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Importance of regions outside the cytoplasmic tail of G-protein-coupled receptors for phosphorylation and dephosphorylation

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Abstract

Two GPCRs (G-protein-coupled receptors), TRHR (thyrotropin-releasing hormone receptor) and β_2AR (β_2-adrenergic receptor), are regulated in distinct manners. Following agonist binding, TRHR undergoes rapid phosphorylation attributable to GRKs (GPCR kinases); β_2AR is phosphorylated by both second messenger-activated PKA (protein kinase A) and GRKs with slower kinetics. TRHR co-internalizes with arrestin, whereas β_2AR recruits arrestin, but internalizes without it. Both receptors are dephosphorylated following agonist removal, but TRHR is dephosphorylated much more rapidly while it remains at the plasma membrane. We generated chimaeras swapping the C-terminal domains of these receptors to clarify the role of different receptor regions in phosphorylation, internalization and dephosphorylation. β_2AR with a TRHR cytoplasmic tail (β_2AR–TRHR) and TRHR with a β_2AR tail (TRHR–β_2AR) signalled to G-proteins normally. β_2AR–TRHR was phosphorylated well at the PKA site in the third intracellular loop, but poorly at GRK sites in the tail, whereas TRHR–β_2AR was phosphorylated strongly at GRK sites in the tail (Ser^{355}/Ser^{356} of the β_2AR). Both chimaeric receptors exhibited prolonged, but weak, association with arrestin at the plasma membrane, but high-affinity arrestin interactions and extensive co-internalization of receptor with arrestin required a phosphorylated TRHR tail. In contrast, swapping C-terminal domains did not change the rates of phosphorylation and dephosphorylation or the dependence of TRHR dephosphorylation on the length of agonist exposure. Thus the interactions of GPCRs with GRKs and phosphatases are determined not simply by the amino acid sequences of the substrates, but by regions outside the cytoplasmic tails.

Keywords

arrestin; β_2-adrenergic receptor; G-protein-coupled receptor (GPCR); G-protein-coupled receptor kinase (GRK); phosphatase; thyrotropin-releasing hormone receptor (TRHR)

INTRODUCTION

GPCRs (G-protein-coupled receptors), the largest known class of transmembrane proteins, transmit extracellular signals into intracellular responses through the binding of endogenous ligands to an orthosteric binding pocket. Ligand binding triggers GPCR conformational changes that allow receptor coupling to heterotrimeric G-proteins and subsequent signalling events. The regulation of GPCR activity is of critical importance for normal physiology.

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AUTHOR CONTRIBUTION
Austin Gehret and Patricia Hinkle designed and performed experiments, analysed data and wrote the paper.

Desensitization of GPCRs is initiated by the phosphorylation of serine and threonine residues in the intracellular loops and C-terminal domain by second messenger-activated kinases, such as PKA (protein kinase A) and GRKs (GPCR kinases) [1]. The phosphorylation of GPCRs is generally a prerequisite for binding of the cytosolic protein arrestin [2-4]. Arrestin serves multiple purposes, including the steric preclusion of subsequent coupling interactions between receptor and G-protein and the targeting of receptors to clathrin-coated pits for endocytosis [5].

For most GPCRs, receptor resensitization includes arrestin dissociation, receptor dephosphorylation and recycling to the membrane. Previous work on the prototypical β_2AR (β_2-adrenergic receptor) established that blocking internalization disrupts the ability of β_2AR to resensitize [6,7], an effect attributed to impaired receptor dephosphorylation [6,8]. These results led to a model of resensitization whereby ligand dissociation from internalized receptor is facilitated in acidified endosomes, resulting in the dissociation of arrestin and dephosphorylation by an endosomally associated phosphatase [9]. This model has been refined in recent years through experiments using phosphosite-specific antibodies which show that dephosphorylation of GRK phosphosites can occur at the plasma membrane, whereas dephosphorylation of the PKA phosphosite slows when internalization is inhibited [10]. Although no specific phosphatase has been identified, β_2AR dephosphorylation is sensitive to okadaic acid and calyculin A, inhibitors of PP (protein phosphatase) 2A and PP1 [6,11].

Some GPCRs, designated class A receptors, have higher affinity for arrestin3 than arrestin2 and internalize without arrestin; class A receptors recycle rapidly. Class B receptors co-internalize together with arrestin2 or arrestin3 and recycle slowly, in many cases undergoing extensive intracellular degradation [12]. Previous work utilizing chimaeras between the β_2AR, a class A receptor, and the vasopressin V2 receptor, a class B receptor, demonstrated that regions in the C-terminal domain of the V2 receptor confer a tight interaction with arrestins [13]. More importantly, swapping the C-terminal domains caused complete class switching of the two receptors, reversing the kinetics of dephosphorylation, recycling and resensitization [13].

TRHR [TRH (thyrotropin-releasing hormone) receptor] does not behave as either a typical class A or class B receptor. The TRHR is rapidly phosphorylated by GRKs at a number of sites and remains strongly phosphorylated in the presence of hormone, but it does not undergo phosphorylation by second messenger-activated kinases [14]. The receptor internalizes extensively with arrestins 2 and 3 [15]. The rapid phosphorylation and sustained interaction with arrestin suggest that the TRHR spends much of its existence in a desensitized state, but the receptor can be dephosphorylated at the plasma membrane or in internal compartments once hormone is removed [14].

Whereas it is clear that the cytoplasmic tail plays an important role in GPCR regulation, the molecular mechanisms that control GPCR phosphorylation, dephosphorylation and trafficking remain incompletely understood. In order to dissect the importance of the helical regions and intracellular loops of the GPCR compared with the cytoplasmic tail, we generated chimaeras between the TRHR and the β_2AR and studied their behaviour following agonist binding. These receptors were selected because highly specific phosphosite-specific antibodies are available to differentiate between GRK and PKA sites in the β_2AR and identify GRK sites in the TRHR. The availability of reliable convenient ELISA methods allowed us to study subtle differences in the interactions of receptors with GRKs, arrestins and GPCR phosphatases. We show that, although the arrestin association profiles are influenced by the cytoplasmic tails of the chimaeric receptors, phosphorylation and

dephosphorylation of these receptors are largely independent of the composition of their C-termini.

MATERIALS AND METHODS

Materials

HEK (human embryonic kidney)-293 and CHO (Chinese-hamster ovary) cells from the A.T.C.C. (Manassas, VA, U.S.A.) were maintained in DMEM (Dulbecco's modified Eagle's medium)/Ham's F12 containing 5 % (v/v) fetal bovine serum and transfected with Lipofectamine™ (Invitrogen) or FuGENE™ HD (Roche) 24–48 h before use. HEK-293 cells stably expressing receptors were created by selecting for G418-resistant cells after transfection. HEK-293 cells were plated on poly-L-lysine-coated culture dishes. Rabbit polyclonal antibodies against β_2AR pSer^{355} and pSer^{356} were purchased from Santa Cruz Biotechnology. Purified mouse monoclonal antibody against the β_2AR PKA site pSer^{262} (Clone 2G3) was generously provided by Dr Richard B. Clark (University of Texas Medical School, Houston, TX, U.S.A.). Plasmid encoding arrestin3–GFP (green fluorescent protein) was provided by Dr Marc Caron (Duke University Medical Center, Durham, NC, U.S.A.) and arrestin-null mouse embryonic fibroblasts from Dr Robert Lefkowitz (Duke University Medical Center, Durham, NC, U.S.A.). Affinity-purified rabbit polyclonal antibodies against TRHR GRK phosphosites were as described previously [14]. Sources of other materials were: BD Falcon multiwell plates from BD Biosciences; isoprenaline (isoproterenol) from Sigma, propranolol from Spectrum Chemicals, TRH from Bachem; HRP (horseradish peroxidase)-linked goat anti-mouse and anti-rabbit antibodies from Bio-Rad Laboratories; and [^{3}H]inositol and [*methyl*-^{3}H]TRH from PerkinElmer Life Sciences.

DNA constructs

The human β_2AR (ADRB2) with three N-terminal HA (haemagglutinin) epitopes was obtained from Missouri S&T cDNA Resource Center. The type 1 rat TRHR tagged at the N-terminus with two HA epitopes was as described previously [16]. To create the receptor chimaeras of the β_2AR and the TRHR, a PCR stitching protocol was employed. The β_2AR–TRHR chimaera was created by amplifying β_2AR from ADRB2 using primers 5′-ATGTCGTAACAACTCCGCCCCATTG-3′ (pcDNAup) and 5′-CTTCTGCTTGCAATTGCAGCACAGAAGCTCCTGGAAGG-3′ (β_2ARΔtailreverse) and the rat TRHR C-terminus from Cys^{335} to Ile^{412} using 5′GAATGACACCTACTCAGACAATGCGAT-3′ (pcDNAdown) and 5′CCTTCCAGGAGCTTCTGTGCTGCAATTGCAAGCAGAAG-3′ (rTRHR-tailforward). The TRHR–β_2AR chimaera was generated by amplifying TRHR from Met^1–Cys^{337} using pcDNAup and 5′-GGCCTTCAAAGAAGACCTTCGCAGGCAATTGCAGAGCTTCCTG-3′ (TRHRΔtailreverse); β_2AR from Leu^{342} to Leu^{413} using pcDNAdown and 5′CAGGAAGCTCTGCAATTGCCTGCGAAGGTCTTCTTTGAAGGCC-3′ (β_2ARtailforward). All four PCR products were gel-purified and then mixed with the appropriate primers in two new PCRs to create the respective chimaeras by amplifying with both pcDNAup and pcDNAdown. Both chimaeric receptors were subcloned into pcDNA3 using HindIII and XbaI and verified by sequencing. Site-directed mutagenesis was completed using QuikChange® Lightning kits (Stratagene).

Fixed-cell ELISA assays

Cells grown on 24-well plates were rinsed once with PBS and equilibrated in 200 μl of HBSS (Hanks balanced salt solution with 20 mM Hepes) (pH 7.4) at 37°C for 20 min. Ligands were added in 100 μl of 3× concentrated solutions in HBSS. To monitor dephosphorylation, the medium was aspirated and the wells were rinsed three times with

HBSS and incubated for various times. To stop the assay, plates were put on ice and fixed with 1:1 ice-cold methanol/acetone for 5 min and air-dried. Each well was washed with PBS and incubated for 20 min with 200 μ1 of 5 % (w/v) non-fat dried skimmed milk powder in RIPA buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS and 0.5 % sodium deoxycholate). Phosphorylated receptors were probed using 1:1000 rabbit polyclonal anti-pTRHR [14], 1:500 rabbit polyclonal anti-pβ_2AR (Ser^{355}/Ser^{356}) or 1:200 mouse monoclonal anti-pβ_2AR (Ser^{262}) in 5 % (w/v) non-fat dried skimmed milk powder/RIPA for 90 min, followed by 1:5000 HRP-labelled anti-rabbit or 1:3000 HRP-labelled anti-mouse IgG for 45 min in 5 % (w/v) non-fat dried skimmed milk powder/RIPA buffer. Following washes, TMB (3,3′,5,5′-tetramethylbenzidine) ELISA substrate (Sigma) was applied for various times. The reaction was terminated with sulfuric acid, transferred to a 96-well plate and read at 450 nm. Unless noted, Ab 6959, which recognizes phosphosites between residues 355 and 365, was used to quantify phosphorylation of the TRHR. Stable cell lines were used in experiments testing PKA site phosphorylation because of high backgrounds in transiently transfected cells. Receptor expression was quantified in phosphorylation and dephosphorylation experiments on parallel wells using ELISAs to measure the N-terminal HA epitope tags on receptors. These assays confirmed that cells did not detach in any of the experimental protocols used.

Receptor internalization assay

HEK-293 cells were grown to confluence on 24-well plates. Cells were rinsed once with PBS, and incubated in 200 μl of HBSS for 20 min at 37°C. To initiate internalization, 100 μl of 3 concentrated ligand solution was added to the wells for various times. Internalization was measured by adding 1:1000 anti-HA antibody (Covance) to unpermeabilized cells to identify cell-surface receptors as described previously [17].

[^{3}H]Inositol phosphate accumulation

Stable or transfected HEK-293 cells were metabolically labelled overnight with 3 μCi/ml *myo*-[^{3}H]inositol in inositol-free DMEM/Ham's F12 with 5 % fetal bovine serum and grown to confluence on 12-well plates pre-coated with 0.001 % poly-L-lysine. Cells were treated with 10 mM LiCl with or without the indicated concentrations of TRH for 30 min at 37°C. Cells were placed on ice, washed three times with ice-cold saline, and then incubated for 30 min in 50 mM formic acid at 4°C. [^{3}H]Inositol phosphates were measured following isolation by ion-exchange chromatography [18].

[*methyl*-^{3}H]TRH binding

Binding of [*methyl*-^{3}H]TRH to live cells was measured in 12-well plates as described previously [19]. HEK-293 cells were transfected with wild-type or chimaeric TRHR. To determine the effect of arrestin on [*methyl*-^{3}H]TRH binding, mouse embryonic fibroblasts from arrestin2/3-knockout mice were transfected with receptor plus either GFP or arrestin3–GFP. After 24 h, cells were incubated for 90 min at 37°C with 0.8–25 nM [*methyl*-^{3}H]TRH in serum-free medium, washed three times with ice-cold saline and solubilized in 0.1 % SDS, and radioactivity was counted. Non-specific binding was measured identically in mock-transfected cells and subtracted. K_d values were determined by Scatchard analysis.

Luciferase reporter assay for cAMP accumulation

CHO cells were plated in 96-well CulturPlate-96 (PerkinElmer) and transiently transfected the next day with DNA encoding receptor and a cAMP-responsive luciferase (Luc) reporter plasmid, RIP1-CRE-Luc, using FuGENE™ HD. The reporter construct contains a non-palindromic CRE (cAMP-response element) from the RIP1 (rat insulin 1 gene promoter) [20]. Transfection mixes were removed from wells 24 h later and replaced by various

concentrations of isoprenaline or 20 μM forskolin in DMEM/Ham's F12 containing 0.1 % BSA for 4 h at 37°C. Agonist was removed and 100 μl of NF-Luc reagent (NanoLight Tech) was added to each well for 10 min before signal was measured in a Victor 420 multilabel plate reader (PerkinElmer). Agonist-stimulated signals were normalized to forskolin-stimulated signals. CHO cells were used in luciferase assays because they gave undetectable basal activity.

Fluorescence microscopy

HEK-293 cells were plated on coverslips and transfected with DNA encoding TRHR, β_2AR, TRHR–β_2AR or β_2AR–TRHR and arrestin3–GFP 24 h before experimentation. Coverslips were rinsed once with PBS and transferred to a microscope chamber. Cells were incubated in HBSS on a 37°C-heated stage. Arrestin3–GFP fluorescence was monitored on an Nikon epifluorescence microscope with 100× 1.3 NA (numerical aperture) objective.

Data analysis

All experiments were repeated at least three times except for the signalling curves in Figure 2(B), which were performed twice. Points represent the mean ± range or S.E.M. for two to nine determinations. Where error bars are not visible, they fell within the symbol size. Phosphorylation data were fitted for one-phase association, dephosphorylation data were fitted for one-phase exponential decay, and signalling data were fitted for sigmoidal dose–response using GraphPad Prism.

RESULTS

Characterization of chimaeric receptors

Studies were designed to examine whether the characteristics of GPCR phosphorylation and dephosphoryation are determined strictly by the amino acid sequences of the phosphorylation sites, or whether other receptor regions contribute. Towards this end, we developed C-terminal domain swaps between the type 1 rat TRHR and the human β_2AR. TRHR–β_2AR denotes the rat TRHR containing the β_2AR C-terminus (residues Leu^{342}–Leu^{413}) attached after Cys^{337} of the TRHR, which has palmitoylation sites at Cys^{335} and Cys^{337} (Figure 1A). The Leu^{342}–Leu^{413} region of the β_2AR cytoplasmic tail was shown previously to confer β_2AR properties on the V2 vasopressin receptor [13]. We also constructed the reverse chimaera, β_2AR–TRHR, which contains the region of the TRHR tail from Cys^{335} to Ile^{412} attached to the first 341 amino acids of β_2AR (Figure 1B). Cys^{335}–Ile^{412} of the TRHR was shown previously to confer desensitization on the mammalian gonadotropin-releasing hormone receptor, which lacks a natural cytoplasmic tail [21].

The TRHR–β_2AR chimaera functionally coupled to $G_{q/11}$ as was evident from its ability to generate inositol phosphates in response to TRH (Figure 1C). The chimaera was slightly less effective at coupling to $G_{q/11}$ than the wild-type TRHR with a 5-fold higher EC_{50} (Table 1). This difference was explained by a reduced affinity for [*methyl*-^{3}H]TRH in the chimaera (Figures 1E and 1F). The β_2AR–TRHR chimaera demonstrated competent G_s-coupling as was evident by the isoprenaline-dependent stimulation of cAMP generation (Figure 1D). The β_2AR–TRHR chimaera exhibited an EC_{50} for cAMP production 3-fold lower than that for wild-type β_2AR (Table 1). These results prove that both chimaeric receptors bind ligand and signal to G-proteins normally.

Characterization of β_2AR phosphorylation using phospho-ELISA protocols

To characterize phosphorylation of these chimaeric receptors, we developed ELISAs to quantify phosphorylation of β_2AR using phosphosite-specific antibodies against $pSer^{355}$/$pSer^{356}$ in the cytoplasmic tail (GRK sites) and $pSer^{262}$ in the third intracellular loop (PKA

site) [10,22]. In HEK-293 cells transiently transfected with β_2AR, isoprenaline stimulated strong GRK site phosphorylation within 5 min (Figure 2A). A β_2AR containing mutations at the GRK sites (S355A and S356A) showed no ligand-dependent phosphorylation at these sites; the two receptors were expressed at comparable levels (Figures 2A and 2B). Multiple GRK and PKA sites in the β_2AR are phosphorylated in response to agonist, and mutation of all of these sites impairs receptor desensitization [23,24]. Previous work establishes Ser^{355} and Ser^{356} as the major sites of GRK phosphorylation in HEK-293 cells, and mutation of these residues in a PKA site-null receptor abolishes receptor desensitization [25,26].

Isoprenaline-stimulated PKA site phosphorylation was inhibited 80 % by H89, a PKA inhibitor (Figure 2C). Phosphorylation of the PKA site was nearly saturated at 1 nM isoprenaline, whereas maximal phosphorylation at the GRK sites required 1 μM or higher concentrations (Figure 2D). The EC_{50} for isoprenaline was approx. 200-fold lower for phosphorylation at the PKA site than at the GRK site (Table 1), in complete agreement with the difference reported previously for adrenaline (epinephrine) stimulation [22].

Characterization of β_2AR–TRHR and TRHR–β_2AR phosphorylation

The chimaeric β_2AR–TRHR exhibited strong phosphorylation at the PKA site in the third intracellular loop in response to isoprenaline (Figure 3A). This was not surprising, since isoprenaline stimulated a robust increase in cAMP. The EC_{50} for phosphorylation measured at 5 min, 0.64 nM, was similar to the EC_{50} for cAMP generation measured at 4 h, 0.16 nM (Table 1). To quantify phosphorylation of the TRHR tail, we used several well-characterized phosphosite-specific antibodies that identify phosphorylation at the major phosphosite at Thr^{364} (Ab 6959) and at several weaker sites downstream (Abs 5025, 5211 and 5213). In contrast with the phosphorylation seen at the PKA site in the third intracellular loop of the β_2AR–TRHR, the tail of the chimaeric receptor was inefficiently phosphorylated at all GRK sites in the TRHR C-terminus (Figure 3B). The same defect in GRK phosphorylation was found when the Cys^{341} palmitoylation site at the original splice junction was removed or when the splice junction was placed right after the NPXXY motif of the β_2AR (results not shown).

Results were different for the chimaeric receptor with the β_2AR tail fused to the TRHR. The wild-type TRHR underwent strong phosphorylation at GRK sites in the cytoplasmic tail (Figure 3C). The TRHR–β_2AR chimaera demonstrated strong TRH-dependent phosphorylation of the β AR GRK sites Ser^{355} and Ser^{356}, and this response occurred at the same concentrations of TRH as phosphorylation at natural sites in the TRHR tail. The EC_{50} values for TRH stimulation of TRHR tail and the β_2AR tail were 8 and 7 nM respectively (Figure 3C and Table 1).

Phosphorylation of the cytoplasmic tail of the TRHR appears to be independent of the C-terminus

In agreement with published results [10,14,22], we found that phosphorylation of GRK sites in the TRHR tail took place much more rapidly than phosphorylation of GRK sites in the β_2AR tail ($t_{\frac{1}{2}}$=0.20 and 1.0 min respectively) when agonists were added at saturating concentrations (Figures 4A and 4B, and Table 1). Interestingly, the TRHR–β_2AR chimaera achieved half-maximal phosphorylation 0.3 min after addition of TRH (Figure 4C and Table 1), typical of TRHR phosphorylation. The rate of TRHR phosphorylation therefore appears to be independent of the sequence of the cytoplasmic tail of the receptor.

PKA site phosphorylation of β_2AR appears to be independent of the C-terminus

Low concentrations of isoprenaline (1 nM) caused a slow, but sustained, increase in phosphorylation of the β_2AR at the PKA site in the third intracellular loop, whereas 1 μM

isoprenaline evoked rapid, but transient, phosphorylation (closed symbols in Figures 5A and 5B). At high concentrations of isoprenaline, the rates of phosphorylation of β_2AR at GRK and PKA sites were similar (Table 1), but never approached the rapid rates of phosphorylation exhibited by the TRHR. As expected, phosphorylation of the β_2AR at GRK sites required higher concentrations of agonist than did phosphorylation at PKA sites (Figures 5A and 5B).

The defect in GRK site phosphorylation of the β_2AR–TRHR chimaera could not be overcome by high concentrations of isoprenaline after 1 h of stimulation (Figure 5D). At the PKA site, 1 nM isoprenaline caused slow phosphorylation of the β_2AR–TRHR that was maintained for at least 1 h, whereas 1 μM isoprenaline led to rapid, but transient, phosphorylation (Figures 5C and 5D). Thus the phosphorylation patterns of the β_2AR–TRHR at the PKA site are the same as those of the β_2AR (Table 1) and apparently independent of the sequence of the C-terminus.

Internalization and arrestin binding of β_2AR, TRHR and chimaeric receptors

Most phosphorylation on the β_2AR induced by low concentrations of isoprenaline occurs at the PKA site [27]. To determine whether PKA site phosphorylation is sufficient to drive β_2AR and β_2AR–TRHR endocytosis, we measured the amount of receptor on the plasma membrane at various times after addition of 1 nM or 1 μM isoprenaline (Figures 6A and 6B). Both the wild-type β_2AR (Figure 6A) and the β_2AR–TRHR (Figure 6B) displayed low, but significant, internalization at 1 nM isoprenaline, which induces much stronger phosphorylation at the PKA site than at the Ser^{355}/Ser^{365} GRK site of the natural β_2AR tail or the GRK sites in the TRHR tail of the chimaeric receptor. Both receptors exhibited 2-fold more internalization in response to 1 μM isoprenaline, which also stimulates GRK site phosphorylation in the β_2AR. In agreement with published studies [26], we found that the S355A/S356A mutant of the β_2AR internalized about half as well as the wild-type receptor in response to 1 μM isoprenaline (results not shown), similar to the extent of internalization attributed to PKA site phosphorylation. The β_2AR–TRHR chimaera was internalized more effectively at the higher concentration of agonist, even though it was not detectably phosphorylated at GRK sites in its cytoplasmic tail.

As shown above, the cytoplasmic tails of both the TRHR and TRHR–β_2AR become phosphorylated in response to TRH, although the phosphosites have completely different amino acid sequences. We took advantage of this to ask how the composition of the tail affects internalization. When stimulated with hormone, both receptors underwent significant endocytosis with 60 % of the TRHR and 40 % of the TRHR–β_2AR internalized after 1 h (Figures 6C and 6D).

We examined the interaction of receptors with arrestin directly by monitoring the localization of arrestin3–GFP in live cells. Micrographs taken at 0, 5 and 30 min after agonist addition are shown in Figure 7; 5 min points were selected because dephosphorylation rates were measured at this time, as shown below. All receptors recruited arrestin3–GFP to the membrane, visible as the formation of puncta at or near the plasma membrane within a few minutes. On the basis of surface ELISAs, the relative concentrations of the receptors on the plasma membrane were 1.0 for TRHR, 0.73 for β_2AR, 0.82 for TRHR–β_2AR, and 0.49 for β_2AR–TRHR. Despite the similar concentrations of the various receptors and their similar internalization patterns (Figure 6), the TRHR appeared to recruit arrestin3–GFP much more robustly than the TRHR–β_2AR, β_2AR or β_2AR–TRHR. The TRHR also demonstrated sustained association with arrestin3– GFP following internalization, indicative of a class B receptor. The β_2AR exhibited class A behaviour, recruiting arrestin3–GFP to the surface, but internalizing without it. Arrestin association did not strictly correlate with phosphorylation when tail domains were exchanged. The presence

of the β_2AR C-terminal tail in the TRHR–β_2AR chimaera converted the receptor into a typical class A GPCR, i.e. the TRHR–β_2AR chimaera recruited arrestin3–GFP but did not internalize with it (Figure 7). This suggests that the phosphorylated TRHR tail is necessary for the class B behaviour of the TRHR.

Bound arrestin increases the affinity of the TRHR and other GPCRs for agonist [15,19,28]. As a further test of the ability of arrestin to interact with the chimaeric TRHR–β_2AR,weexpressed the TRHR and TRHR–β_2AR with or without arrestin in arrestin-null mouse embryo fibroblasts and carried out equilibrium [*methyl*-^{3}H]TRH-binding studies in live cells (Figure 1F). In the presence of arrestin, both receptors bound with apparent K_d values close to those measured in HEK-293 cells, which express endogenous arrestin. As reported previously [15,19], in the absence of arrestin, the affinity of TRHR was much lower. Arrestin had only a small effect on the affinity of the chimaeric receptor, confirming microscopic data suggesting that arrestin binds less avidly to the activated TRHR–β_2AR despite good phosphorylation of the tail.

The behaviour of the β_2AR–TRHR chimaera appeared to be intermediate between class A and class B. Addition of the TRHR tail to the β_2AR was insufficient to cause the strong sustained association with arrestin seen with the TRHR, but some arrestin3–GFP was visible in puncta in cells expressing the β_2AR–TRHR chimaera (Figure 7), even though the cytoplasmic tail was poorly phosphorylated.

Dephosphorylation of TRHR and TRHR–β_2AR appears independent of the C-terminus

The rate of TRHR dephosphorylation decreases as the receptor undergoes endocytosis following hormone binding [14]; conversely, the rate of β_2AR dephosphorylation does not change during the first 20 min of isoprenaline treatment [29]. To assess whether these properties are determined by the C-terminal tails, we measured dephosphorylation rates at the GRK sites of the β_2AR, TRHR and TRHR–β_2AR following agonist removal after 5 min, when most receptors remained on the plasma membrane, or after 1 h, when much of the receptor was internalized. For all three receptors, the amount of phosphorylated receptor was about the about same after exposure to ligand for 5 or 60 min (see Figures 4A–4C). In agreement with previous studies, we found that the rate of β_2AR GRK site dephosphorylation was the same after 5 min or 1 h of agonist treatment (Figure 4D). In contrast, the rate of TRHR and TRHR–β_2AR dephosphorylation decreased by half if hormone was removed after 1 h compared with 5 min (Figures 4E and 4F). The TRHR–β_2AR was dephosphorylated more slowly than the TRHR or the β_2AR (Table 1). This establishes that it is some other feature of the receptor and not simply the sequence of the phosphorylated cytoplasmic tail that controls the dephosphorylation rate.

DISCUSSION

The novel finding of the present study is that regions outside the C-terminal domains of the TRHR and β_2AR set the rate of GRK-mediated phosphorylation, the rate of dephosphorylation and the dependence of dephosphoryation on subcellular localization. In contrast, and in agreement with previous studies, we found that sequences in the C-terminal domains of TRHR and β_2AR are important for interactions with arrestin.

The speed of agonist-dependent phosphorylation of TRHR by GRKs is exceptional for a GPCR. GRK phosphorylation of the TRHR proceeds faster than either second messenger-or GRK-mediated phosphorylation of the β_2AR. Despite the fact that GRKs catalyse phosphorylation at sites in the cytoplasmic tails of these receptors, their activity rates appear to be determined by other regions of the receptor. It is uncertain whether the differences in phosphorylation of the two receptors are due to distinct GRK subtypes acting on each or

different extents of activation of the same kinase. There is considerable evidence that both GRK2 and GRK5 are capable of phosphorylating the TRHR and β_2AR [14,30], but the precise roles of individual kinases are not known and probably depend on the cell context, the receptor and perhaps the ligand. One potential explanation for the rapid phosphorylation exhibited by the TRHR and the TRHR–β_2AR chimaera is that both receptors couple to G_q and activate PKC (protein kinase C). PKC potentiates the activity of GRK2 [31] by increasing recruitment of the kinase to the plasma membrane [32]. This seems unlikely to play a major role in GRK-dependent phosphorylation of TRHR and TRHR–β_2AR, however, because PKC inhibition has little effect on agonist-dependent TRHR phosphorylation [14].

The results suggesting that the helical domains or intracellular loops of the TRHR are critical for rapid phosphorylation by GRKs agree with previous work demonstrating that a C-terminally truncated rhodopsin mutant increases the activity of rhodopsin kinase toward a peptide substrate [33]. Intracellular loops I and II of rhodopsin have been shown to interact directly with rhodopsin kinase [34], and peptides from intracellular loops of the β_2AR have been shown to inhibit GRK2 activity [3]. We reported recently that positively charged residues in the helix 8 region of the TRHR, between the seventh transmembrane domain and palmitoylation sites, are important for GRK activation [17]. Crystallographic structures of rhodopsin kinase and several other GRKs are available and have led to models in which the kinases dock with GPCRs in a manner that provides for a substantial gap between the plane of the membrane and the kinase domain. Conceptual models allow for an extended C-terminal domain of the docked or an adjacent receptor or a peptide substrate to fit in the active site of the enzyme [35-37]. The substrate flexibility proposed in such models helps support our results suggesting that the C-terminal domain sequence is not critical for the rate of phosphorylation.

A specific serine cluster identified within the C-terminus of the vasopressin V2 receptor undergoes GRK phosphorylation and mediates prolonged association with arrestin during receptor endocytosis [13]. The prolonged association with arrestin appears to prevent dephosphorylation following agonist removal, and this property can be imparted on the β_2AR by switching tail domains [13]. The TRHR possesses multiple serine and threonine residues that are potential GRK phosphorylation sites, but these are not clustered in the same manner as those in the V2 receptor. We have reported previously that serine and threonine residues between residues 355 and 365 of the TRHR tail are key for high-affinity arrestin binding [14]. TRHR binds arrestin strongly enough to internalize with it, but unlike the V2 receptor, the TRHR is rapidly dephosphorylated when hormone is removed.

In the present study, we showed that the phosphorylated TRHR co-internalizes with arrestin while the phosphorylated TRHR–β_2AR chimaera does not. This result suggests that the amino acid sequence of the phosphorylated region of the receptor, and perhaps the number of phosphorylated residues, are important determinants of the strength of the arrestin-binding interaction. This conclusion is consistent with previous studies emphasizing the importance of the cytoplasmic tail of a GPCR for arrestin binding [13]. One important caveat in interpreting our studies is that we only measured phosphorylation of the β_2AR tail at Ser^{355}/Ser^{356}; we would not have detected phosphorylation at other sites. Although Ser^{355} and Ser^{356} are the major sites of GRK phosphorylation in HEK-293 cells [25,26], multiple sites in the β_2AR can undergo phosphorylation in response to agonist [23,24].

Some GPCRs that can bind arrestin nonetheless internalize via arrestin-independent pathways [38-40], but the β_2AR and TRHR internalize predominantly through arrestin-dependent mechanisms [15,39]. Multiple phosphorylation sites are required for strong arrestin binding to rhodopsin [41,42], and most rhodopsin-like GPCRs must undergo activation and phosphorylation at several sites to bind arrestin tightly [4]. Both the TRHR

and β_2AR have been found to require phosphorylation at several sites for full agonist-mediated internalization and short-term desensitization [14,23,26]. Given these considerations, the simplest explanation for our results is that a multiply phosphorylated TRHR tail is responsible for high-affinity arrestin binding and a class B internalization profile.

The rate of dephosphorylation of both the TRHR and TRHR–β_2AR decreased at longer times of agonist exposure. After 5 min, both receptors were associated with arrestin, and after 30 min both receptors had internalized, but only the TRHR had internalized with arrestin (Figure 7). This suggests that the dependence of receptor dephosphorylation on the time of agonist exposure is not due to arrestin association, but is more likely to be due to subcellular localization. This conclusion agrees with previous studies showing that the TRHR is dephosphorylated at the same rate whether arrestin is present or not [15], but more rapidly when internalization is blocked [14]. The β_2AR can also undergo dephosphorylation when clathrin-mediated internalization is inhibited [10], and its dephosphorylation rate at the GRK sites remains remarkably stable over 1 h of agonist exposure (Figure 4D). Although the TRHR, β_2AR and chimaeric receptors are maximally internalized after 1 h, the receptors may be localized distinctly, accounting for different rates of dephosphorylation. In fact, a mutant β_2AR lacking ubiquitination sites experiences both a change in subcellular localization away from lysosomes and an accelerated dephosphorylation compared with wild-type [43].

The stability of PKA site phosphorylation of β_2AR is inversely correlated with the concentration of isoprenaline used to activate it [10]. PKA site phosphorylation is stable at low concentrations of isoprenaline that do not stimulate GRK site phosphorylation. It has been proposed that arrestin association triggered by GRK phosphorylation at high concentrations of isoprenaline prevents continued access of PKA to the receptor, leading to the decline in phosphorylation [10]. The β_2AR–TRHR chimaera exhibits the same inverse relationship between isoprenaline concentration and PKA phosphorylation in the absence of much GRK site phosphorylation, however. An alternative explanation for the loss of PKA phosphorylation at high isoprenaline concentrations is that PKA activity decreases as the β_2AR switches from G_s- to G_i-coupled pathways in a PKA-dependent manner [44]. Phosphatases have been shown to associate constitutively with some receptors through scaffolds such as A-kinase-anchoring proteins [45]. The possibility remains that phosphatase activity towards the β_2AR PKA site is somehow enhanced at high agonist concentrations via a scaffolding protein.

In summary, using a combination of phosphosite-specific antibodies and an ELISA-based platform for monitoring receptor phosphorylation, we were able to analyse relatively modest differences in the regulation of two distinct GPCRs and to differentiate PKA- and GRK-dependent phosphorylation of the well-studied β_2AR. The kinetics and concentration-dependence of phosphorylation and dephosphorylation of the β_2AR and TRHR were in strong agreement with published results. Chimaeric receptors provided new insight into the interactions of receptors with GRKs, arrestin and phosphatases. Whereas arrestin binding by the β_2AR/TRHR chimaeras was strongly influenced by the sequence of the phosphorylated C-terminus, the rates of GRK phosphorylation and GRK site dephosphorylation were independent of the actual sequence of the phosphorylation sites in the cytoplasmic tails, but dependent upon other regions of the receptors. This is concordant with data indicating that there are multiple contacts between a GPCR and a GRK outside of the active-site region [36,37]. Although the phosphatases responsible for TRHR and β_2AR dephosphorylation have not been identified and their mode of activation is unknown, our data imply that GPCR phosphatases, like GRKs, interact in a complex manner with different regions of the substrate.

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Abbreviations used

β_2AR	β_2-adrenergic receptor
CHO	Chinese-hamster ovary
DMEM	Dulbecco's modified Eagle's medium
GFP	green fluorescent protein
GPCR	G-protein-coupled receptor
GRK	GPCR kinase
HA	haemagglutinin
HBSS	Hanks balanced salt solution with 20 mM Hepes
HEK	human embryonic kidney
HRP	horseradish peroxidase
PKA	protein kinase A
PKC	protein kinase C
PP	protein phosphatase
TRH	thyrotropin-releasing hormone
TRHR	TRH receptor

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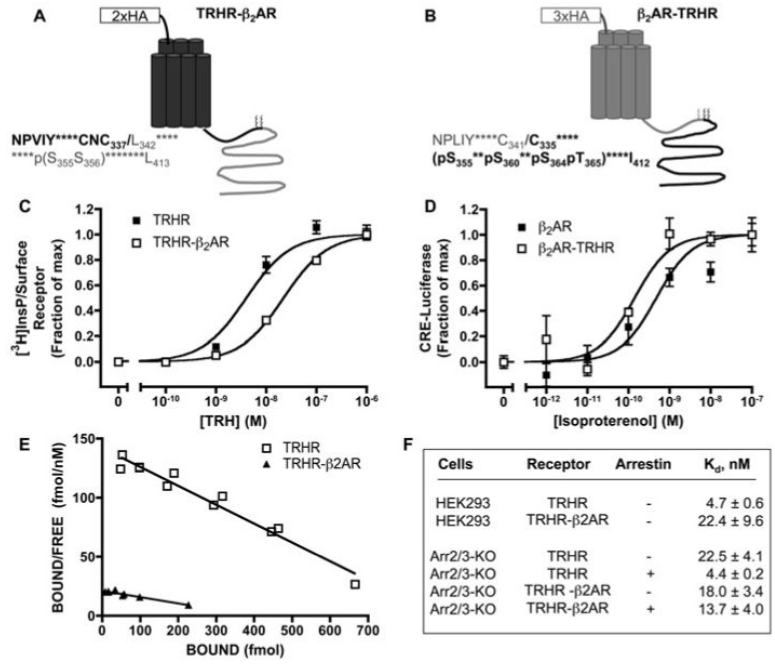

Figure 1. Characterization of receptor chimaera signalling

Schematic representation of the (**A**) TRHR–β_2AR and (**B**) β_2AR–TRHR chimaeras. (**C**) HEK-293 cells were transiently transfected with DNA encoding TRHR or TRHR–β_2AR. Cells were metabolically labelled overnight with [^{3}H]inositol and incubated with 10 mM LiCl and the indicated concentrations of TRH for 30 min when total [^{3}H]inositol phosphates ([^{3}H]Ins*P*) were quantified. Surface receptor levels were measured by ELISA using antibody against receptor N-terminal HA epitopes. [^{3}H]Ins*P* production was normalized to surface receptor expression. (**D**) CHO cells were transiently transfected with DNA encoding β_2AR or β_2AR–TRHR and a plasmid encoding RIP1-CRE-Luc [19], a cAMP-responsive promoter. Cells were treated with 20 μM forskolin or with the indicated concentrations of isoprenaline (isoproterenol) in vehicle (1 mM thiourea and 0.1 mM ascorbic acid, pH 7) or with vehicle alone for 4 h when luciferase activity was measured. Luciferase activity was normalized to the activity with forskolin. (**E**) Scatchard plot and (**F**) K_d values for equilibrium binding of [*methyl*-^{3}H]TRH to HEK-293 cells transfected with TRHR or TRHR–β_2AR or to arrestin-null mouse embryo fibroblasts (Arr2/3-KO) transfected with TRHR or TRHR–β_2AR with or without arrestin3–GFP. Results are means ± range or S.E.M. for two to four determinations.

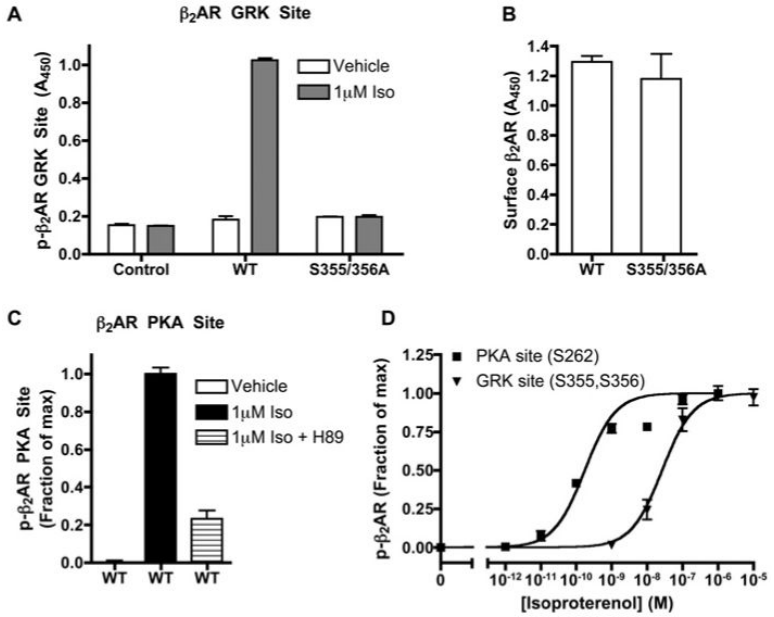

Figure 2. Characterization of β_2 AR phosphorylation

(**A** and **B**) HEK-293 cells were transiently transfected with control DNA or DNA encoding wild-type β_2AR (WT) or a mutant β_2AR harbouring mutations S355A/S356A. (**A**) Cells were stimulated with 1 μM isoprenaline (Iso) or vehicle alone for 5 min and then fixed; phosphorylated receptors were measured using a p-β_2AR (Ser^{355}/Ser^{356}) antibody specific for GRK sites. (**B**) Surface receptor expression was measured in unpermeabilized cells. (**C**) HEK-293 cells stably expressing β_2AR were incubated for 5 min with vehicle or isoprenaline (Iso) as shown. PKA site phosphorylation was quantified using an antibody against $pSer^{262}$ of the β_2AR. To test the effects of PKA inhibition, cells were pre-incubated with 10 μM H89 or DMSO for 30 min before and during the 5 min incubation with 1 μM isoprenaline. H89 has been reported to act as a β_2AR antagonist, but the antagonism caused by 10 μM H89 is largely overcome at 1 μM isoprenaline [46]. (**D**) HEK-293 cells stably expressing β_2AR were incubated with various concentrations of isoprenaline (isoproterenol) for 5 min when the PKA site and GRK site phosphorylation were measured.

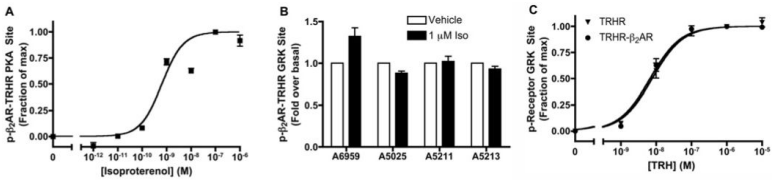

Figure 3. Characterization of receptor chimaera phosphorylation

(**A**) HEK-293 cells stably expressing β_2AR–TRHR chimaeras were incubated for 5 min with various concentrations of isoprenaline (isoproterenol). (**B**) HEK-293 cells stably expressing the β_2AR–TRHR chimaera were incubated for 5 min with or without 1 μM isoprenaline (Iso) and then assayed for phosphorylation using a panel of antibodies recognizing different phosphorylated regions of the TRHR C-terminus. (**C**) HEK-293 cells stably expressing the TRHR or TRHR–β_2AR chimaera were incubated with the indicated concentrations of TRH for 5 min and then fixed; phosphorylation at GRK sites in the TRHR or β_2AR tails was measured as described in the Materials and methods section.

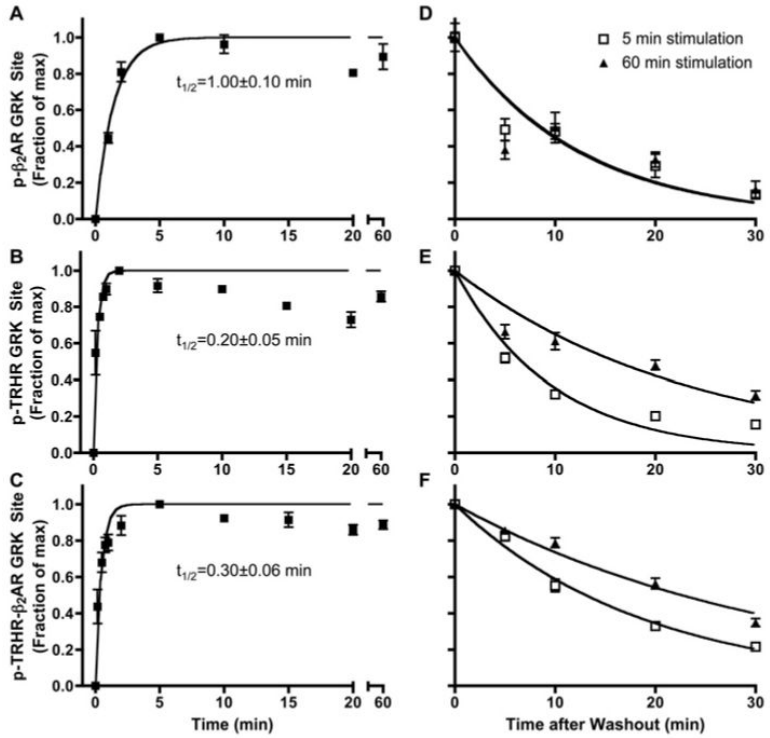

Figure 4. Kinetics of receptor GRK site phosphorylation and dephosphorylation

HEK-293 cells stably expressing β_2AR (**A** and **D**), TRHR (**B** and **E**) or TRHR–β_2AR (**C** and **F**) were used. (**A**–**C**) Cells were treated for various times with either (**A**) 1 μM isoprenaline or (**B** and **C**) 100 nM TRH and then fixed and permeabilized to measure phosphorylation. (**D**) Cells were incubated with 1 μM isoprenaline for 5 or 60 min and then washed three times and incubated for the indicated times with 1 μM propranolol before assessing phosphorylation at GRK sites. (**E** and **F**) Cells were incubated with 100 nM TRH for 5 or 60 min and then washed three times and incubated for the indicated times in buffer alone before measuring phosphorylation at GRK sites in the (**E**) TRHR or (**F**) β_2AR tails.

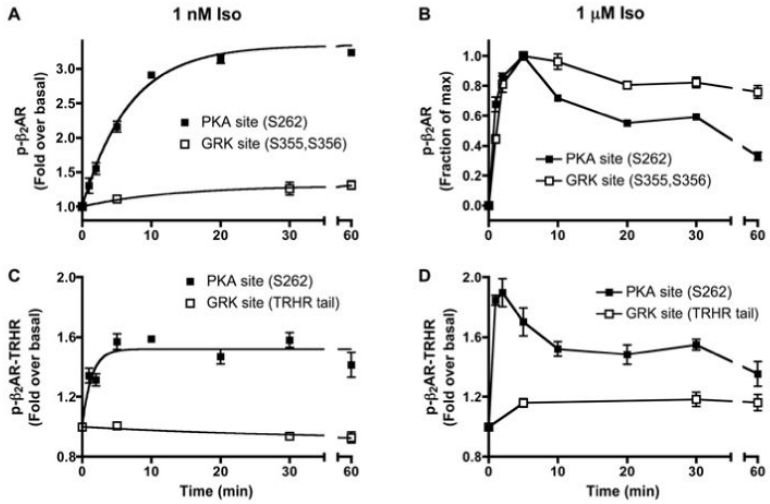

Figure 5. Kinetics of receptor PKA site phosphorylation

HEK-293 cells stably expressing β_2AR (**A** and **B**) or β_2AR–TRHR (**C** and **D**) were incubated with 1 nM or 1 μM isoprenaline (Iso), as shown, for the times indicated when phosphorylation at both the PKA and GRK sites was measured.

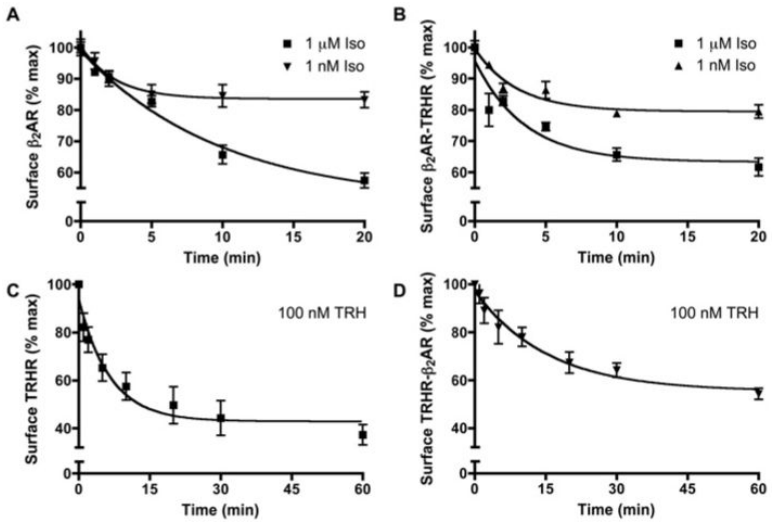

Figure 6. Internalization of receptors in response to low or high concentrations of isoprenaline

HEK-293 cells stably expressing (**A**) β_2AR, (**B**) β_2AR–TRHR, (**C**) TRHR or (**D**) TRHR–β_2AR were treated with (**A** and **B**) 1 nM or 1 μM isoprenaline (Iso) or (**C** and **D**) 100 nM TRH for the indicated times. Unpermeabilized cells were probed with an anti-HA antibody to assess surface receptor expression.

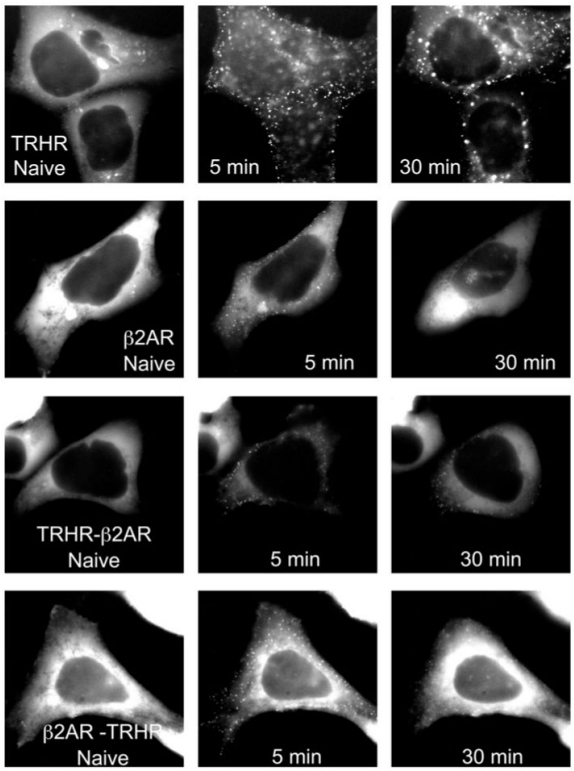

Figure 7. Arrestin3–GFP recruitment to membranes in response to agonist

HEK-293 cells were transiently transfected with DNA encoding receptor and arrestin3–GFP in a 3:1 ratio. Cells were treated with either 100 nM TRH or 1 μM isoprenaline for the indicated times at 37°C and arrestin3–GFP localization was monitored in live cells.

Table 1

Characterization of receptor phosphorylation and dephosphorylation

Results from Figures 1-4 were analysed as described in the Materials and methods section, and are means ± S.E.M. NA, not applicable.

Receptor	EC_{50} (signalling) (nM)	EC_{50} (GRK site phosphorylation) (nM)	EC_{50} (PKA site phosphorylation) (nM)	GRK site phosphorylation rate ($t_{\frac{1}{2}}$) (min)	PKAsite phosphorylation rate ($t_{\frac{1}{2}}$) (min)		GRKsite dephosphorylation rate ($t_{\frac{1}{2}}$) (min)	
					1 nM Isoprenaline	1 μM Isoprenaline	5 min agonist	60 min agonist
TRHR	4.6 ± 1.0	8.3 ± 1.9	NA	0.20 ± 0.05	NA	NA	6.8 ± 0.8	16.2 ± 1.9
TRHR–β_2AR	23.0 ± 2.0	7.0 ± 1.2	NA	0.30 ± 0.06	NA	NA	13.1 ± 1.0	22.9 ± 1.9
β_2AR	0.46 ± 0.09	40 ± 11	0.19 ± 0.04	1.0 ± 0.1	4.7 ± 0.3	0.70 ± 0.10	8.6 ± 1.4	8.8 ± 1.9
β_2AR–TRHR	0.16 ± 0.05	NA	0.64 ± 0.04	NA	0.90 ± 0.01	0.40 ± 0.10	NA	NA