ARRESTIN BINDS TO DIFFERENT PHOSPHORYLATED REGIONS OF THE TRH RECEPTOR WITH DISTINCT FUNCTIONAL CONSEQUENCES

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Abstract

Arrestin binding to agonist-occupied phosphorylated G protein-coupled receptors typically increases the affinity of agonist binding, increases resistance of receptor-bound agonist to removal with high acid/salt buffer, and leads to receptor desensitization and internalization. We tested whether TRH receptors lacking phosphosites in the C-terminal tail could form stable and functional complexes with arrestin. Fibroblasts from mice lacking arrestins 2 and 3 were used to distinguish between arrestin-dependent and -independent effects. Arrestin did not promote internalization or desensitization of a receptor that had key Ser/Thr phosphosites mutated to Ala (4Ala receptor). Nevertheless, arrestin greatly increased acid/salt resistance and the affinity of 4Ala receptor for TRH. Truncation of 4Ala receptor just distal to the key phosphosites (4AlaStop receptor) abolished arrestin-dependent acid/salt resistance but not the effect of arrestin on agonist affinity. Arrestin formed stable complexes with activated wildtype and 4Ala receptors, but not with 4AlaStop receptor, as measured by translocation of arrestin-GFP to the plasma membrane or chemical cross-linking. An arrestin mutant that does not interact with clathrin and AP2 did not internalize receptor but still promoted high affinity TRH binding, acid/salt resistance, and desensitization. A sterically restricted arrestin mutant did not cause receptor internalization or desensitization, but did promote acid/salt resistance and high agonist affinity. The results demonstrate that arrestin binds to proximal or distal phosphosites in the receptor tail. Arrestin binding at either site causes increased agonist affinity and acid/salt resistance, but only the proximal phosphosites evoke the necessary conformational changes in arrestin for receptor desensitization and internalization.

INTRODUCTION

G protein-coupled receptors (GPCRs)[1] compose the largest family of cell-surface proteins and transduce a multiplicity of extracellular signals across the plasma membrane. Their signaling and localization are regulated, in part, by a much smaller and structurally conserved family of cytosolic proteins known as arrestins (Ferguson, 2001; Moore et al., 2007). Receptor binding by arrestins is enhanced several fold when the receptor is both agonist-bound (i.e. "active") and phosphorylated (Gurevich and Gurevich, 2006). Vertebrates express four arrestins: two are confined to rods or cones, and two, known as arrestins 2 and 3 (or β-arrestin 1 and 2, respectively) are ubiquitously expressed. How only two arrestins can regulate the very diverse family of GPCRs is an ongoing question.

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Arrestins were originally identified as proteins that stop signaling by binding receptors. Numerous proteins that bind arrestin, in addition to GPCRs, have since been identified, revealing arrestin as a signaling scaffold and not only a steric inhibitor of receptor-G protein binding (DeWire et al., 2007). Several GPCRs activate extracellular signal-regulated kinase 1/2 (ERK1/2) via arrestin, and arrestin 2 has recently been shown to translocate to the nucleus where it regulates gene expression (reviewed in (DeWire et al., 2007)). Additionally, direct interaction between arrestin and clathrin and AP-2 is required for internalization of many GPCRs (Ferguson, 2001; Moore et al., 2007), and arrestin-dependent ubiquitination is necessary for normal post-endocytic degradation of receptors (Shenoy, 2007).

While arrestin is often required for GPCR desensitization, internalization, and extracellular signal-regulated kinase activation, it is now clear that some receptors require arrestin for only a subset of these behaviors. For example, arrestin is required for protease-activated receptor 1 to desensitize but not to internalize (Paing et al., 2002), whereas arrestin is dispensable for uncoupling N-formyl peptide receptor from G protein but not for receptor recycling to the plasma membrane (Bennett et al., 2001; Vines et al., 2003). Indeed, as reviewed by Gurevich and Gurevich (2006), numerous combinations of arrestin-dependence and -independence have been described for GPCRs, making it clear that the consequences of arrestin binding are not "all or nothing."

The type 1 thyrotropin-releasing hormone (TRH) receptor is expressed in the anterior pituitary, where it controls synthesis and secretion of thyrotropin. When bound to TRH, the TRH receptor activates $G\alpha_{q/11}$, leading to the production of inositol 1,4,5-trisphosphate and diacylglycerol by phospholipase Cβ. Downstream signaling includes the release of calcium from internal stores and the activation of protein kinase C. Through the use of phosphosite-specific antibodies and site-directed mutagenesis, we previously defined a region in the TRH receptor C-terminal tail that is phosphorylated in response to agonist binding and is essential for receptor internalization and desensitization (Jones et al., 2007). Because arrestin is important for TRH receptor desensitization and internalization (Jones and Hinkle, 2005), we hypothesized that receptors lacking these key phosphosites would be defective in other arrestin-dependent behaviors due to an overall inability to bind arrestin. We co-expressed TRH receptors with or without arrestins in fibroblasts from mice lacking both arrestins 2 and 3 (Arr2/3KO MEFs) to distinguish between arrestin-dependent and -independent effects. Contrary to our expectation, we report that a mutant receptor lacking key phosphosites remains coupled to G protein even though it recruits and stably interacts with arrestin. We also provide evidence that desensitization and internalization require a conformational change in the arrestin molecule that is induced by specific receptor-bound phosphates.

MATERIALS AND METHODS

Cell Culture and Transfection

Mouse embryo fibroblasts (MEFs) lacking arrestins 2 and 3 were from Dr. Robert Lefkowitz (Duke University, Durham, NC). HEK293 cells were from ATCC (Manassas, VA). Cells were grown in DMEM/F12, 5% FBS (Invitrogen, Carlsbad, CA). MEFs were transfected with LipofectAMINE (Invitrogen, Carlsbad, CA) and HEK293 cells with FuGene HD (Roche Diagnostics, Indianapolis, IN) following manfacturers' instructions. Plasmid encoding arrestin 2 ΔLIELD/F391A was from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA); arrestin 3-GFP was from Dr. Marc Caron (Duke University, Durham, NC); all other arrestin-encoding plasmids were based on arrestin 3 and obtained from Dr. Vsevolod Gurevich (Vanderbilt University, Nashville, TN). All arrestins were bovine except intact Flag-tagged arrestins, which were rat. N-terminal hemagglutinin (HA)-tagged TRH receptors were described previously (Jones et al., 2007). When tested, expression levels of wildtype and 4Ala receptors were found to be similar and not affected by co-expression with different arrestins,

as determined by labeling cell-surface receptors with anti-HA antibody or total receptor on western blot, whereas surface expression of 4AlaStop receptor was typically 50–70% that of wildtype (data not shown).

TRH Receptor Internalization

MEFs expressing TRH receptors with two N-terminal HA tags were treated with or without 100 nM TRH for up to 30 min and fixed with 3% paraformaldehyde. Fixed cells were incubated with 1:1000 monoclonal anti-HA antibody (Covance, Berkeley, CA) in 5% goat serum in PBS without detergent to label only receptors remaining at the surface, then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad, Hercules, CA) and BM Blue POD Substrate (Roche). The colorimetric reaction was terminated with 5% sulfuric acid and the absorbance at 450 nm was measured. Internalization was defined as the percent of receptor lost from the surface after addition of TRH. Background obtained without antibody or in untransfected cells was normally less than 10% of total signal and was subtracted.

Arrestin Translocation

Arr2/3KO MEFs grown on glass coverslips were transiently transfected with TRH receptor and arrestin-3-GFP. Cells were placed in Hank's Balanced Salt Solution, 15 mM HEPES, pH 7.4 at room temperature and imaged before and after addition of 1 μM TRH. GFP was detected by excitation with a 488-nm argon laser, 543-nm bandpass emission filter, on a Nikon C1 visible light laser scanning confocal microscope with a 60x (1.4 NA) oil immersion objective. All images were processed identically using Metamorph Imaging Software (Molecular Devices, Downingtown, PA).

Co-immunoprecipitation and immunoblotting

HEK293 cells in 6 cm culture dishes were transiently transfected with HA-tagged TRH receptor and FLAG-tagged arrestin, incubated in HBSS, 15 mM HEPES, pH 7.4 at room temperature, and stimulated with or without 1 μM TRH for 2 min. Proteins were cross-linked with 2 mM dithiobis(succinimidyl)propionate (Pierce, Rockford, IL), 30% DMSO for 30 min at room temperature. HEK293 cells were used because the transfection efficiency of Arr2/3KO MEFs was low and the MEFs did not produce enough protein for Western blot. The reaction was quenched by washing with 20 mM Tris, 500 mM NaCl, pH 7.4 and cells were lysed on ice in 1 ml RIPA buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 10 mM NaF, 100 nM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, pH 8.0) plus 1:1000 protease inhibitor cocktail III (Calbiochem, La Jolla, CA). Lysates were vortexed twice and centrifuged at $10,000 \times g$ for 10 min at 4 °C. Receptor complexes were immunoprecipitated from the supernatant with 1:5000 anti-HA antibody and 20 μl protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA). Complexes were denatured and cross-linker was cleaved by boiling in LDS sample buffer (Invitrogen) plus 100 mM dithiothreitol and proteins were resolved by SDS-PAGE on PAGEr Gold precast gels (Lonza, Rockland, ME). FLAG-arrestin was immunoblotted with 1:5000 M2 anti-FLAG antibody (Sigma, St. Louis, MO) and 1:1000 TrueBlot anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (eBioscience, San Diego, CA), which does not recognize denatured IgG heavy chain that runs near FLAG-arrestin (~50 kDa), followed by chemiluminescence detection with Western Lightning (PerkinElmer, Waltham, MA). Gels were subsequently incubated in 0.1% sodium azide and reprobed for HA-TRH receptor using 1:5000 anti-HA primary antibody and 1:5000 goat anti-mouse secondary antibody (Bio-Rad) to control for differences in receptor expression and gel loading. Lysates from untreated cells were also resolved by SDS-PAGE to normalize differences in arrestin expression. Densitometry was performed using Scion Image (Scion, Frederick, MD). For immunoblotting, cell lysates were resolved on 10% gels and blotted with polyclonal antibodies against cyclophilin B (1:10,000) or arrestin (Ab2913, 1:500), both from

Abcam (Cambridge, MA). The amino acid sequence recognized by the arrestin antibody is identical in mouse, rat and bovine arrestins but differs between arrestins 2 and arrestin 3.

Other

Measurement of inositol phosphate production, specifically-bound [^{3}H]MeTRH in acid/salt-resistant form, and affinity for [^{3}H]MeTRH by Scatchard analysis were performed as described (Jones and Hinkle, 2005). [^{3}H]MeTRH at 0.625 to 25 nM was used for Scatchard analysis. All experiments were performed at least three times, unless noted, and error bars show mean ± S.E. of triplicate determinations. Some error bars fell within symbol size. Differences were considered significant at $p < 0.05$ or 0.01, determined by one- or two-way analysis of variance and *post hoc* Tukey's test or Student's unpaired *t* test, as appropriate.

RESULTS

TRH Receptor Signaling

Arrestins sterically inhibit GPCR signaling through G proteins by binding to the phosphorylated receptor. Several studies indicate that this involves a conformational change in the arrestin molecule (Gurevich and Gurevich, 2006; Nobles et al., 2007) whereby its N- and C-domains close around the receptor like a clamp. A 12-residue "hinge" region links the N- and C-domains of arrestin; deletion of seven amino acids in the hinge (ΔHinge) inhibits its flexibility, greatly diminishing binding to receptors (Vishnivetskiy et al., 2002) but not to other proteins, such as microtubules (Hanson et al., 2006), JNK3, and Mdm2 (Song et al., 2006). We tested whether ΔHinge arrestin could inhibit receptor signaling through G proteins by co-expressing receptor and arrestin in Arr2/3KO MEFs and measuring total inositol phosphate production in cells metabolically labeled with [^{3}H]inositol. ΔHinge arrestin was unable to desensitize TRH receptor, while wildtype arrestin caused a dramatic decrease in inositol phosphate production (Fig. 1A). Arrestin was not required for TRH stimulation of extracellular signal-regulated kinase at 5 or 35 min in Arr2/3KO MEFs (data not shown).

We previously identified phosphosites in the TRH receptor C-terminal tail that are required for internalization and desensitization (Jones et al., 2007). Mutation of these sites to Ala (4Ala receptor) reduces overall phosphorylation of the receptor by half. Truncation of 4Ala receptor just distal to this region (4AlaStop receptor) removes an additional 14 potential phosphosites, including several phosphorylated in response to TRH (Jones et al., 2007). 4Ala and 4AlaStop receptors were not desensitized by wildtype or ΔHinge arrestins (Fig. 1A and data not shown and (Jones et al., 2007)). 4Ala receptors were desensitized, however, by an arrestin mutant, R169E, that binds activated receptors even if pthey are not phosphorylated (Fig. 1A). This indicates that the desensitization defect of the 4Ala receptor is due primarily to the absence of key phospho-Ser/Thr residues. R169E arrestin did not desensitize the 4AlaStop receptor, which is expected to have no arrestin binding sites in the cytoplasmic tail (Fig. 1A). Transfected arrestins were expressed at concentrations similar to those found for endogenous arrestins in wildtype MEFs (Fig. 1B).

TRH Receptor Internalization

The C-terminal tail of receptor-bound arrestin interacts with clathrin and AP2, thus recruiting the receptor to clathrin-coated pits in the plasma membrane. We previously reported that an arrestin that lacks clathrin and AP2 binding sites, ΔLIELD/F391A arrestin, does not promote receptor endocytosis even though it effectively desensitizes the receptor (Jones and Hinkle, 2005). We asked whether a conformational change in the hinge region of arrestin is required for receptor endocytosis by expressing receptor and ΔHinge arrestin in Arr2/3KO MEFs. Cells were stimulated for various times with TRH and receptor remaining on the surface was quantified by ELISA using an antibody against an N-terminal (extracellular) epitope on the

receptor. A substantial fraction of TRH receptor was internalized in cells co-transfected with receptor and wildtype arrestin, but not in cells co-transfected with ΔLIELD/F391A arrestin, ΔHinge arrestin, or vector control (Fig. 2).

Acid/salt Resistance

Ligand bound to receptors at the plasma membrane is often subject to removal with acid/salt buffer, whereas internalized ligand is protected, and formation of an acid/salt-resistant complex has therefore been used as a measure of receptor internalization (Bohm et al., 1997; Drmota and Milligan, 2000; Hunyady et al., 1994; Innamorati et al., 1998; Kalatskaya et al., 2004; McGraw and Maxfield, 1990; Prossnitz et al., 1999). Because ΔLIELD/F391A and ΔHinge arrestins do not support receptor internalization, we expected them to be deficient in promoting acid/salt resistance. Surprisingly, both arrestin mutants increased acid/salt resistance in Arr2/3KO MEFs co-transfected with TRH receptor (Fig. 3A). Our results clearly indicate that acid/salt-resistant ligand is not necessarily internalized.

The two concave domains of arrestin differ in sequence and function, but both domains contain receptor-binding elements: the N domain houses important phosphate-binding elements whereas the C domain acts as the activation sensor (Hanson and Gurevich, 2006). The two domains of ΔHinge arrestin cannot "clamp" around the receptor, suggesting that perhaps only one of the arrestin domains needs to interact with the receptor to promote high affinity binding (described below) and acid/salt resistance. We expressed the N and C domains of arrestin either separately or together in Arr2/3KO MEFs to ask if either domain alone could bind to the TRH receptor. When expressed together, the domains behaved essentially like wildtype arrestin. Neither domain on its own, however, had altered ligand binding or acid/salt resistance (Fig. 3B and C). The data suggest that, despite steric restraints, both domains of ΔHinge arrestin interact with the receptor. The Flag-tagged N- and C-domains were expressed at high levels, particularly when co-expressed, relative to Flag-tagged full-length arrestins 2 and 3 (Fig. 3F).

Neither 4Ala receptor nor 4AlaStop receptor is desensitized by arrestin (Fig. 1A) or internalized in response to TRH (Jones et al., 2007), although the internalization defect of 4Ala receptor is partially overcome when arrestin is overexpressed (data not shown). We therefore expected that these mutant receptors would not form acid/salt-resistant complexes. While 4AlaStop receptor behaved as predicted, 4Ala receptor showed very strong acid/salt resistance, albeit slightly reduced in comparison to wildtype receptor (Fig. 3D).

Some GPCRs bind preferentially to arrestin 3 and others, including the TRH receptor, bind equally to arrestin 2 and 3 (Oakley et al., 2000). Because there is precedent for the arrestin binding preferences of a receptor changing after mutation of potential phosphosites (Qiu et al., 2007), we asked whether the phosphosite substitutions in the 4Ala and 4AlaStop receptors caused either to prefer one of the arrestins. We co-expressed wildtype or mutant receptor with wildtype arrestin 2 or 3 and measured acid/salt resistance and found that none of the receptors showed a strong preference for either arrestin. The slightly reduced acid/salt resistance with the 4Ala receptor was seen whether it was co-expressed with arrestin 2 or arrestin 3 (Fig. 3E). 4AlaStop receptor did not exhibit acid-salt resistance regardless of co-expression of arrestin 2 or 3 (Fig. 3D and data not shown).

Receptor-ligand Affinity

Arrestin preferentially binds to phosphorylated, agonist-bound receptors, thereby stabilizing the interaction between receptor and ligand (Gurevich et al., 1997). We examined the possibility that acid/salt resistance results simply from the formation a high affinity agonist-receptor complex. We measured the affinity of TRH receptors for radioligand by Scatchard analysis in Arr2/3KO MEFs expressing receptor with or without arrestin. Wildtype, ΔLIELD/

F391A, and ΔHinge arrestins all substantially increased the affinity of the wildtype receptor for TRH (Table 1). The affinities of wildtype and 4Ala receptors were identical in the absence of arrestin, and arrestin caused the same ~13-fold increase in affinity when co-expressed with either receptor. This result shows that acid/salt resistance is not merely a reflection of enhanced affinity for ligand.

Interestingly, wildtype arrestin increased the affinity of the 4AlaStop receptor 4.9-fold even though arrestin could not rescue this receptor's inability to form an acid-resistant complex with TRH (Fig. 3B). R169E arrestin, which binds even in the absence receptor phosphorylation, was slightly more effective (Table 1). The N- and C-domains of arrestin had little or no effect on agonist affinity when expressed alone, but promoted high affinity binding as effectively as wildtype arrestin when expressed together (Table 1).

Arrestin Translocation and Co-immunoprecipitation

To visualize arrestin recruitment to the plasma membrane, we co-expressed arrestin-GFP with wildtype TRH receptor in Arr2/3KO MEFs. Arrestin rapidly moved to the plasma membrane in response to TRH (Fig. 4B). 4Ala receptor also recruited arrestin (Fig. 4D), but 4AlaStop receptor did not (Fig. 4F).

To measure TRH-induced receptor clustering, we exposed cells to 100 nM TRH for one minute, then added antibody to an N-terminal receptor epitope to label cell surface receptors exclusively and examined cells using immunofluorescence microscopy. Receptor clustering was not detected in the absence of arrestin. In the presence of arrestin, strong clustering of wildtype receptors, less intense clustering of 4Ala receptors, and negligible clustering of 4AlaStop receptors was observed (data not shown). Receptor aggregation therefore follows the same pattern as recruitment of arrestin-GFP (Fig. 4).

To monitor arrestin-receptor interaction biochemically, we transfected cells with HA-tagged TRH receptors and FLAG-tagged arrestins and co-immunoprecipitated after chemically cross-linking proteins. Wildtype arrestin co-immunoprecipitated with wildtype receptor in cells incubated with TRH, but less arrestin was recovered with 4Ala receptor (Fig. 5A and B). We saw no co-immunoprecipitation of arrestin with 4AlaStop receptor, consistent with this receptor's failure to recruit arrestin to the plasma membrane (Fig. 4F).

Because ΔHinge arrestin increased receptor affinity for ligand and acid/salt resistance, we asked whether this arrestin could also be co-immunoprecipitated with wildtype receptor. While ΔHinge arrestin co-immunoprecipitated with wildtype receptor, the amount was reduced in comparison to wildtype arrestin (Fig. 5C and D). In summary, 4AlaStop receptor is severely deficient in recruiting and binding to arrestin, whereas the 4Ala receptor forms intermediately stable complexes. Likewise, ΔHinge arrestin-receptor binding is reduced, but not abolished, in comparison to wildtype arrestin.

DISCUSSION

Arrestins recognize and interact with a large family of cell-surface receptors. Despite the apparent flexibility of the arrestin-receptor complex, certain features are believed to be universal: arrestins bind preferentially to the agonist-bound form of receptors; arrestins undergoes conformational changes that are essential for high affinity binding when they bind to negative charges on receptors, usually in the form of phosphate groups attached to Ser or Thr residues; and arrestins block signaling through G proteins when they bind. Our report challenges this paradigm, because we identified two situations where receptors that clearly bound to arrestins were not uncoupled from G protein signaling. To our knowledge, this is the

first report of a receptor that binds arrestin but does not desensitize, something previously thought impossible.

G protein uncoupling is believed to precede internalization of most GPCRs (Ferguson, 2001). We previously showed that TRH receptor desensitization is dependent on arrestin binding but not endocytosis, because receptor expressed with ΔLIELD/F391A arrestin does not internalize but desensitizes normally (Jones and Hinkle, 2005). Nevertheless, desensitization and internalization were strongly correlated for every TRH receptor that we expressed with wildtype arrestin (Table 2), suggesting that desensitization and internalization of the TRH receptor require a similar—if not identical—interaction with arrestin. Conversely, over-expression of dominant negative GRK2 results in a normal rate of β_2-adrenergic receptor internalization but decreased desensitization, indicating that different phosphosites regulate the two events (Kong et al., 1994). Furthermore, mutation or deletion of key phosphosites in the m2 muscarinic, CB1 cannabinoid, n-formyl peptide, B$_2$ bradykinin, complement 5a, and μ-opioid receptors leads to receptors that internalize but have reduced desensitization (Blaukat et al., 2001; Celver et al., 2004; Christophe et al., 2000; Jin et al., 1999; Maestes et al., 1999; Pals-Rylaarsdam et al., 1995).

Although there is no crystal structure of an arrestin-receptor or G protein-receptor complex, the fact that G proteins and arrestins share some of the same receptor interaction sites suggests that arrestin inhibits G protein activation at least in part by direct competition. Two regions on receptors are crucial for both G protein and arrestin binding: first, the conserved Asp/Glu-Arg-Tyr motif, which is required for normal G protein activation, is also necessary for arrestin binding (Marion et al., 2006); second, a cavity between helices on the receptor's cytoplasmic side opens upon agonist binding, whereupon part of arrestin or Gα is inserted (Gurevich and Gurevich, 2006). Receptors are stabilized in an active conformation when elements from either Gα or arrestin bind in the transmembrane helix cavity.

By stabilizing a receptor's active conformation, arrestin increases its affinity for agonist (Gurevich et al., 1997; Jorgensen et al., 2005; Key et al., 2001; Key et al., 2003; Martini et al., 2002). As shown in Table 1, arrestin caused a profound increase in agonist affinity for all of the TRH receptors studied. This was predictable for the wildtype and 4Ala receptors, because both receptors recruit arrestin-GFP to the plasma membrane and co-immunoprecipitate chemically cross-linked arrestin. The ability of arrestin to increase agonist affinity of the 4AlaStop receptor was unexpected, because no interaction between this receptor and arrestin could be detected.

Arrestin is thought to sequentially probe, bind, and stabilize relevant elements of the receptor's active conformation, and to undergo structural changes in its final receptor-bound state (Gurevich and Gurevich, 2004). In the case of the n-formyl peptide receptor, stepwise interactions with arrestin result in the formation of a ternary complex of ligand, receptor, and arrestin with high agonist affinity (Key et al., 2003). Key et al. proposed that the receptor first binds to arrestin through an activation-dependent binding site and through proximal phosphosites on the receptor tail, causing the release of the arrestin C-tail that otherwise constrains arrestin in an "inactive" conformation. With its C-tail released, arrestin is able to bind distal phosphosites on the receptor C-tail, inducing additional conformational changes in arrestin that stabilize receptor-ligand binding. Thus, high affinity ligand binding comes last in a series of interactions between n-formyl peptide receptor and arrestin. Our results with mutant receptors reveal a very different affinity spectrum, because the initial, weak interaction between arrestin and 4AlaStop receptor was sufficient to promote high receptor affinity for agonist but none of the other effects of arrestin (Table 2). Alternatively, arrestin may act indirectly to regulate receptor affinity.

Recent work has shown that the affinity of phosphorylated rhodopsin for arrestin increases dramatically with the number of phosphates on rhodopsin (Vishnivetskiy et al., 2007). Phosphosites in both proximal (355-365) and distal (370-412) regions of the TRH receptor tail are sufficient for strong arrestin binding, as shown by the ability of both 4Ala receptor (no proximal sites) and receptor truncated at residue 370 (no distal sites) to translocate arrestin-GFP and undergo chemical cross-linking to arrestin (Figs. 4 and 5 and data not shown). A more complete understanding of the role of arrestin will require future studies capable of quantifying the avidity of different arrestin-receptor interactions.

Even though distal sites in the TRH receptor are phosphorylated (Jones et al., 2007), only the proximal sites enable arrestin-dependent desensitization. Mutating the phosphosites in the proximal 355-365 region of intact or truncated receptor produced receptors that failed to undergo arrestin-mediated uncoupling or receptor endocytosis. Likewise, an arrestin mutant (ΔHinge) strongly increased agonist affinity but was completely ineffective at desensitizing or internalizing receptor (Table 2). ΔHinge arrestin is intact except for its inability to undergo phosphoreceptor-induced conformational changes. Our results suggest that the structural rearrangements that require flexibility in the hinge region of arrestin are not required to stabilize receptor-ligand binding but are essential to prevent interaction with G protein and expose AP2 and clathrin binding sites in arrestin. Incompletely phosphorylated receptors either lack the necessary phosphosites or bind arrestin in an alternate conformation that is unable to evoke all of the normal changes in arrestin. In effect, binding of wildtype arrestin to 4Ala receptor or ΔHinge arrestin to wildtype receptor "stalls" in its early stages, resulting in increased ligand affinity and acid/salt resistance but not desensitization or internalization (Fig. 6).

Development of acid/salt resistance and receptor endocytosis are distinct, since two arrestin mutants, ΔLIELD/F391A and ΔHinge, which were completely ineffective at sequestering receptor, produced a substantial increase in acid/salt resistance. Acid/salt resistance does not simply reflect enhanced agonist affinity, because overexpression of wildtype arrestin did not increase acid/salt resistance of TRH bound to 4AlaStop receptor but did increase ligand affinity ~5-fold. We conclude that phosphosites in either the proximal (355-365) or distal (370-412) half of the receptor's C-terminal tail are essential for acid/salt resistance but not for high agonist affinity (Table 2).

In addition to extending our understanding of TRH receptor-arrestin interaction, our report yields insight into how arrestins interact with GPCRs in general. Arrestin-receptor binding proceeds in a stepwise manner (Gurevich and Benovic, 1993; Gurevich and Gurevich, 2004) involving dramatic conformational changes in arrestin (Nobles et al., 2007; Vishnivetskiy et al., 2002) and possibly in the receptor as well (Kisselev et al., 2004). Our data demonstrate that each stage in this process has distinct functional consequences for the TRH receptor. The processive nature of arrestin-receptor interaction helps to explain why certain GPCRs bind arrestin but fail to display all of the canonical arrestin-dependent behaviors.

Acknowledgements

List of nonstandard abbreviations

TRH

thyrotropin-releasing hormone

GPCR

G protein-coupled receptor

HA

hemagglutinin

Arr2/3KO MEFs

mouse embryo fibroblasts from arrestin 2 and 3 knockout animals

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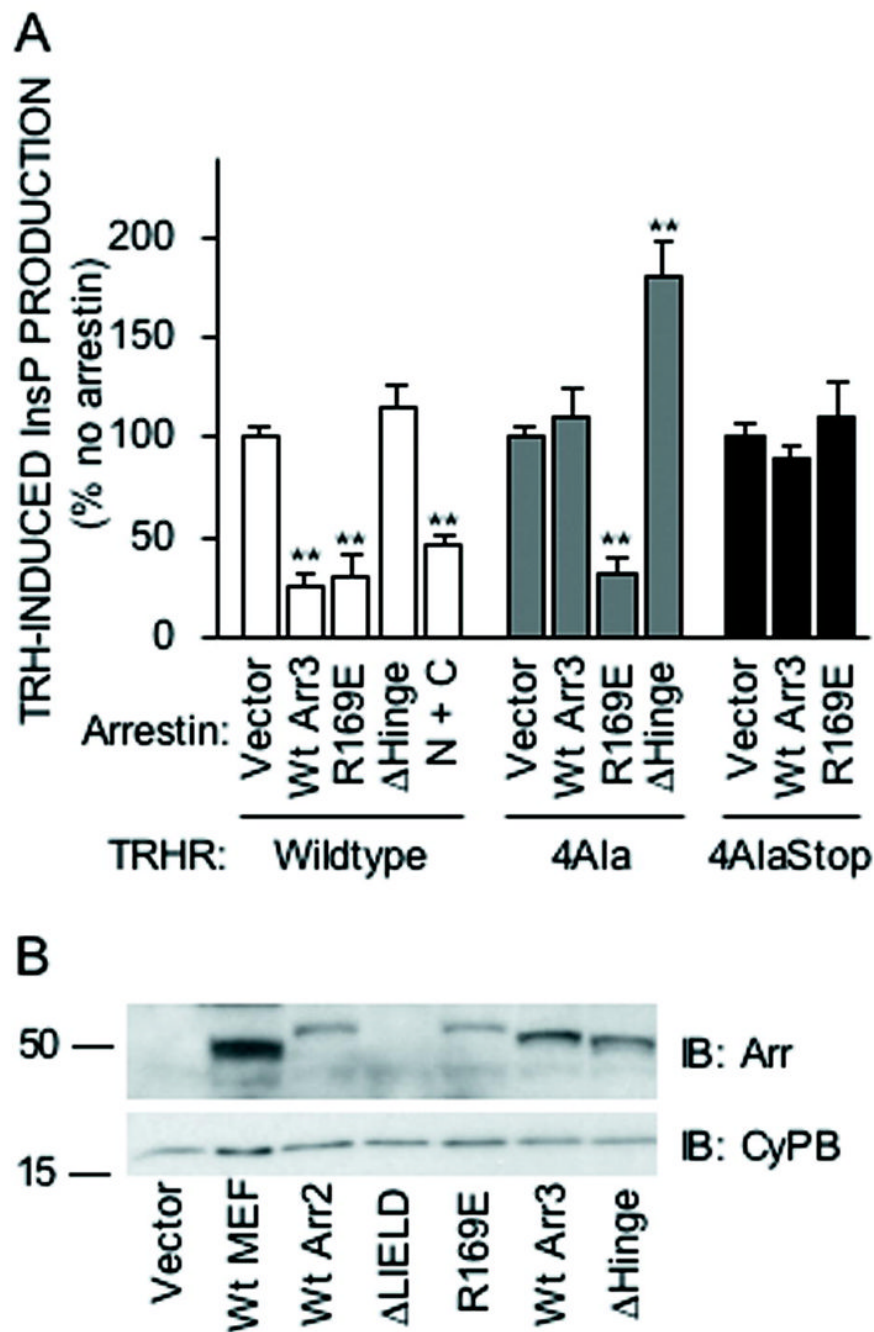
Fig. 1.
Arrestin desensitizes TRH receptor signaling. *A*, Arr2/3KO MEFs were co-transfected with TRH receptors and arrestins or empty vector, labeled with [^{3}H]inositol overnight, and incubated for 30 min with 10 mM LiCl with or without 1 μM TRH when total [^{3}H]InsPs were measured. Shown are TRH-induced InsPs as a percentage of InsPs with receptor plus vector. Arrestin expression did not alter basal InsP formation and counts with either mutant receptor plus vector were within 5% of those for wildtype receptor plus vector. **, $p < 0.01$ *versus* receptor plus vector. *B*, Cell lysates from cells transfected as in *A (lanes 1, 3–7)*, or from MEFs from wildtype animals endogenously expressing arrestins 2 and 3 (lane 2), were separated by SDS-PAGE and immunoblotted with antibodies against arrestin 2 and 3 (*top*) or cyclophilin

B (*bottom*). The arrestin antibody recognizes residues in the mutated region of arrestin ΔLIELD/F391A.
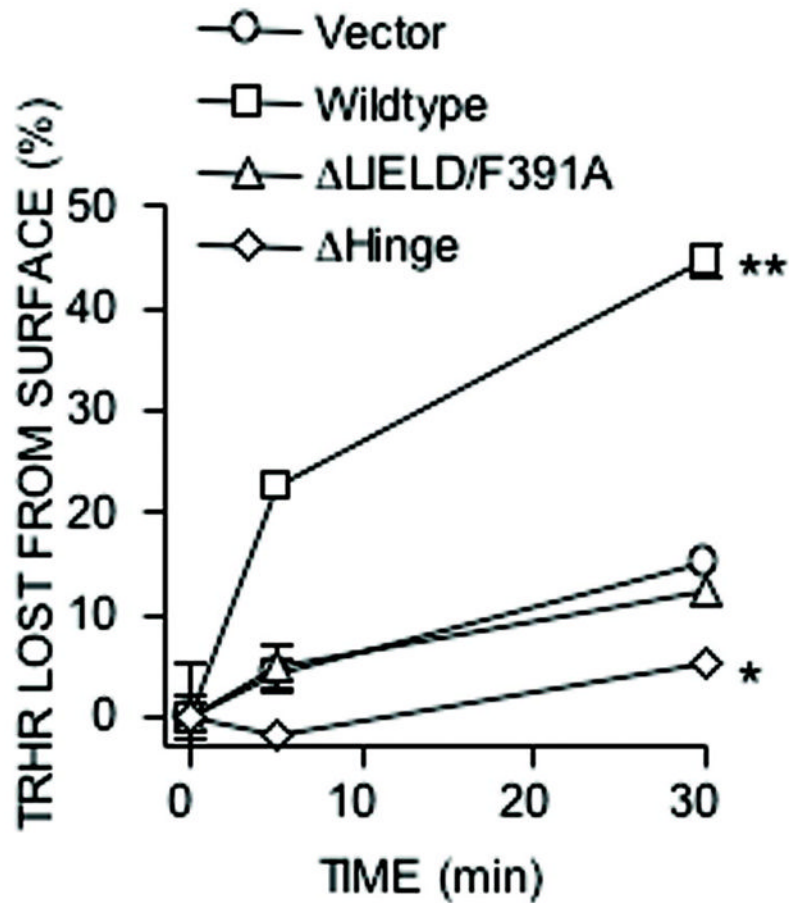
Fig. 2.
TRH receptor internalization is arrestin-dependent. Arr2/3KO MEFs were co-transfected with TRH receptor, arrestins or vector control as shown. Cells were incubated with or without 100 nM TRH and surface receptor quantified by ELISA against an N-terminal epitope on the receptor. Shown is percent loss of receptor from the surface after addition of TRH. Where not visible, error bars (mean ± range of duplicate determinations) are within symbol size. Arrestin co-expression did not significantly alter receptor number. *, $p < 0.05$ and **, $p < 0.01$, time course significantly different *versus* vector control by two-way ANOVA.
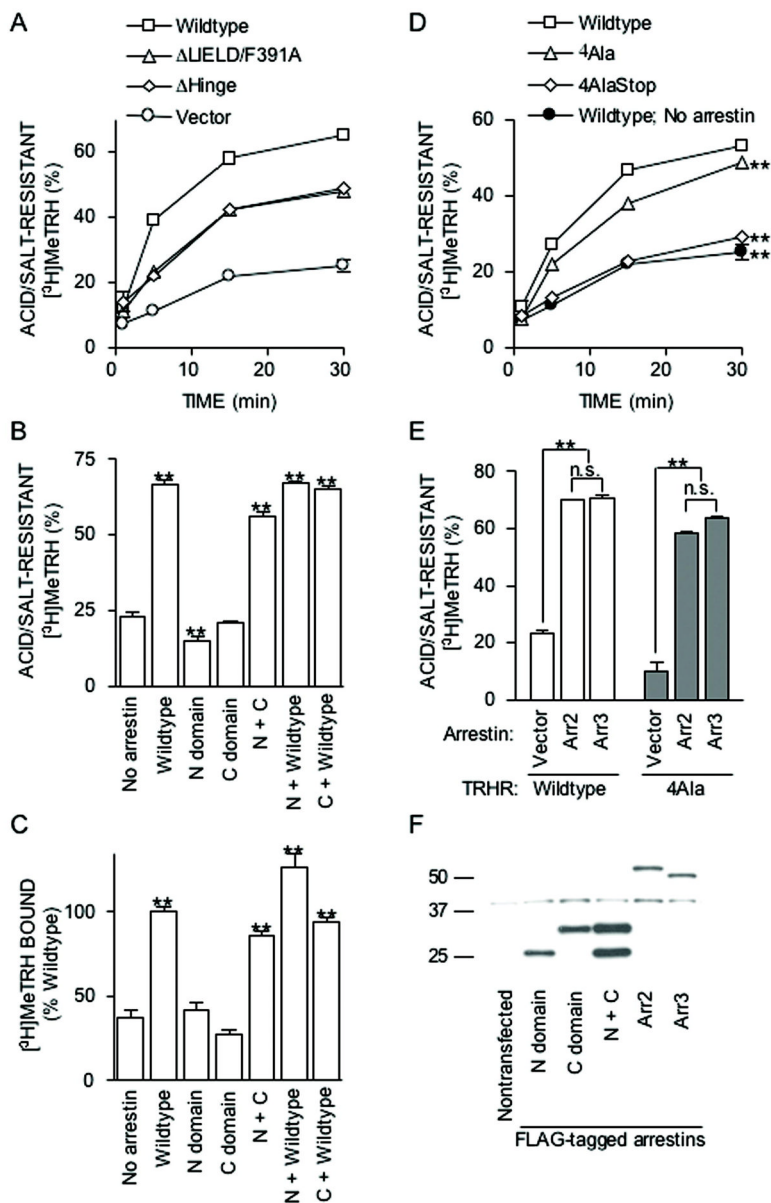
Fig. 3.
Mutant arrestins promote resistance of receptor-bound agonist to removal with acid/salt buffer. Arr2/3KO MEFs were transiently transfected and incubated with 5 nM [^{3}H]MeTRH for 1–30 min. Shown in *A–B* and *D–E* is percent of specifically-bound radioligand in acid/salt-resistant form and in *C* total radioligand binding. Where not visible, error bars are within symbol size. Cells were transfected with TRH receptors, arrestins and/or arrestin N and/or C domains, or vector as shown. Arrestins used in panels B, C and F were FLAG-tagged. *A*, Differences were significant ($p < 0.01$, two-way ANOVA) between wildtype and mutant arrestins and also between all arrestins and vector control. *B* and *C*, **, $p < 0.01$ *versus* no arrestin. *D*, **, $p < 0.01$, time course significantly different *versus* wildtype receptor plus arrestin by two-way ANOVA. *E*, **, $p < 0.01$. *F*, Cell lysates were separated by SDS-PAGE and immunoblotted with antibody against the C-terminal FLAG epitope on each arrestin.
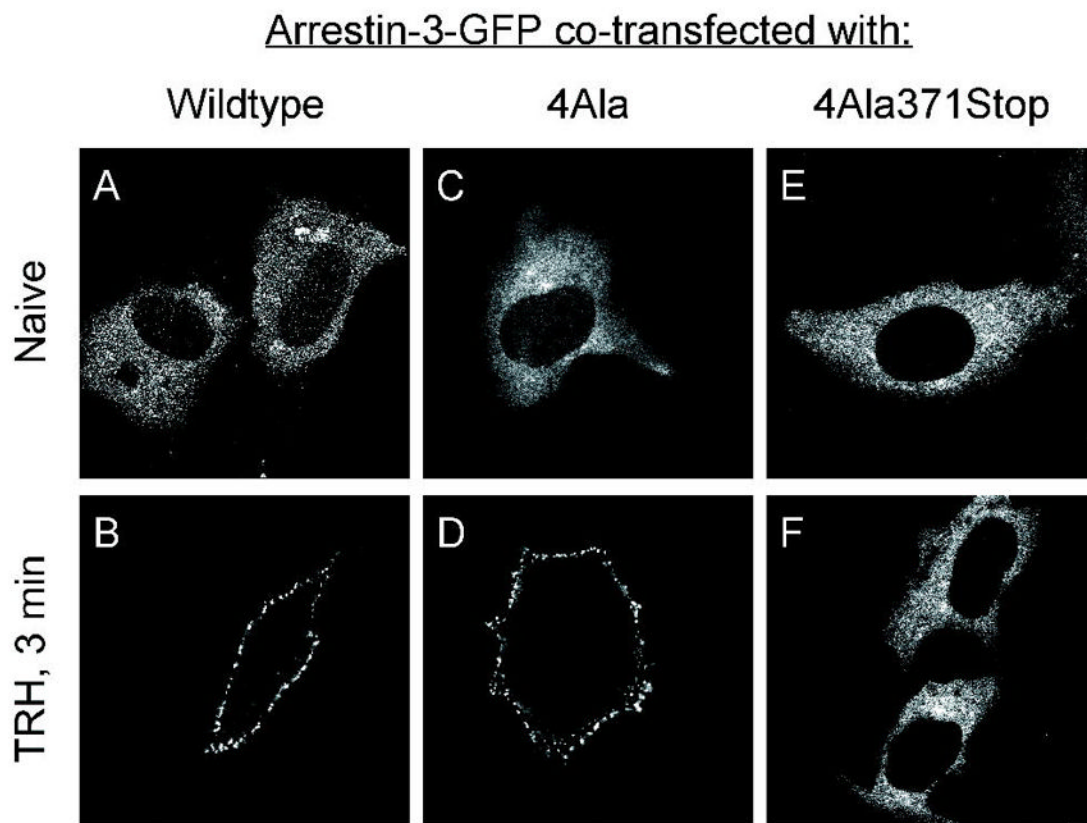
Arrestin-3-GFP co-transfected with:

Fig. 4.
TRH receptor recruits arrestin to the plasma membrane in response to TRH. Arr2/3KO MEFs were co-transfected with TRH receptors and arrestin-3-GFP and imaged by confocal microscopy before and after addition of 1 μM TRH for 3 min. Shown are typical cells from at 3 to 6 separate experiments. Arrestin-GFP did not move if TRH receptor was not co-expressed (data not shown).
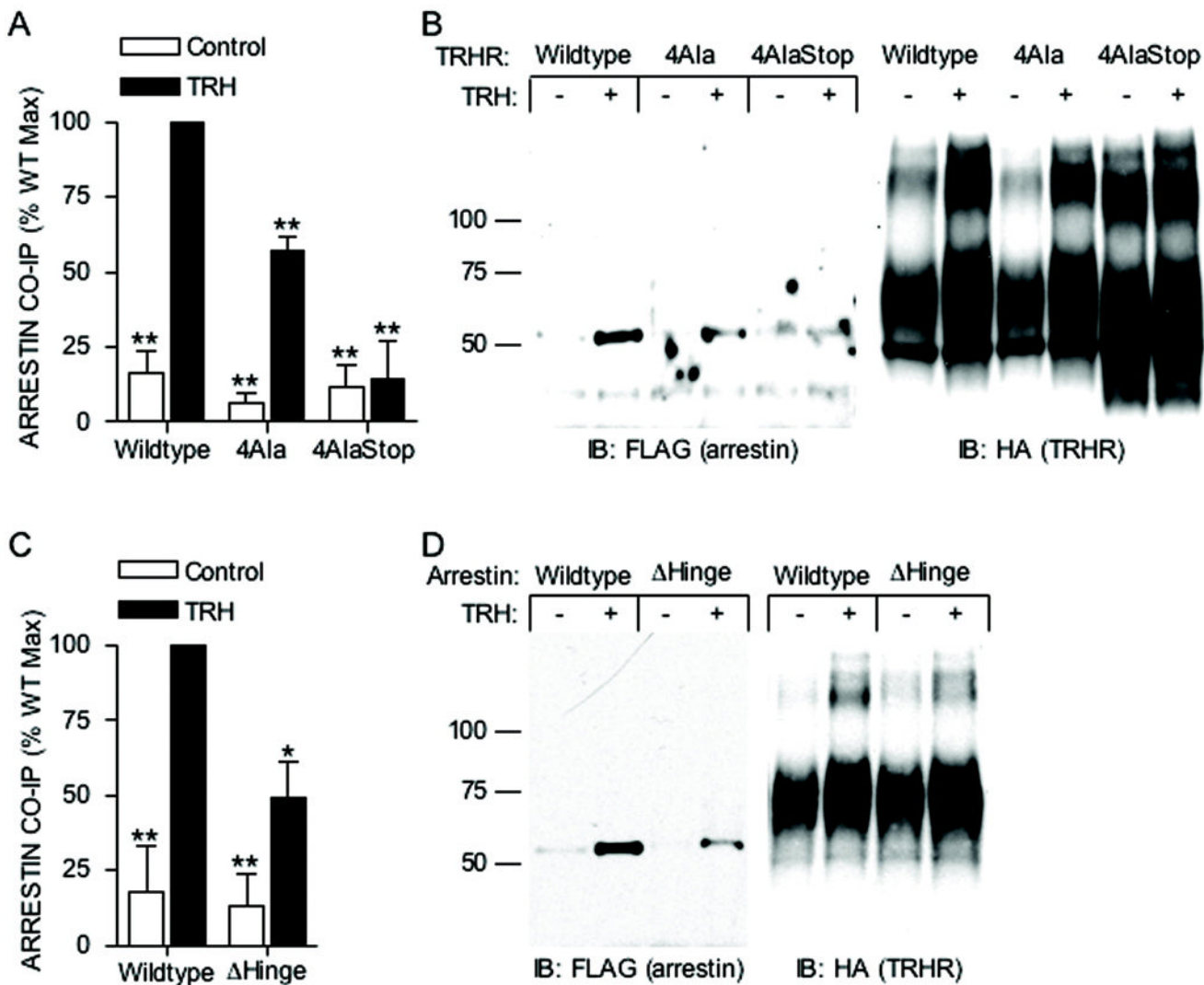
Fig. 5.
Arrestin binding to TRH receptor is impaired by mutation or deletion of key phosphosites. HEK293 cells transiently co-transfected with HA-tagged TRH receptors and FLAG-tagged wildtype or ΔHinge arrestin 3 were treated for 2 min with or without 1 μM TRH before proteins were cross-linked, immunoprecipitated with anti-HA antibody, and resolved on SDS-PAGE. Gels were immunoblotted for FLAG-arrestin and then reprobed for HA-TRH receptor. *A* and *C*, Densitometric analysis of 3 to 4 independent experiments showing arrestin immunoprecipitated with TRH receptor as a percent of wildtype arrestin immunoprecipitated with wildtype receptor, normalized for differences in protein expression. *, $p < 0.05$; **, $p < 0.01$ *versus* TRH stimulated wildtype receptor plus wildtype arrestin. *B* and *D*, representative immunoblots of *A* and *C*, respectively. The small amount of arrestin that apparently co-immunoprecipitated from cells not stimulated with TRH (e.g. *B*, lane 1) was also seen in cells transfected with FLAG-tagged arrestin alone (data not shown).
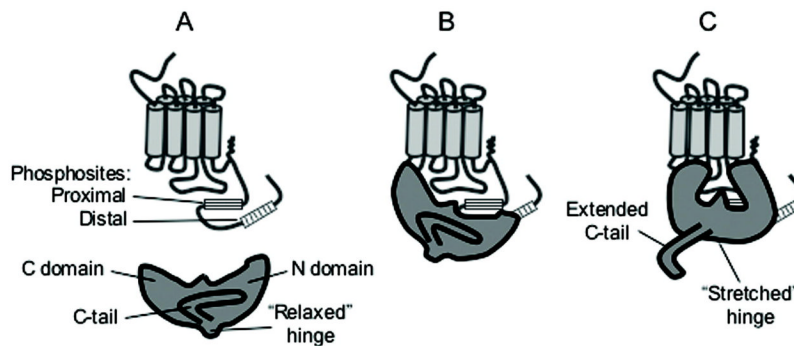
Fig. 6.
Model depicting arrestin-TRH receptor interactions. *A*, TRH receptor phosphorylated on proximal and/or distal sites recruits arrestin from the cytosol to the plasma membrane. *B*, Arrestin initially interacts with receptor in multiple low-affinity conformations that procede in a step-wise manner (only one is depicted). Contact sites are in the arrestin C domain (activation sensor) and N domain (phosphorylation sensor) and receptor intracellular loops and phosphorylated C-tail. *C*, Only the proximal phosphosites in the receptor C-tail can cause the conformational changes in arrestin that lead to receptor desensitization and internalization.

Table 1

Arrestin increases the affinity of TRH receptor. Arr2/3KO MEFs were co-transfected with TRH receptors and arrestins and incubated with [^{3}H]MeTRH. Ligand binding affinity was calculated by Scatchard analysis. Table shows mean ± S.E. or range from 2–4 independent experiments.

TRH Receptor	*Arrestin*	n	K_d (nM [^{3}H]MeTRH)
Wildtype			
	None	4	20.1 ± 2.6
	Wildtype	4	1.4 ± 0.2[**]
	ΔLIELD/F391A	3	1.4 ± 0.3[**]
	ΔHinge	3	3.8 ± 0.8[**]
	N domain	3	17.8 ± 2.8
	C domain	3	10.5 ± 2.3[*]
	N + C domains	2	1.8 ± 0.2[**]
371Stop			
	None	3	n.d.[a]
	Wildtype	3	1.2 ± 0.2
4Ala			
	None	3	23.8 ± 4.3
	Wildtype	3	1.8 ± 0.1[**]
4AlaStop			
	None	3	23.7 ± 5.8
	Wildtype	4	4.4 ± 0.7[**]
	R169E	2	2.1 ± 0.4[**]

[a] Affinity of 371Stop receptor without arrestin was >300 nM.

[*] $p < 0.05$ or

[**] $p < 0.001$ *versus* receptor without arrestin.

Table 2

Summary of the effects of TRH receptor or arrestin mutation. *n.d.*, not determined.

	Increased agonist affinity	Recruits arrestin-GFP	Increased acid/salt-resistance	Co-IPs	Arrestin-dependent desensitization	Increased internalization
TRH receptors co-expressed with wildtype arrestin						
Wildtype	++++	++++	++++	++++	++++	++++
371Stop	++++	++++	++++	++++	++++	++++
4Ala	+++	+++	+++	++	-	-
4AlaStop	++	-	-	-	-	-
Arrestins co-expressed with wildtype TRH receptor						
Wildtype	++++		++++	++++	++++	++++
ΔLIELD/F391A	++++		++	n.d.	++++	-
ΔHinge	++		++	++	-	-