre, Davidson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK; [¶1]Leibniz-Institut für Molekulare Pharmakologie (FMP), Campus Berlin-Buch, Robert-Rössle-Str. 10, 13125 Berlin, Germany; University of Alabama at Birmingham, Comprehensive Cancer Center, Birmingham, Alabama 35294-3300, USA; [†]Department of Chemistry, Heriot-Watt University, Riccarton Campus, Edinburgh EH14 4AS, Scotland, UK.

[§] *Address correspondence to*:- Professor Miles Houslay, Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK; tel +44(0)-141-330-4624; fax +44(0)-141-330-4365; email M.Houslay@bio.gla.ac.uk

*Running title*:- PDE4D5 interaction with βarrestin N- and C-domains

*Keywords*:- phosphodiesterase-4, PDE4; cAMP; βarrestin; $\beta_2$-adrenoceptor; desensitization; peptide array; compartmentalisation; compartmentation.

*Abbreviations*:- PDE, cAMP phosphodiesterase; PDE4, family 4 cAMP specific phosphodiesterase; PDE4D5, PDE4 sub-family D, isoform 5 with this human isoform having GenBank Accession Number AF012073; βarrestin2, isoform2 of βarrestin2, with this human isoform having GenBank Accession Number NM004313; MEFs, mouse embryo fibroblasts; $\beta_2$-AR, $\beta_2$-adrenoceptor; GRK, G-protein receptor kinase; GPCR, G-protein coupled receptor; PKA, cAMP dependent protein kinase A; cAMP, cyclic 3'5' adenosine monophosphate.



© 2007 The Authors Journal compilation © 2007 Biochemical Society

**Abstract**

$\beta_2$-adrenoceptors ($\beta_2$-AR) become rapidly desensitised upon recruitment of cytosolic βarrestin. The cAMP phosphodiesterase-4D isoform, PDE4D5 can be recruited in complex with βarrestin, whereupon it regulates PKA phosphorylation of the $\beta_2$-AR. Here we use novel technology, employing a library of overlapping peptides (25-mers) immobilized on cellulose membranes that scan the entire sequence of βarrestin2, to define the interaction sites on βarrestin2 for binding of PDE4D5 and the cognate long isoform, PDE4D3. We identify a binding site in the βarrestin2 N-domain for the common PDE4D catalytic unit and two regions in the βarrestin2 C-domain that confer specificity for PDE4D5 binding. Alanine scanning peptide array analysis of the N-domain binding region identified severely reduced interaction with PDE4D5 upon Arg26Ala substitution and reduced interaction upon either Lys18Ala or Thr20ala substitution. Similar analysis of the βarrestin2 C-domain identified Arg286 and Asp291, together with the Leu215-His220 region, as being of importance in binding PDE4D5 but not PDE4D3. Transfection with wild-type βarrestin2 profoundly decreased isoprenaline-stimulated PKA phosphorylation of the $\beta_2$-AR in mouse embryo fibroblasts (MEFs) lacking both βarrestin1 and βarrestin2. This effect was negated using either the Arg26Ala or Arg286Ala mutant forms of βarrestin2 or one with substitution of Leu215-His220 for an alanine cassette, which showed little/no PDE4D5 binding but were still recruited to the $\beta_2$-AR upon isoprenaline challenge. These data show that the interaction of PDE4D5 with both the N- and C- domains of βarrestin2 are essential for $\beta_2$-AR regulation.



© 2007 The Authors Journal compilation © 2007 Biochemical Society

## Introduction

Plasma membrane localised, transmembrane G-protein coupled receptors (GPCRs) provide a pivotal and ubiquitous system for cellular signal transduction as well as targets for therapeutics and understanding the molecular pathology of various diseases (se e.g. [1-3]). Routinely, the regulation of GPCR functioning is characterised by a rapid and transient desensitization process [4]. This is mediated by their initial phosphorylation through the action of G-protein receptor kinases (GRKs) [5], which allows for the recruitment of cytosolic arrestins that sterically interdict the association of GPCRs with their functional G-protein [4, 6, 7]. Although multi-site phosphorylation of GPCRs by GRK is required for arrestin binding, the prime 'phosphate' sensor is a single arginine residue located in a 'polar core' located between the arrestin N- and C-domains [6]. Each of these arrestin domains are formed from a β-strand sandwich where two β-strand sheets are annealed through hydrophobic interactions between side chains inside the sandwich [6, 8].

Studies of the $\beta_2$-adrenoceptor ($\beta_2$-AR) have played a fundamental role in dissecting out the functional significance of arrestin in regulating GPCR signalling [1, 4, 7]. Indeed, this system provides a critical paradigm given the importance of selective $\beta_2$-AR agonists in the treatment of inflammatory lung disease, notably asthma [9], and that changes in $\beta_2$-AR functioning, expression and polymorphisms relate to heart failure [1, 2, 10, 11]. Mechanistically, agonist occupancy of the $\beta_2$-AR allows it to couple to $G_s$, which leads to activation of adenylyl cyclase and the generation of the key intracellular second messenger, cAMP [1, 4]. GRK phosphorylation of the $\beta_2$-AR initiates recruitment of βarrestins, which attenuates $\beta_2$-adrenoceptor-$G_s$ coupling, thereby lowering adenylyl cyclase activity [1, 4, 12].

It is now well-appreciated that cAMP signalling in cells is compartmentalised [13-17], with gradients of cAMP shaped through spatially confined degradation achieved by the action of specifically tethered cAMP phosphodiesterases [18, 19]. Isoforms encoded by the phosphodiesterase-4 (PDE4) family play a pivotal role in shaping compartmentalised cAMP responses in many cell types, being sequestered to specific signalling complexes and membranes within cells [13, 18, 20-24]. Thus dominant negative [25, 26], siRNA-mediated knockdown [27] and targeted gene knockout approaches [28] have demonstrated that PDE4 isoforms and sub-families can have unique, non-redundant functional roles. The key roles that PDE4 isoforms play in regulating key processes in various cell types associated with the immune system and brain, for example, undoubtedly, underpins the promising therapeutic use of PDE4 selective inhibitors for treating various inflammatory diseases, depression and as cognitive enhancers [9, 22, 29, 30].



© 2007 The Authors Journal compilation © 2007 Biochemical Society

Four PDE4 genes encode around 20 different isoforms [20, 22, 31, 32]. Each isoform is characterised by a unique N-terminal region that has been shown in many instances to be involved in targeting to signalling complexes, membranes or the cytoskeleton [20, 23]. It has been shown that PDE4 enzymes can associate with βarrestin in a variety of cell types [33] and that the βarrestin/PDE4 complex can be recruited to the $\beta_2$-AR upon agonist occupancy [34]. The increased level of cAMP, occurring upon agonist occupancy, elicits the protein kinase A (PKA)-mediated phosphorylation of the $\beta_2$-AR [35]. Mediating this is a sub-population of the RII form of PKA that is tethered to the $\beta_2$-AR itself by sequestration to the AKAP79 scaffolding protein [27, 36]. One functional consequence of PKA phosphorylation of the $\beta_2$-AR, which is observed in various cell types, is to switch its coupling from the $G_s$-mediated activation of adenylyl cyclase to the $G_i$-mediated activation of ERK [35]. βarrestin-recruited PDE4 acts to regulate specifically the phosphorylation of the $\beta_2$-AR by AKAP79-sequestered PKA, presumably by lowering localised cAMP levels below the threshold required for activation by this spatially restricted pool of PKA [13, 25, 27, 33, 34]. βarrestin-recruited PDE4 also acts to regulate synaptic release probability and presynaptic inhibition by opioids [37] and whilst PDE4 inhibitors have potential for treating asthma [22, 29, 38], βarrestin2 has been implicated in the development of allergic asthma [39].

PDE4 isoforms from all four families have the potential to interact with βarrestin due to the presence of a common interacting site located on the surface of helix-17 of their catalytic unit [33, 40]. However, in a variety of cell types expressing the PDE4D5 isoform, which include cardiac myocytes, then it is this isoform that is found preferentially sequestered to βarrestin [33]. Underpinning this is the presence of a further binding site for βarrestin across a broad region of the isoform-unique N-terminal region of PDE4D5 in addition to the common interaction site found in it catalytic unit [33, 40]. Thus, the presence of these two binding sites, presumably, allows βarrestin to straddle PDE4D5 and transport it to the $\beta_2$-AR upon agonist challenge. Such a delivery allows βarrestin-recruited PDE4D5 to attenuate PKA phosphorylation of the $\beta_2$-AR and desensitize $G_i$ signalling to ERK as βarrestin desensitizes signalling to through $G_s$ [13, 25].

Previous 2-hybrid analyses of βarrestin2 truncates, indicated that both the N- and C-domains of βarrestin2 contributed to the binding of PDE4D5 [33]. Here we explore the interaction of PDE4D5 with βarrestin and suggest that the common PDE4 catalytic unit interacts with the extreme N-terminal region of βarrestin, whilst the N-terminal region of PDE4D5 interacts with the C-domain of βarrestin.

**Experimental procedures**



© 2007 The Authors Journal compilation © 2007 Biochemical Society

*Materials*

A rabbit polyclonal antibody against the $\beta_2$AR (sc-569) and PKA P-$\beta_2$AR ser345/346 (sc-16718) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG M2 mouse monoclonal antibody (F3165), plus a conjugate of the same antibody to agarose (A2220). VSV antibody (V5507), VSV-agarose (A1970), isoprenaline (I6504) were from Sigma-Aldrich (Poole, Dorset, UK). Protein A beads used in immunoprecipitation reactions were from Invitrogen (15918-014). Antisera detecting βarrestin1/2 and mouse embryo fibroblasts MEFs) from animals lacking both βarrestin1 and βarrestin2 [34, 41] were from Dr Robert Lefkowitz (Howard Hughes Medical Center, Duke University, NC, USA). HEK293B2 cells, which stably express a GFP- and Flag-tagged $\beta_2$-AR, were as described before by one of us [42]. ECL kits were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Cyclic AMP determinations were carried out on cell lysates using the cAMP HTS immunoassay kit from Upstate (17-418)

*Cell Culture*

HEK293 and HEK293B2 cells were cultured as before [25, 27, 34, 40]. Transfections were performed using Polyfect (Qiagen 301107) according to the manufacturer's instructions. The mouse embryonic fibroblast cell line derived from βarrestin1/2 double knock-out animals was transfected using Lipofectamine (GIBCO-BRL) following the manufacturers instructions and maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum for HEK293 cells and 10% New Born Calf serum for HEKB2 cells, both with 1 x penicillin/streptomycin. Cells were treated with isoprenaline (10μM) for 5 minutes, as indicated.

*Immunopurification studies*

Briefly, detergent-soluble proteins were isolated from cells by disruption in lysis buffer (1% (v/v) Triton X-100, 50 mM HEPES buffer, pH 7.2, 10 mM EDTA, 100 mM $NaH_2PO_4$, $2H_2O$) containing Complete (Roche) protease inhibitor mixture to 8% volume. Detergent-insoluble proteins were removed by centrifugation at 10,000 x $g_{av}$ for 10 min, and the soluble fraction was retained. Equal volumes of cell lysate containing 500 μg of protein were cleared by incubation with 30 μl of protein A slurry. The beads were then removed by centrifugation at 10,000 x $g_{av}$ for 10 min at 4 °C, and cleared lysate was incubated at 4 °C for 2 h with constant agitation with a volume of antiserum determined to immunoprecipitate all $\beta_2$AR from mouse embryo fibroblast (ME βarrestin1(-/-):βarrestin2(-/-) cells. Immunoglobulins were then isolated by incubation with protein-A-coated Sepharose beads for 1 h before retrieval by refrigerated centrifugation at 10,000 x $g_{av}$ for 5 min. A similar protocol was used to isolate FLAG tagged constructs, however lysates were precleared with VSV-agarose and immunopurifications carried



© 2007 The Authors Journal compilation © 2007 Biochemical Society

out using FLAG-tagged agarose. Immunopurified proteins were run on SDS PAGE and immunoblotted as described previously [25, 27, 34, 40].

*Site-directed Mutagenesis*

This was performed by the circular mutagenesis method. All mutagenesis and deletion constructs were confirmed by DNA sequencing prior to use.

*Mammalian Cell Expression Constructs*

Human PDE4D5 cDNA (GenBank™ AF012073) with a carboxyl-terminal VSV epitope tag, was cloned into pcDNA3 (Invitrogen, Paisley, Scotland, UK) as before [33, 40]. βarrestin2 (GenBank™ BC007427) with a carboxyl-terminal FLAG epitope tag, was cloned into pcDNA3 (Invitrogen, Paisley, Scotland, UK).

*Expression of Fusion Proteins in Escherichia coli*

Full-length PDE4D5 and PDE4D3 were each expressed as amino-terminal glutathione-S-transferase (GST) fusion proteins and purified to apparent homogeneity as before [40, 43].

*SPOT synthesis of Peptides and Overlay Experiments*

Peptide libraries were produced by automatic SPOT synthesis [44] and synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc-chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany) [44, 45]. The interaction of spotted peptides with GST and GST fusion proteins was determined by overlaying the cellulose membranes with 10 µg/ml of recombinant protein. Bound recombinant proteins were detected with specific rabbit antisera and detection performed with secondary anti-rabbit horseradish peroxidase coupled antibody (1:2,500 dilution; Dianova) and visualisation by ECL, as in immunoblotting.

## Results

*Probing a βarrestin2 peptide array with PDE4D5 and PDE4D3*

βarrestin2 is a 418 amino acid protein that consists of two distinct sub-domains, called the N-domain and the C-domain, which are linked by a polar core (Fig. 1). In co-immunoprecipitation, pull-down and 2-hybrid analyses it has been shown to bind to the cAMP phosphodiesterase-4D5 (PDE4D5) isoform [25, 33, 40]. Here, this interaction was explored using peptide array analysis, which provides a novel and powerful technology for gaining insight into the basis of specific protein-protein interactions [44, 45]. In order to do this a library of overlapping peptides (25 mers), each shifted by 5 amino acids across the entire sequence of βarrestin2, was spot-synthesised on cellulose membranes. This immobilized peptide library was



© 2007 The Authors Journal compilation © 2007 Biochemical Society

probed with purified, recombinant GST fusion proteins of both PDE4D5 and PDE4D3, whose binding was assessed immunologically with positive interactions identified as dark spots (Fig. 1). PDE4D5 and PDE4D3 are long PDE4 isoforms from the same gene family (*PDE4D*) and differ only in their un-related, isoform-specific N-terminal region, which for PDE4D5 is 88 amino acids long and for PDE4D3 is 15 amino acids long [46]. Probing the βarrestin2 peptide library with PDE4D3-GST, positive reactions were only obtained with the first three peptide spots (Fig. 1), representing the sequence at the extreme N-terminus of βarrestin2 (amino acids Met1-Val35). Probing the βarrestin2 peptide library with PDE4D5-GST, positive reactions were obtained not only with the first three peptide spots but also with two regions in the C-domain of βarrestin2 (Fig. 1). One of these additional binding sites was within K206-Ser235 and the other within R286-Gly310 (Fig. 1).

Purified GST did not bind to any spot within the βarrestin2 peptide array (data not shown). PDE4D5-GST and PDE4D3-GST failed to bind to any spots other than those shown in Fig 1 within the βarrestin2 peptide array (data not shown).

*PDE4D5-GST binding to a scanning alanine substitution array of Gly6-Asp30*

To define further the amino acids involved in allowing PDE4D5 to bind to the region of βarrestin2 to which PDE4D3 also binds (Fig. 1). To do this we screened a family of peptides derived from a 25-mer parent peptide whose sequence reflected amino acids Gly6-Asp30 of βarrestin2. The 25 progeny of this parent peptide each had a single substitution, to alanine, of successive amino acids in the sequence to form a scanning peptide array (Fig. 2a). A severely reduced interaction was evident for Arg26Ala substitution, with the dual Lys25Ala:Arg26Ala substitution essentially ablating PDE4D5-GST interaction (Fig. 2a). In contrast, paired Lys11:Lys12 substitution, with alanine, failed to affect binding of PDE4D5-GST (Fig. 2a). We noted reduced interaction of PDE4D5-GST upon alanine substitution at Lys18 and Thr20 (Fig. 2a). No effect on PDE4D5-GST binding was observed upon substitution of the Ser13:Ser14 pairing to either alanine or aspartate, or their individual substitution to aspartate (Fig. 2a).

Using a 1-25 βarrestin2 peptide we also probed the region from Met1 to Pro5 in βarrestin2 employing single and multiple replacements with alanine (Fig. 2b). However, none of these altered the binding of the PDE4D5 probe. Interestingly, however, charge substitution by Glu3Arg replacement ablated PDE4D5 interaction with the 1-25 βarrestin2 peptide, suggesting that although a negative charge at this position is not required for PDE4D5 binding, the presence of a positive charge is inhibitory. Unfortunately the crystal structure of βarrestin2 has no electron density in this region, which precludes any potential structural insight.



© 2007 The Authors Journal compilation © 2007 Biochemical Society

*PDE4D5-GST binding to a scanning alanine substitution array of Lys206-Lys230*

To gain insight into the amino acids in the first region of the C-domain of βarrestin2 to which PDE4D5 binds specifically we performed scanning alanine substitution of the Lys206-Lys230 region (Fig. 2c) encompassed by spot-42 (Fig. 1). In doing so, we found that alanine substitution of any single one of a stretch of amino acids from Leu215-His220 (sequence LNVNVH) severely attenuated / ablated interaction with PDE4D5-GST (Fig. 2c). Consistent with this, substitution of Leu215 with aspartate also severely attenuated / ablated interaction with PDE4D5-GST (Fig. 2c). However, substitution of the positively charged Lys227 with the negatively charged aspartate, as with alanine substitution, did not affect interaction with PDE4D5-GST (Fig. 2c).

*PDE4D5-GST binding to a scanning alanine substitution array of Arg286-Gly310*

In order to gain insight into the amino acids in the second region of the C-domain of βarrestin2 to which PDE4D5 binds specifically (Fig. 1) we performed scanning alanine substitution of the Arg286-Gly310 region (Fig. 2d) encompassed by spot-58 (Fig. 1). In doing so, we found that alanine substitution of either Arg286 or Asp291 severely attenuated / ablated interaction with PDE4D5-GST (Fig. 2d). Dual alanine substitution of the Lys293:Lys295 pairing, however, had no effect on PDE4D5-GST interaction (Fig. 2d). As with single substitution of Arg286Ala, peptides with triple alanine substitution of either Arg286:Lys293:Lys295 or Arg286:Lys293:Lys308 did not noticeable interact with PDE4D5-GST (Fig. 2d). No effect on PDE4D5-GST interaction was observed upon substitution of either Lys308 with aspartate or Glu309 with lysine or upon their dual substitution, Lys308Asp:Glu309Lys (Fig. 2d). Substitution of the Ile306:Val307 pairing with alanine did not prevent interaction with PDE4D5-GST (Fig. 2d).

*The three regions of βarrestin2 identified in peptide array analyses are each involved in conferring PDE4D5 binding*

Our peptide array analyses identified three putative sites in βarrestin2 involved in conferring specificity for binding to PDE4D5. One in the N-domain deduced as allowing interaction with the PDE4(D) catalytic unit and two in the C-domain interacting with the unique N-terminal region of PDE4D5. To explore this further we generated defined mutations in each of these regions where scanning substitution peptide arrays had identified amino acids whose replacement with alanine led to compromised interaction with PDE4D5 (Fig. 2). In the first analysis we co-transfected COS cells with VSV-tagged PDE4D3 and either wild-type or the indicated mutant forms of FLAG-tagged βarrestin2. Using an antibody against the FLAG epitope



© 2007 The Authors Journal compilation © 2007 Biochemical Society

tag we were able to immunoprecipitate recombinant βarrestin2 specifically and determine whether or not recombinant PDE4D3 interacted by immunoblotting for the VSV epitope tag (Fig. 3a, b). Doing this we found that vsv-PDE4D3 was co-immunoprecipitated with wild-type βarrestin2 and with the Arg286Ala mutant and a mutant where the stretch of amino acids from 215-220 in βarrestin2 was replaced by a 5 residue alanine cassette (Fig. 3a, b). However, it was not co-immunoprecipitated with the Arg26Ala mutant form of βarrestin2 (Fig. 3a, b). Repeating this, but co-transfecting with vsv epitope-tagged PDE4D5, we noted that whilst vsv-PDE4D5 was co-immunoprecipitated with wild-type βarrestin2, its interaction was severely compromised for all of these various mutant forms of βarrestin2 (Fig. 3c, d). These data support the peptide array analyses that indicated all these three regions were involved in PDE4D5 interaction, whilst only that in the N-domain of βarrestin2 was involved in conferring interaction with PDE4D3. This suggests that the isoform-directed specificity of the interaction is conferred/determined by the C-domain of βarrestin.

*Probing mutations in the three regions of βarrestin2 involved in PDE4D5 interaction for functional consequences*

The $\beta_2$-AR becomes PKA phosphorylated in MEFs and various other cell types due to the action of an associated membrane-bound PKA whose activity is attenuated consequent upon the recruitment of PDE4D5, in complex with βarrestin2, to the $\beta_2$-AR [25, 27, 40]. We exploited this property in order to determine whether the functional impact of PDE4D5 on the $\beta_2$-AR is attenuated in MEFs transfected with forms of βarrestin2 mutated so as to compromise their interaction with PDE4D5.

In order to evaluate this, cells were utilized that lack endogenous βarrestins so as to incorporate mutant, recombinant species, and compare their functioning with wild-type recombinant βarrestin2 in this system. βarrestin2 is the predominant form of βarrestin in MEFs [41]. However, MEFs generated from 'double knockout' mice, by the disruption of genes encoding both βarrestin1 and βarrestin2 [41], exhibit no immunologically detectable forms of either of these two proteins [34, 41]. MEFs from 'double knockout' βarrestin1(-/-):βarrestin2(-/-) mice were transfected to allow similar levels of expression with either wild-type βarrestin2 or the indicated mutant species (Fig. 4b). MEFs express endogenously both PDE4D3 and PDE4D5, as shown from immunoblots of lysates from these cells (Fig. 4c). However, as seen with other cells [40], it is PDE4D5 that preferentially interacts with βarrestin2, demonstrated here by its selective immunoprecipitation with βarrestin2 (Fig. 4c). Disruption of any of the three sites on βarrestin2, suggested from scanning peptide arrays, with either Arg26Ala mutation or Arg286Ala mutation



© 2007 The Authors Journal compilation © 2007 Biochemical Society

or replacement of the LNVNVH motif with an alanine cassette ablated interaction with PDE4D5 as indicated by failure to co-immunoprecipitate (Fig. 4c).

Challenge of MEFs from βarrestin1(-/-):βarrestin2(-/-) double knockout animals with isoprenaline (10 μM for 5 min) caused a profound increase in PKA phosphorylation status of the $\beta_2$-AR as detected using a phospho-antiserum (Fig. 4a). However, transfection of these cells with wild-type βarrestin2 substantially decreased the PKA phosphorylation status of the $\beta_2$-AR upon isoprenaline challenge (Fig. 4a). In contrast to this, mutations to disrupt the interaction of PDE4D5 with βarrestin2, either in its N-domain (Arg26Ala) or in the first site in the C-domain (215-216 Ala cassette) or at the second site in the C-domain (Arg286Ala), all negated the inhibitory effect seen for wild-type βarrestin2 on isoprenaline-stimulated PKA phosphorylation of the $\beta_2$-AR (Fig. 4a).

Thus transfection of wild-type, recombinant βarrestin2 ablates the isoprenaline-induced PKA phosphorylation status of the $\beta_2$-AR by allowing recruitment of PDE4D5. In contrast, such an action is not seen when using forms of βarrestin2 engineered so as to compromise their ability to bind PDE4D5 (Fig. 4a).

It is possible that the differences between wild-type βarrestin2 and the various engineered species could also have arisen if such mutation had ablated their isoprenaline-dependent recruitment to the $\beta_2$-AR. Addressing this, we show here that wild-type, recombinant, FLAG-tagged βarrestin2 is recruited to the $\beta_2$-AR upon challenge of MEFs with isoprenaline and that similar, or even greater, levels of recruitment are evident with the various mutants examined here (Fig. 5a). Such analyses were done with similar levels of expression of the various recombinant βarrestin2 forms (Fig. 5b) and analysis of immunoprecipitates containing equal amounts of $\beta_2$-AR (Fig. 5c). A key function of βarrestin2 is to cause desensitization of $\beta_2$-AR-stimulated adenylyl cyclase, thereby attenuating cAMP generation [4, 7, 47]. Here we see that transfection of MEFs with wild-type, recombinant βarrestin2 does indeed attenuate cAMP generation after 5min challenge with isoprenaline and that the various mutant forms of βarrestin2 act similarly (Fig. 5d). Thus our various βarrestin2 mutants, although unable to bind PDE4D5, appear to both be recruited similarly to the $\beta_2$-AR and similarly desensitize isoprenaline-stimulated adenylyl cyclase activity.

**Discussion**

βarrestins, as well as causing desensitization by binding to GRK-phosphorylated GPCRs, can bind various other proteins involved in mediating cell signalling actions [4, 6, 7, 12],



© 2007 The Authors Journal compilation © 2007 Biochemical Society

indicating that βarrestins act as signal scaffolding proteins. Indeed, it now appears that far more proteins have potential to interact with βarrestin than could simply be accommodated upon any one molecule, suggesting competition for binding. This warrants determination of the 'footprint' that individual partners place upon the surface of βarrestins in order to gain insight into which combinations of proteins may be allowed to scaffold with βarrestins in any one complex.

Members of the 20+ strong family of PDE4 cAMP phosphodiesterases play a key role in underpinning compartmentalised cAMP signalling [13, 18, 24, 27, 48]. The first demonstration that a particular PDE isoform could exert a specific functional role in cells came from the use of dominant negative and siRNA knockdown strategies targeted at PDE4D5 [25-27, 48]. These uncovered the ability of βarrestin-complexed PDE4D5 to control the phosphorylation of $\beta_2$-AR via a spatially restricted PKA population. One functional consequence of this is to allow switching of $\beta_2$-AR signalling to ERK activation [35]. This process, which we have characterised in both HEK293 cells and in primary cardiac myocytes [25], depends upon the recruitment of a cytosolic βarrestin/PDE4D5 complex to the plasma membrane-located $\beta_2$-AR. There the local delivery of cAMP hydrolyzing PDE regulates a PKA subpopulation, spatially constrained through tethering to the $\beta_2$-AR by the scaffold protein AKAP79 [27].

PDE4D5 selectively interacts with βarrestins in cells where it is expressed it [33]. Such selectivity occurs because PDE4D5 not only has a βarrestin binding site on its conserved catalytic unit, through which all PDE4 isoforms can potentially interact with βarrestin, but it has an additional βarrestin binding site over an extended surface of its isoform-specific, N-terminal region [40]. We used a novel peptide array technology to identify these binding sites, which were confirmed by biochemical analysis [40]. Here, we have used such an approach to gain insight into how PDE4D5 interacts with βarrestin2. In order to do this we probed a library of overlapping 25-mer peptides that scanned the entire sequence of βarrestin2 with, not only PDE4D5, but also with the cognate PDE4D3 long isoform. Through this we anticipated being able to identify regions on βarrestin2 involved in anchoring the 'common' PDE4(D) catalytic site as well as those involved in anchoring the isoform-specific N-terminal region of PDE4D5.

Both PDE4D3 and PDE4D5 probes recognized spots 1-3, encompassing the first 35 amino acids of βarrestin2 (Fig. 1). This sequence contributes β-strands 1 and 2 in a surface-exposed β sheet motif within the arrestin N-domain [49], and its recognition by both PDE4D3 and PDE4D5 indicates that it is the conserved catalytic unit of PDE4 that interacts with this sequence. Focusing on peptide-2 (Gly6-Asp30), we generated a family of peptides in which successive amino acids in the sequence were singly substituted by alanine so as to form a scanning peptide array (Fig. 2a).



© 2007 The Authors Journal compilation © 2007 Biochemical Society

Analysis of this array identified reduced interaction of the PDE4D5 probe upon alanine substitution of three amino acids, namely Lys18, Thr20 and Arg26, suggesting them to be of potential importance in conferring interaction with the PDE4 catalytic unit (Fig. 2a). Alanine substitution of the positively charged Lys25, adjacent to Arg26, by itself had no effect on PDE4D binding (Fig. 2a). The X-ray crystal structure of bovine βarrestin2 reflects its basal conformation, being associated neither with PDE4 nor GPCR, [49], shows that Lys18 and Thr20 are located on β-strand 2 proximal to the β-1/2 hairpin and that both residues are prominently surface exposed (Fig. 6). Lys25 and Arg26, which are also surface exposed, lie on a loop C-terminal to β-strand 2 and in the crystal structure the side chains of Lys25 and Arg26 are presented in diametrically opposed directions that, if mimicked in the peptide, may contribute to the selectively of PDE4D interaction with these adjacent positively charged species. However, we note that the dual Lys25Ala:Arg26Ala substitution completely ablated interaction with PDE4D (Fig. 2a), which suggests it likely that conformational flexibility at the C-terminal end of peptide-2 may allow Lys25 to facilitate, to some small extent, the interaction with PDE4D that Arg26 provides. We substantiated the role of Arg26 in full-length βarrestin2, where substitution to alanine ablated interaction with PDE4D5, as evinced by the failure of the two proteins to co-immunoprecipitate (Fig. 3c, d).

In the βarrestin2 basal conformation (Fig. 6 a, b), Arg26 is partially occluded by folding of the arrestin C-terminal tail across the surface of β-strands 1 and 2. Indeed the guanidinium functionality of Arg26 interacts with three residues in this tail, forming a salt bridge to Glu389 and H-bonds to the main chain carbonyl groups of Phe391 and Ala392. Significantly, the recruitment of βarrestin to phospho-GPCR is associated with a hinged re-orientation of the βarrestin N- and C-domains in a conformational change that requires displacement of the C-terminal sequence from being folded over β-strands 1 and 2 in the basal state [6, 12, 47]. We suggest that the βarrestin C-terminal sequence may be similarly displaced upon βarrestin-PDE4D5 interaction and, in this way, the side chain of Arg26 might be more accessible for interaction with PDE4 (Fig. 6c).

The difference between peptide-3, to which βarrestin2 binds, and peptide-4, to which it does not, is the sequence KKSSP (Fig. 2a). This sequence is surface-exposed in the βarrestin2 crystal structure. However, substitution of any of these amino acids individually to alanine did not affect βarrestin2 binding (Fig. 2a). Additionally, the dual Lys11Ala:Lys12Ala substitution had no effect, eliminating these positively charged residues from a potential interaction with the PDE4D catalytic unit. Indeed, Lys11 and Lys12 are thought to 'guide' the GRK-phosphorylated receptor



© 2007 The Authors Journal compilation © 2007 Biochemical Society

to the key phosphate sensor provided by Arg170 located in a buried polar core within βarrestin2 [6, 12, 47]. Similarly, substitution of Ser13 and Ser14, either singly or together, with either alanine or aspartate, had little evident effect (Fig. 2a) on the interaction of peptide-3 with PDE4D5. The importance of the KKSSP sequence in peptide-3, therefore, most likely arises from its impact on the peptide's conformation, as these residues contribute to β-strand 1 and pair anti-parallel with the sequence containing Lys18/Thr20 in β-strand 2. Thus, although individual residues within the KKSSP sequence may not interact directly with the PDE4D catalytic unit, the removal of this sequence in peptide-4 may destroy conformational control over the presentation of the Lys18/Thr20 side chains and/or the organization of these two residues relative to Arg26.

We propose that the PDE4D catalytic unit forms a contact surface spanning Lys18 to Arg26 and running approximately along the axis of β-strand 2 of βarrestin2. It is conceivable that other residues on β-strands 1 and 2 may contribute direct interactions with the enzyme's surface, but where the extent of the interaction is insufficient to compromise binding of PDE4D5 to peptide-2 when residues are replaced individually by alanine. Given the potential importance of Lys18 and Arg26 for conferring binding to the PDE4 catalytic unit, we note from analysis of the PDE4D catalytic unit the presence of a negatively charged glutamate in the motif, FxFELxL that is required for βarrestin2 binding [40]. It is tempting to suggest that this glutamate in the PDE4D catalytic unit may form an ion pair with residues in the βarrestin2 N-domain when the proteins interact. However, we also note that a cluster of negatively charged amino acids that abut this motif in the PDE4 catalytic unit, as in FqFELtLeed, and these may provide the locus of a second negative charge able to accommodate the positive charged pairing of Lys18 and Arg26 in the N-domain of βarrestin2.

Analysis of the βarrestin2 peptide arrays identified two sets of PDE4D5-specific interacting peptides in the protein's C-domain (Fig. 1). One was spot 58 (Arg286 – Gly310) and the other encompassed by spots 42 and 43 (Lys206 – Ser235). The selectivity for PDE4D5 suggests that it is the unique N-terminal sequence of PDE4D5 that binds to the βarrestin2 C-domain. Scanning alanine substitution with peptide-42 showed that the motif, 215-LNVNVH-220 may be of importance for conferring βarrestin2 binding because single substitution of any of these residues reduced interaction with PDE4D5 (Fig. 2c). The LNVNVH sequence is located on the convex surface of the C-domain β-sandwich (Fig. 6). Significantly, this is the opposite face to that implicated in the recruitment of βarrestin to GRK-phosphorylated receptors [12], consistent with the ability of βarrestin to associate simultaneously with both the phospho-receptor and PDE4D5. Of the LNVNVH sequence, the side chains of amino acids N216, N218 and H220 are



© 2007 The Authors Journal compilation © 2007 Biochemical Society

surface exposed and thus most likely to interact with PDE4D5. The side chains of L215, V217 and V219 are orientated into the core structure and form a hydrophobic interface with the inner side of the β-sheet that forms the concave surface of the protein's C-domain. Direct interaction of these side chains with the PDE4D5 N-terminus is difficult to envisage without invoking major structural reorganization of βarrestin.

Scanning analysis of peptide-58 identified Arg286, whose substitution to either alanine or aspartate ablated interaction with PDE4D5 (Fig. 2d). This contrasted with alanine substitutions of the other positively charged residues in this peptide (Lys293, Lys295, Lys308), which had no effect on PDE4D5 interaction (Fig. 2d). The sequence corresponding to peptide-58 threads through the core structure with residues 286-300 surface exposed on one side (proximal to Arg26) and residues 306-310 surface exposed on the other (Fig. 6d). The Arg286 side chain is prominently exposed, does not engage other residues in βarrestin2 and likely presents an excellent target for interaction with PDE4D5.

Interestingly, peptide-57 (spanning residues 281 – 305) failed to produce a positive spot with the PDE4D5 probe. This peptide encompasses the region containing Arg286 and the surface-exposed residue group from peptide-58 proximal to Arg26. However, peptide-57 lacks residues 306-IVKEG-310 that form the second exposed surface in the sequence corresponding to peptide-58. This suggested that residues within this locus may also be involved in binding the PDE4D5 N-terminal sequence. However, the double substitution, Ile306Ala:Val307Ala, and charge reversal substitutions (Lys308Asp and Glu309Lys) within this region of peptide-58 failed to affect its interaction with the PDE4D5 probe.

Scanning substitution of peptide-58 also identified Asp291, whose mutation to alanine attenuated interaction with the PDE4D5 probe. This residue is not solvent exposed in the βarrestin basal conformation but is considered to ion pair with the protein's key phosphate sensor, Arg170, in its polar core [6, 12, 47]. Disruption of this salt bridge by pairing of Arg170 with receptor phosphates acts as a switch that allows the conformational transition of arrestin into its high-affinity receptor binding state. Arg170 must become accessible in order to interact with receptor phosphates. Thus, conceivably, Asp291 may become exposed upon activation of this switch, facilitating PDE4D5 binding to receptor-sequestered βarrestin2.

Residues within two distinct regions of the βarrestin C-domain, Leu215-His220 and Arg286/Asp291 are implicated in the interaction with the PDE4D5 N-terminal sequence. Given the possibility that conformational changes may occur in βarrestin upon association with PDE4, the relative disposition of these regions, which are well spaced when mapped onto the βarrestin



© 2007 The Authors Journal compilation © 2007 Biochemical Society

basal conformation (Fig. 6), may well alter. However, it is interesting that scanning peptide array analysis with the unique 88 amino acid N-terminal region of PDE4D5 suggests that an extensive surface from this sequence is involved in binding to βarrestin2 [40]. This would be consistent with the studies shown here, which indicate that two, spatially discrete, surfaces on the βarrestin2 C-domain are required for the specific binding of PDE4D5 (Fig. 1). Significantly, the location of the βarrestin C-domain sites for interaction with the PDE4D5 N-terminus, as well as the protein's N-domain surface for interaction with the conserved PDE4 catalytic unit, require that PDE4D5 docks to a distinct face of βarrestin from that involved in interaction with phospho-GPCR. It is intriguing that, in binding to the protein's N-domain, the PDE4 catalytic unit interacts with a region of βarrestin2 immediately adjacent to residues (Lys11:Lys12) believed to be involved in guiding the GRK-phosphorylated receptor to phosphate sensor at Arg170 in βarrestin2 [6, 12, 47]. The involvement of Asp291 from the phosphate switch, and the spatially proximal Arg286 in binding the N-terminal region of PDE4D5 may be significant. Given that βarrestins can tether a variety of proteins with distinct functional roles [4, 7, 47] it is tempting to speculate that the binding of PDE4D5 to βarrestin2 may influence the fidelity of interaction of βarrestin2 with other partner proteins.

In order to explore the possible functional significance of the three regions identified here to the interaction between PDE4D5 and βarrestin2, we generated a representative mutation from each region in βarrestin2 and analysed these for their ability to interact with PDE4D5 (Fig. 3c, d). The importance of all three regions in the binding of βarrestin2 to PDE4D5 in cells is shown from co-immunoprecipitation studies where the interaction was dramatically attenuated/ablated by disruption of either the N-domain site with Arg26Ala, or the two C-domain sites with either the Arg286Ala mutation for one site or the replacement of LNVNVH with an alanine cassette at the other (Fig. 3c, d). In marked contrast, interaction of PDE4D3 with βarrestin2 was only ablated upon disruption of the N-domain site using the Arg26Ala mutation (Fig. 3a, b). These data are consistent with the peptide arrays studies, indicating that the extreme N-terminus of βarrestin2 contains a site that allows binding of the common PDE4 catalytic unit whilst the C-domain contains two sites involved in conferring specific interaction with the unique N-terminal region of PDE4D5.

We wished to determine if forms of βarrestin2 mutated to compromise their interaction with PDE4D5 would have functional consequences on compartmentalised cAMP signalling, specifically on the PKA sub-population associated with the $\beta_2$-AR whose spatially localised action can be assessed by monitoring the PKA phosphorylation status of the $\beta_2$-AR subsequent to



© 2007 The Authors Journal compilation © 2007 Biochemical Society

agonist challenge with isoprenaline [27]. To evaluate this we required cells that lack endogenous βarrestins so as to be able to incorporate mutant species. Thus MEFs from double βarrestin1(-/-):βarrestin2(-/-) mice were transfected to express similar levels of either wild-type βarrestin2 or mutant species (Fig. 5b). In these double knockout cells, isoprenaline caused a profound PKA phosphorylation of the $\beta_2$-AR (Fig. 4a). However, whilst expression of wild-type βarrestin2 profoundly attenuated this action, this was not evident in βarrestin2 mutants unable to bind PDE4D5 (Fig. 4a), despite such mutants being recruited to the $\beta_2$-AR subsequent to isoprenaline challenge (Fig. 4a). Compromising PDE4D5 binding to βarrestin2 thus has profound functional significance regarding the phosphorylation of the $\beta_2$-AR by the PKA sub-population tethered to it [27, 36].

Here we have used novel scanning peptide array technology to show that the extreme N-domain of βarrestin contains a binding site for the conserved PDE4 catalytic unit, whilst it is the C-domain of βarrestin that contains two surfaces conferring selectivity for PDE4D5. Thus the combined N- and C-domains of βarrestin2 act to straddle PDE4D5 in order to form a functional complex that can be recruited to the agonist occupied $\beta_2$-AR. The activity of such spatially localised PDE4D5 influences local cAMP levels so as to regulate the phosphorylation of the $\beta_2$-AR by AKAP79-tethered PKA. Together, these proteins form a spatially constrained module that reflects a defined facet of compartmentalised cAMP signalling in cells. Future structural and other studies of PDE4/βarrestin2 complexes are required to provide further insight into this system and to determine if, as suggested here, the binding of PDE4D5 engenders a conformational change in βarrestin2 and affects its ability to bind other partner proteins.

**Acknowledgements.**

This work was supported by Medical Research Council (U.K.) grants G8604010 (MDH), and G0400053 (MDH & GM), by National Institutes of Health Grant R01-GM58553 (GBB), by the Deutsche Forschungsgemeinschaft grant Kl1415/2 (EK) and by European Union Grant 037189 (EK and MDH).

**References**

1 Hill, S. J. (2006) G-protein-coupled receptors: past, present and future. Br. J. Pharmacol. **147** Suppl 1, S27-37

2 Tang, C. M. and Insel, P. A. (2004) GPCR expression in the heart; "new" receptors in myocytes and fibroblasts. Trends Cardiovasc. Med. **14**, 94-99



© 2007 The Authors Journal compilation © 2007 Biochemical Society

3 Torrecilla, I. and Tobin, A. B. (2006) Co-ordinated covalent modification of G-protein coupled receptors. Curr. Pharm. Des. **12**, 1797-1808

4 Lefkowitz, R. J. and Shenoy, S. K. (2005) Transduction of receptor signals by beta-arrestins. Science **308**, 512-517

5 Penela, P., Ribas, C. and Mayor, F., Jr. (2003) Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. Cell. Signal. **15**, 973-981

6 Gurevich, V. V. and Gurevich, E. V. (2006) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol. Ther. **110**, 465-502

7 McDonald, P. H. and Lefkowitz, R. J. (2001) Beta-Arrestins: new roles in regulating heptahelical receptors' functions. Cell. Signal. **13**, 683-689

8 Han, M., Gurevich, V. V., Vishnivetskiy, S. A., Sigler, P. B. and Schubert, C. (2001) Crystal structure of beta-arrestin at 1.9 A: possible mechanism of receptor binding and membrane Translocation. Structure **9**, 869-880

9 Barnes, P. J. (2004) New drugs for asthma. Nat Rev Drug Discov **3**, 831-844

10 Baker, J. G., Hall, I. P. and Hill, S. J. (2003) Agonist and inverse agonist actions of beta-blockers at the human beta 2-adrenoceptor provide evidence for agonist-directed signaling. Mol. Pharmacol. **64**, 1357-1369

11 Brodde, O. E. and Leineweber, K. (2005) Beta2-adrenoceptor gene polymorphisms. Pharmacogenet Genomics **15**, 267-275

12 Gurevich, V. V. and Gurevich, E. V. (2004) The molecular acrobatics of arrestin activation. Trends Pharmacol. Sci. **25**, 105-111

13 Baillie, G. S. and Houslay, M. D. (2005) Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes. Curr. Opin. Cell. Biol. **17**, 129-134

14 Fischmeister, R. (2006) Is cAMP good or bad? Depends on where it's made. Circ. Res. **98**, 582-584

15 Tasken, K. and Aandahl, E. M. (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol. Rev. **84**, 137-167

16 Wong, W. and Scott, J. D. (2004) AKAP signalling complexes: focal points in space and time. Nat. Rev. Mol. Cell Biol. **5**, 959-970

17 Zaccolo, M. and Pozzan, T. (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science **295**, 1711-1715

18 Mongillo, M., McSorley, T., Evellin, S., Sood, A., Lissandron, V., Terrin, A., Huston, E., Hannawacker, A., Lohse, M. J., Pozzan, T., Houslay, M. D. and Zaccolo, M. (2004) Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ. Res. **95**, 67-75

19 Mongillo, M., Tocchetti, C. G., Terrin, A., Lissandron, V., Cheung, Y. F., Dostmann, W. R., Pozzan, T., Kass, D. A., Paolocci, N., Houslay, M. D. and Zaccolo, M. (2006) Compartmentalized phosphodiesterase-2 activity blunts beta-adrenergic cardiac inotropy via an NO/cGMP-dependent pathway. Circ. Res. **98**, 226-234

20 Houslay, M. D. and Adams, D. R. (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. Biochem. J. **370**, 1-18

21 Houslay, M. D. and Milligan, G. (1997) Tailoring cAMP-signalling responses through isoform multiplicity. Trends Biochem. Sci. **22**, 217-224

22 Houslay, M. D., Schafer, P. and Zhang, K. Y. (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. Drug Discov. Today **10**, 1503-1519

23 Huston, E., Houslay, T. M., Baillie, G. S. and Houslay, M. D. (2006) cAMP phosphodiesterase-4A1 (PDE4A1) has provided the paradigm for the intracellular




© 2007 The Authors Journal compilation © 2007 Biochemical Society

targeting of phosphodiesterases, a process that underpins compartmentalized cAMP signalling. Biochem. Soc. Trans .**34**, 504-509

24 Baillie, G. S., Scott, J. D. and Houslay, M. D. (2005) Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. FEBS Lett **579**, 3264-3270

25 Baillie, G. S., Sood, A., McPhee, I., Gall, I., Perry, S. J., Lefkowitz, R. J. and Houslay, M. D. (2003) beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi. Proc. Natl. Acad. Sci. U.S.A. **100**, 940-945

26 McCahill, A., McSorley, T., Huston, E., Hill, E. V., Lynch, M. J., Gall, I., Keryer, G., Lygren, B., Tasken, K., van Heeke, G. and Houslay, M. D. (2005) In resting COS1 cells a dominant negative approach shows that specific, anchored PDE4 cAMP phosphodiesterase isoforms gate the activation, by basal cyclic AMP production, of AKAP-tethered protein kinase A type II located in the centrosomal region. Cell. Signal. **17**, 1158-1173

27 Lynch, M. J., Baillie, G. S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., van Heeke, G. and Houslay, M. D. (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. J. Biol. Chem. **280**, 33178-33189

28 Ariga, M., Neitzert, B., Nakae, S., Mottin, G., Bertrand, C., Pruniaux, M. P., Jin, S. L. and Conti, M. (2004) Nonredundant function of phosphodiesterases 4D and 4B in neutrophil recruitment to the site of inflammation. J Immunol **173**, 7531-7538

29 Huang, Z., Ducharme, Y., Macdonald, D. and Robichaud, A. (2001) The next generation of PDE4 inhibitors. Curr. Opin. Chem. Biol. **5**, 432-438

30 O'Donnell, J. M. and Zhang, H. T. (2004) Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). Trends Pharmacol. Sci. **25**, 158-163

31 Bolger, G. B. (1994) Molecular biology of the cyclic AMP-specific cyclic nucleotide phosphodiesterases: a diverse family of regulatory enzymes. Cell. Signal. **6**, 851-859

32 Conti, M., Richter, W., Mehats, C., Livera, G., Park, J. Y. and Jin, C. (2003) Cyclic AMP-specific PDE4 Phosphodiesterases as Critical Components of Cyclic AMP Signaling. J. Biol. Chem. **278**, 5493-5496

33 Bolger, G. B., McCahill, A., Huston, E., Cheung, Y. F., McSorley, T., Baillie, G. S. and Houslay, M. D. (2003) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with beta-arrestins. J. Biol. Chem. **278**, 49230-49238

34 Perry, S. J., Baillie, G. S., Kohout, T. A., McPhee, I., Magiera, M. M., Ang, K. L., Miller, W. E., McLean, A. J., Conti, M., Houslay, M. D. and Lefkowitz, R. J. (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. Science **298**, 834-836

35 Lefkowitz, R. J., Pierce, K. L. and Luttrell, L. M. (2002) Dancing with different partners: protein kinase a phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. Mol. Pharmacol. **62**, 971-974

36 Fraser, I. D., Cong, M., Kim, J., Rollins, E. N., Daaka, Y., Lefkowitz, R. J. and Scott, J. D. (2000) Assembly of an A kinase-anchoring protein-beta(2)-adrenergic receptor complex facilitates receptor phosphorylation and signaling. Curr. Biol. **10**, 409-412

37 Bradaia, A., Berton, F., Ferrari, S. and Luscher, C. (2005) beta-Arrestin2, interacting with phosphodiesterase 4, regulates synaptic release probability and presynaptic inhibition by opioids. Proc. Natl. Acad. Sci. U.S.A. **102**, 3034-3039

38 Lipworth, B. J. (2005) Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. Lancet **365**, 167-175



© 2007 The Authors Journal compilation © 2007 Biochemical Society

39 Walker, J. K., Fong, A. M., Lawson, B. L., Savov, J. D., Patel, D. D., Schwartz, D. A. and Lefkowitz, R. J. (2003) Beta-arrestin-2 regulates the development of allergic asthma. J. Clin. Invest. **112**, 566-574

40 Bolger, G. B., Baillie, G. S., Li, X., Lynch, M. J., Herzyk, P., Mohamed, A., Mitchell, L. H., McCahill, A., Hundsrucker, C., Klussmann, E., Adams, D. R. and Houslay, M. D. (2006) Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, beta-arrestin and RACK1, in cAMP-specific phosphodiesterase PDE4D5. Biochem. J. **398**, 23-36

41 Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A. and Lefkowitz, R. J. (2001) beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. Proc. Natl. Acad. Sci. U.S.A. **98**, 1601-1606

42 McLean, A. J. and Milligan, G. (2000) Ligand regulation of green fluorescent protein-tagged forms of the human beta(1)- and beta(2)-adrenoceptors; comparisons with the unmodified receptors. Br. J. Pharmacol. **130**, 1825-1832

43 Yarwood, S. J., Steele, M. R., Scotland, G., Houslay, M. D. and Bolger, G. B. (1999) The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. J. Biol. Chem. **274**, 14909-14917

44 Kramer, A. and Schneider-Mergener, J. (1998) Synthesis and screening of peptide libraries on continuous cellulose membrane supports. Methods Mol Biol **87**, 25-39

45 Frank, R. (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. J. Immunol. Methods **267**, 13-26

46 Bolger, G. B., Erdogan, S., Jones, R. E., Loughney, K., Scotland, G., Hoffmann, R., Wilkinson, I., Farrell, C. and Houslay, M. D. (1997) Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. Biochem. J. **328**, 539-548

47 Gurevich, E. V. and Gurevich, V. V. (2006) Arrestins: ubiquitous regulators of cellular signaling pathways. Genome Biol **7**, 236

48 Terrin, A., Di Benedetto, G., Pertegato, V., Cheung, Y. F., Baillie, G., Lynch, M. J., Elvassore, N., Prinz, A., Herberg, F. W., Houslay, M. D. and Zaccolo, M. (2006) PGE1 stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: role of compartmentalized phosphodiesterases. J. Cell. Biol. **175**, 441-451

49 Milano, S. K., Pace, H. C., Kim, Y. M., Brenner, C. and Benovic, J. L. (2002) Scaffolding functions of arrestin-2 revealed by crystal structure and mutagenesis. Biochemistry **41**, 3321-3328



© 2007 The Authors Journal compilation © 2007 Biochemical Society

**Figure Legends.**

*Figure 1. Probing a βarrestin2 peptide array with PDE4D5-GST and PDE4D3-GST.*

βarrestin2 is shown schematically with its N- and C-domains. Data shows immobilized peptide 'spots' of overlapping 25-mer peptides each shifted along by 5 amino acids in the entire βarrestin2 sequence probed for interaction with either PDE4D5-GST or PDE4D3-GST and detection by immunoblotting. Positively interacting peptides generate dark spots while none-interacting peptide leave white (blank) spots. In all other sections of the array spots were blank with either probe. Spot numbers relate to peptides in the scanned array and whose sequence is given as indicated. Arrays probed with purified GST did not yield any positively interacting spots. These data are typical of experiments performed three times.

*Figure 2. Scanning binding alanine substitution analysis to probe the binding sites for PDE4D5-GST in the N- and C-domains of βarrestin2.*

These show βarrestin2 peptide arrays probed for PDE4D5-GST binding based upon the indicated 25mer 'parent' βarrestin2 peptide where the indicated amino acids were sequentially and individually substituted with alanine. Ctr refers to the native peptide and all other spots reflect peptide 'progeny'. GST alone did not bind to any peptide spot (data not shown). (a) shows the alanine substitution array for the Gly6-Asp30 peptide whose sequence is in the N-domain of βarrestin2, together with the additional indicated substitutions, which were to either alanine (no label) or aspartate (labeled 'D'); (b) this examines a peptide representing amino acids 1-25 in βarrestin2 with changes made solely in the first five amino acids (native sequence: MGEKP). The alterations in this portion are indicated using single letter amino acid code. These data are typical of experiments performed three times; (c) shows the alanine substitution array for the Lys206-Lys230 peptide whose sequence is in the C-domain of βarrestin2, together with the additional indicated substitutions which were either to aspartate (D) or to arginine (R); (d) shows the alanine substitution array for the Arg286-Gly310 peptide whose sequence is in the C-domain of βarrestin2, together with the additional indicated substitutions which were to either alanine (no label) or aspartate (labeled 'D') or lysine (K). LAL is L288A:A289D:L290A. RKK1 is R286A:K293A:K295A. RKK2 is R286A:K293A:K295A:K308A. KE is K308A:E309A. IV is I306A:V307A.



© 2007 The Authors Journal compilation © 2007 Biochemical Society

*Figure 3. Consequences of mutations in the N- and C-domain binding sites in βarrestin2, based on peptide array data, for the binding of PDE4D3 and PDE4D5.*

(a) HEK293 cells were co-transfected with vsv-epitope-tagged PDE4D3 (L20970) together with the indicated forms of FLAG-tagged βarrestin2, namely either wild-type (Wt) or mutations of Arg26Ala (R26A), Arg286Ala (R296A) and one where the stretch of amino acids from Leu215-His220 in βarrestin2-FLAG were replaced by a 5 residue alanine cassette (βarrestin2-FLAG L215-H220(5A)). The lower panel shows the result of immunoprecipitating the indicated βarrestin2-FLAG construct with an anti-FLAG antibody and probing for co-immunoprecipitated PDE4D3-vsv by immunoblotting with an anti-vsv antibody. (b) quantification, by densitometry, of co-immunoprecipitated PDE4D3-vsv from 3 separate experiments as in (a) with means ± SD and with the indicated control analyzing cells that had not been transfected with βarrestin2-FLAG, but which has been transfected with PDE4D3-vsv. (c) as in (a) but co-transfection with vsv-epitope-tagged PDE4D5 (AF012073) instead of PDE4D3-vsv. (d) as in (b) but co-transfection with PDE4D5-vsv instead of PDE4D3-vsv.

*Figure 4. Functional consequences of mutations disrupting N- and C-domain binding sites in βarrestin2 for PDE4D5.*

(a) The upper panel shows the PKA phosphorylation status of the $\beta_2$-AR, as assessed using a phospho-specific antiserum, in βarrestin1(-/-):βarrestin2(-/-) double knockout MEFs treated with isoprenaline (ISO) (10 μM) for either 0 or 5 min, as indicated. This was assessed in both mock transfected cells and also in cells transfected to express either wild-type βarrestin2-FLAG or the various βarrestin2-FLA mutants as described in the legend to Figure 3. The lower panel shows a loading blot for the $\beta_2$-AR in these cells. (b) shows an immunoblot for the indicated forms of wild-type and mutant βarrestin2 expressed in βarrestin1(-/-):βarrestin2(-/-) double knockout MEFs treated with isoprenaline (ISO) as indicated. (c) shows an immunoblot of endogenous PDE4D species in lysates (L) of MEFs transfected with the indicated wild-type and mutant forms of βarrestin2-FLAG. This detects the presence of endogenous PDE4D3 and PDE4D5 in lysates (L) from these cells. Also shown are PDE4D immunoblots of both FLAG immunoprecipitates (I) and bead control immunoprecipitates (cl), where no anti-FLAG antiserum was added. These data are typical of experiments performed three times.

*Figure 5. Consequences of mutations in the N- and C-domain binding sites in βarrestin2, based on peptide array data, for the binding of PDE4D3 and PDE4D5.*



© 2007 The Authors Journal compilation © 2007 Biochemical Society

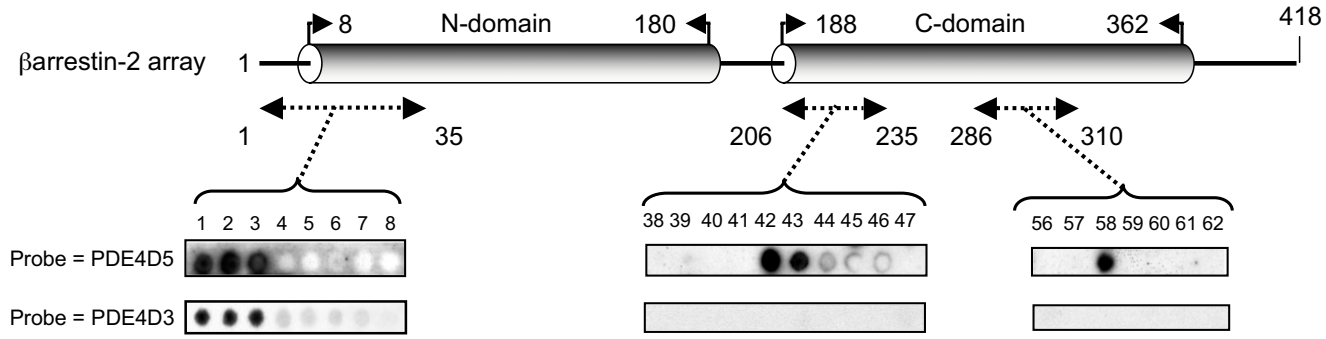
(a) βarrestin1(-/-):βarrestin2(-/-) double knockout MEFs were transfected to express either wild-type βarrestin2-FLAG, the various βarrestin2-FLAG mutants as described in the legend to Figure 3 or transfected with vector alone (mock). Cells were then challenged with isoprenaline (ISO) (10 μM) for the indicated time prior to disruption for immunoprecipitation of the $\beta_2$-AR. Immunoprecipitates from equal quantities of lysate protein were immunoblotted with a FLAG antibody to detect associated βarrestin2-FLAG. (b) shows FLAG immunoblots of equal quantities of lysate protein from the transfection studies described in (a) using the indicated βarrestin2-FLAG constructs. (c) as in (b) but immunoblotting for the $\beta_2$-AR. (d) shows the increase in intracellular cAMP level in βarrestin1(-/-):βarrestin2(-/-) double knockout MEFs transfected to express either wild-type βarrestin2-FLAG, the various βarrestin2-FLAG mutants as described in the legend to Figure 3 or transfected with vector alone (mock), after a 5min challenge with isoprenaline. For comparison the increase in intracellular cAMP is shown relative to that seen in mock transfected cells, which lack both βarrestin1 and βarrestin2. Data are typical of 3 experiments with, in (d), data given as means ± SD given and where mock-transfected cells had 0.96 ± 0.05 pmol cAMP/μg cell lysate protein, rising to 4.28 ± 0.19 pmol cAMP/μg cell lysate protein after 5min challenge with isoprenaline.

Figure 6. *Structure of bovine βarrestin2 and location of residues implicated in PDE4D binding.* **(a)** Showing residues implicated in PDE4D binding mapped onto the solvent accessible surface of bovine βarrestin2 in its basal conformation (Brookhaven: 1JSY); numbering used is taken from human βarrestin2 sequence. N-terminal residues 1-6 and residues 356-382 (preceding the C-terminal sequence) are disordered. Red (residues 7-35) corresponds to array peptides-1 to -3; dark blue (residues 206-235) corresponds to array peptides-42 & -43; green (residues 286-310) corresponds to array peptide-58; arrestin C-terminal sequence is shown purple. Residues whose individual mutation to alanine significantly compromises PDE4D binding are highlighted in yellow. Lys25 and the 11-KKSSP-15 sequence within peptide-2 are highlighted in light blue. **(b)** Ribbon representation of structure viewed as in (a). **(c)** As (a) but with C-terminal sequence displaced. **(d)** As (c) but rotated 45° about the horizontal axis; phospho-GPCR docks from the rear of the structure as viewed.



© 2007 The Authors Journal compilation © 2007 Biochemical Society

Fig. 1




(Spot 42) 206-KELYYHGEPLNVNVHVTNNSTKTVK-230

(Spot 43) 211-HGEPLN VNVHVTNNSTKTVKKIKVS-235

(spot 1) 1-MGEKPGTRVFKKSSPNCKLTVYLGK-25

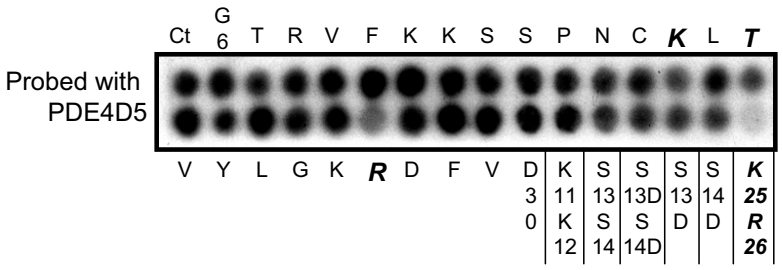
(spot 2) 6-GTRVFKKSSPNCKLTVYLGKRDFVD-30

(spot 3) 11-KKSSPNCKLTVYLGKRDFVDHLDKV-35

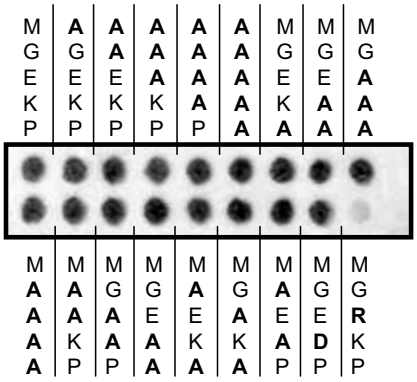
(Spot 58) 286-RGLALDGKLKHEDTNLASSTIVKEG-310

© 2007 The Authors Journal compilation © 2007 Biochemical Society

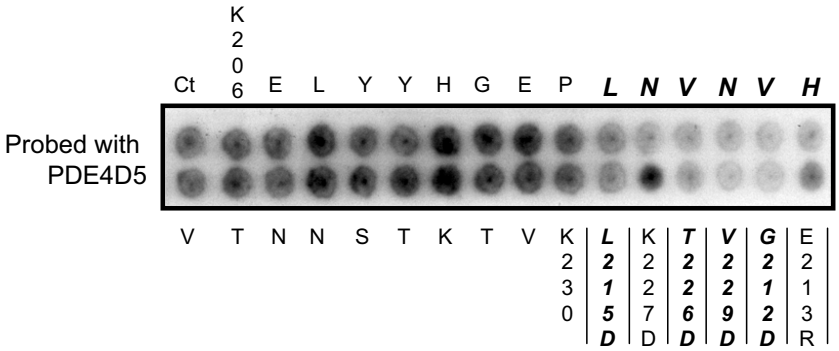
(a) βarrestin-2 Spot-2 scanning substitution array (G6-D30)




(b) βarrestin-2 substitution array of M1 to P5




(c) βarrestin-2 Spot-42 scanning substitution array (K206-K230)




(d) βarrestin-2 Spot 58 scanning substitution array (R286 -G310)

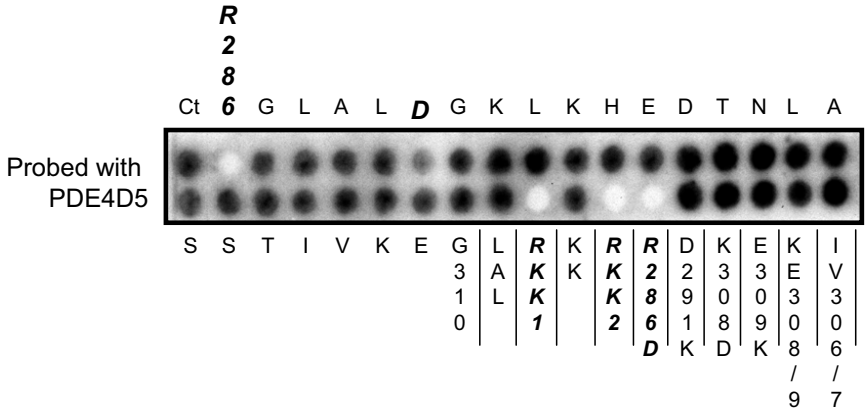
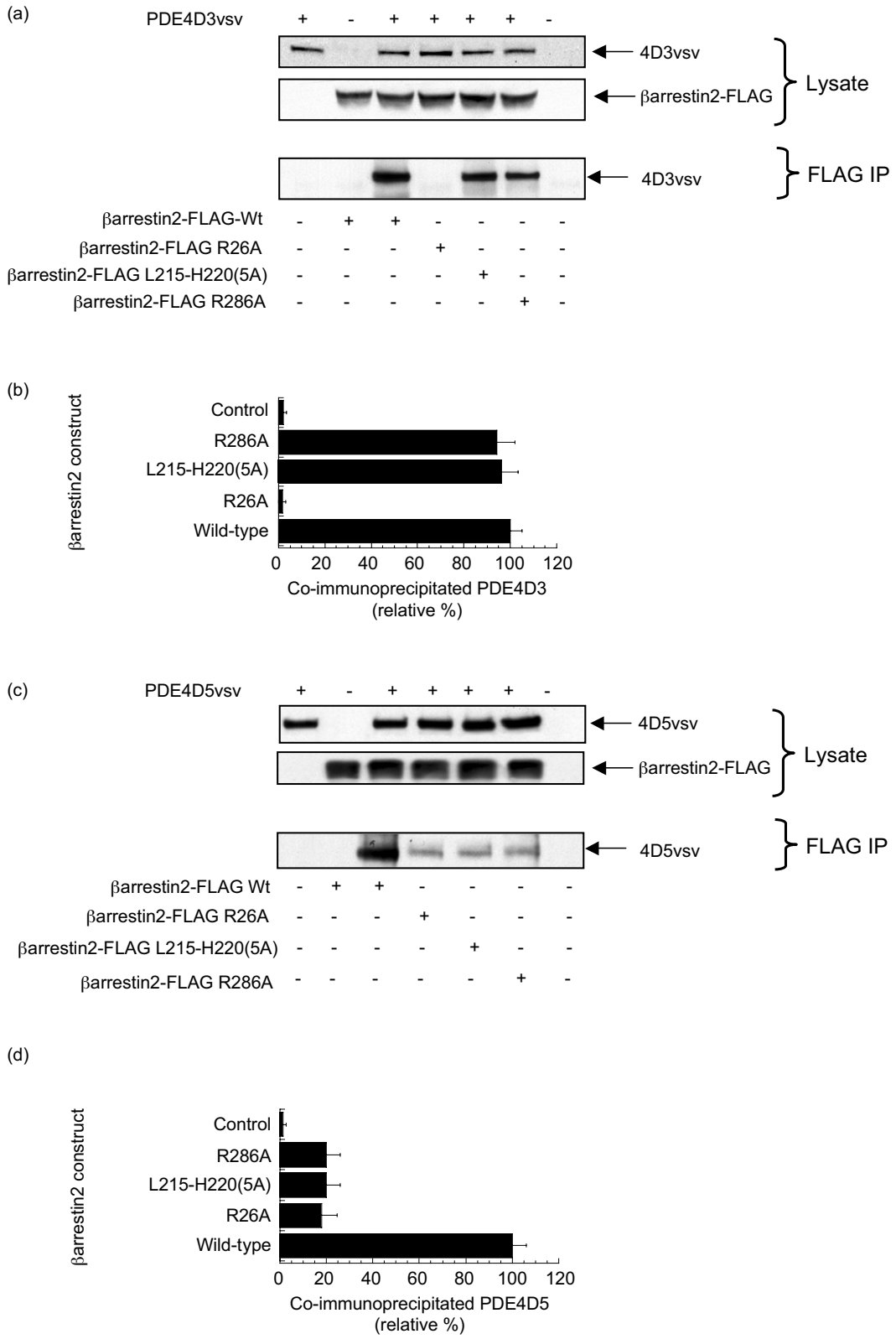


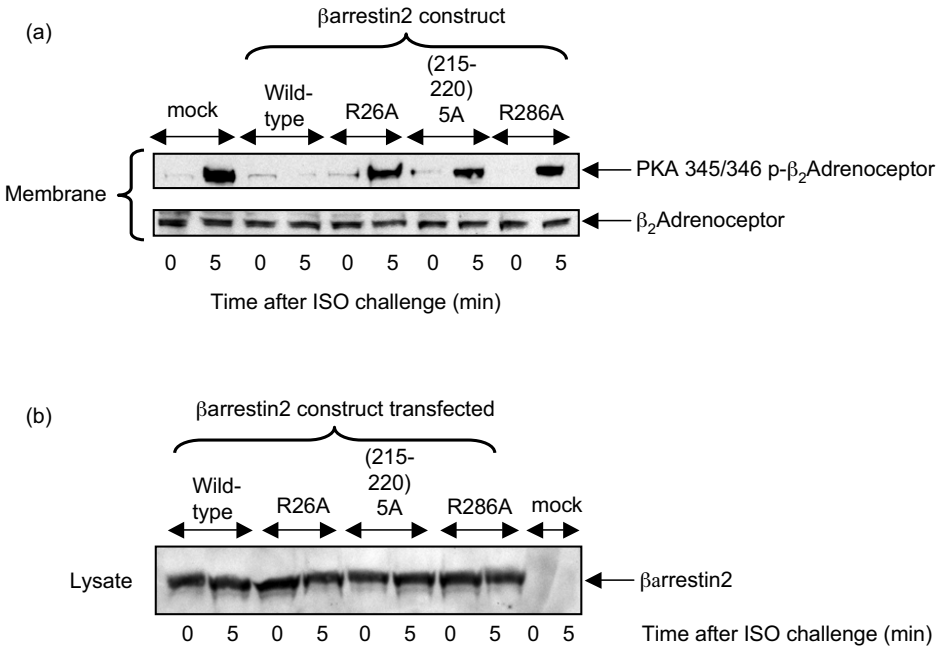

Fig. 2

© 2007 The Authors Journal compilation © 2007 Biochemical Society



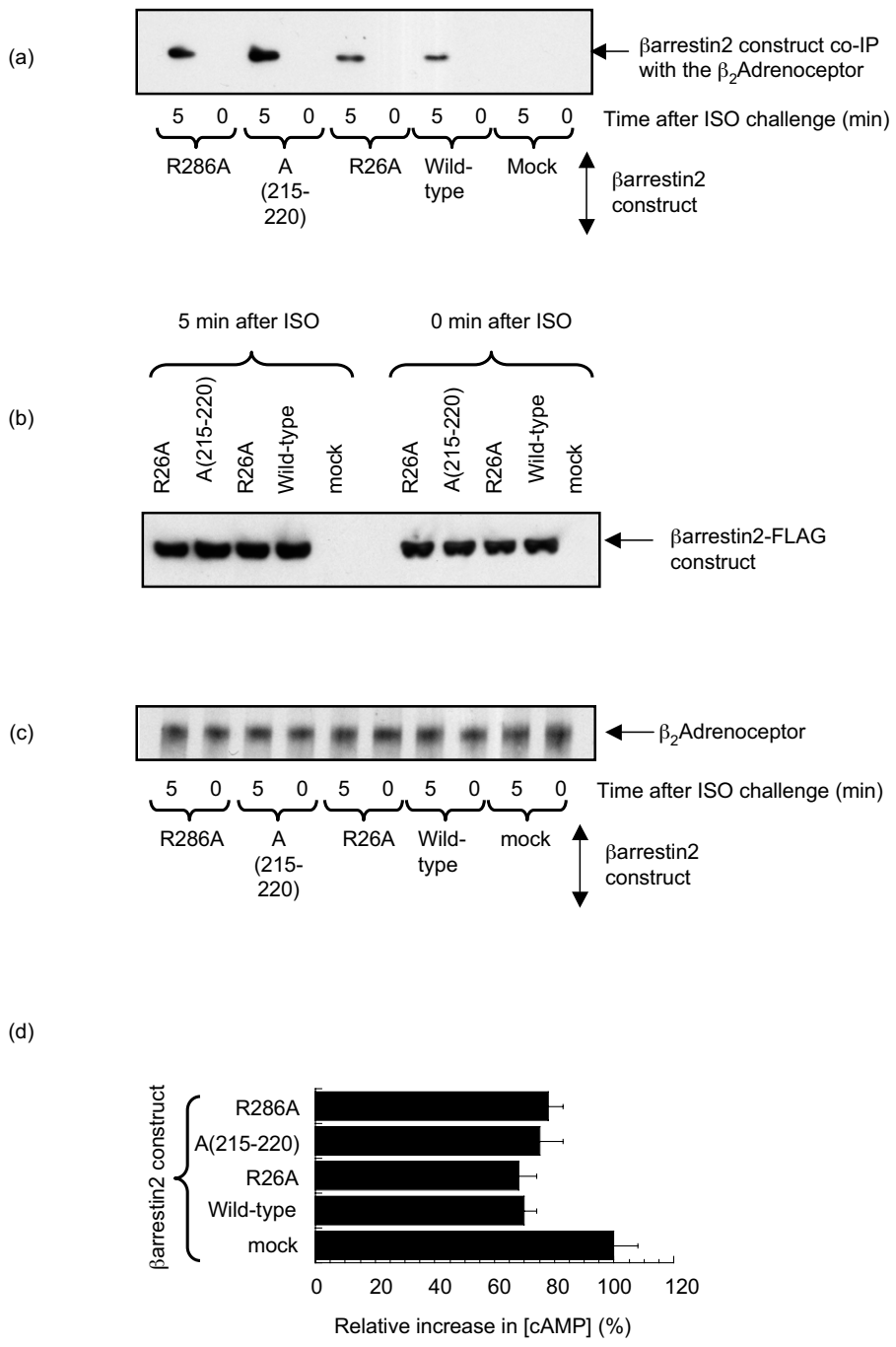

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Fig. 4

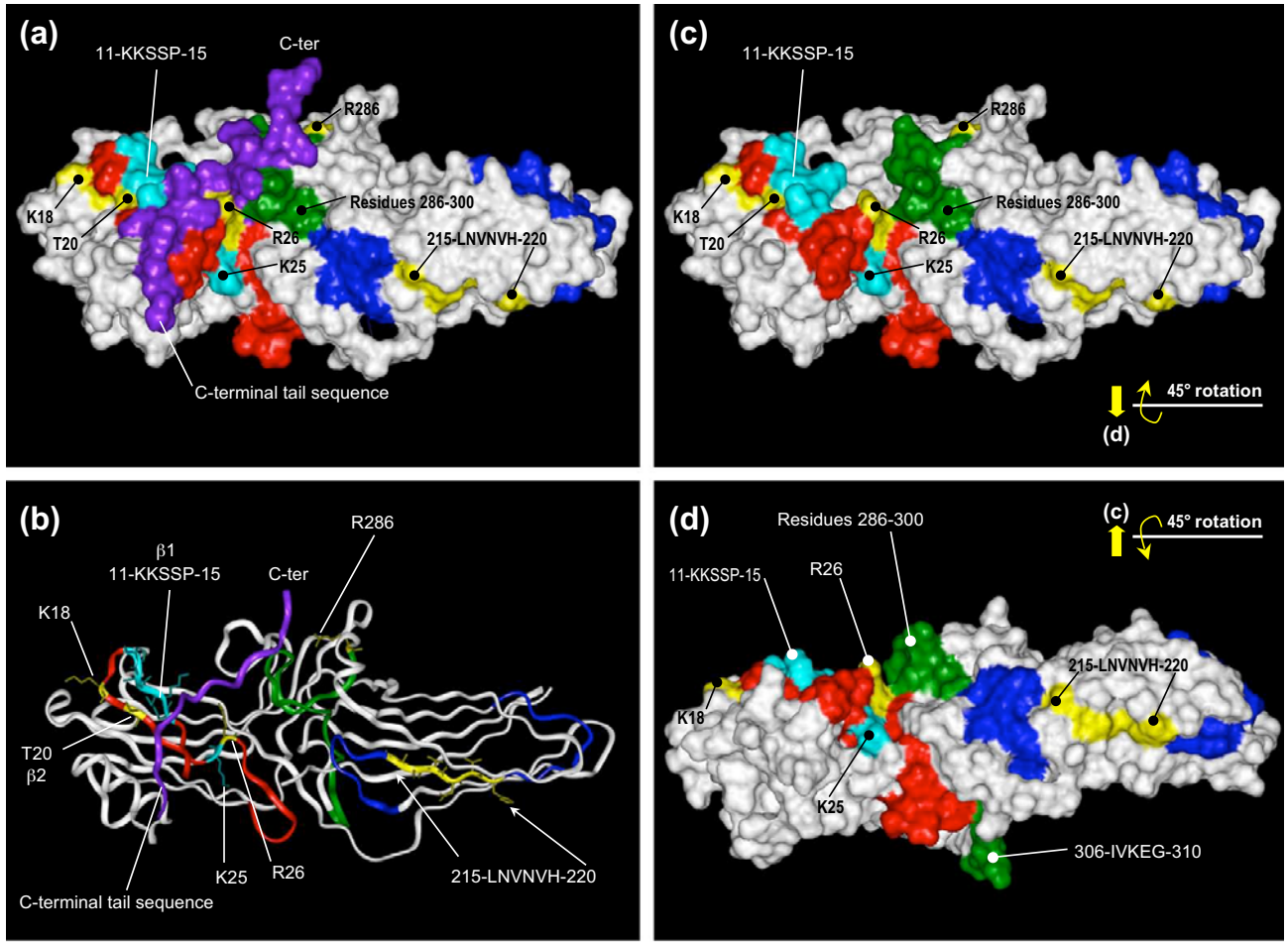



© 2007 The Authors Journal compilation © 2007 Biochemical Society

Fig. 5




© 2007 The Authors Journal compilation © 2007 Biochemical Society




© 2007 The Authors Journal compilation © 2007 Biochemical Society