Molecular Determinants Underlying the Formation of Stable Intracellular G Protein-coupled Receptor-β-Arrestin Complexes after Receptor Endocytosis*

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β-Arrestins bind agonist-activated G protein-coupled receptors (GPCRs) and mediate their desensitization and internalization. Although β-arrestins dissociate from some receptors at the plasma membrane, such as the β2 adrenergic receptor, they remain associated with other GPCRs and internalize with them into endocytic vesicles. Formation of stable receptor-β-arrestin complexes that persist inside the cell impedes receptor resensitization, and the aberrant formation of these complexes may play a role in GPCR-based diseases (Barak, L.S., Oakley, R.H., Laporte, S.A., and Caron, M.G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 93–98). Here, we investigate the molecular determinants responsible for sustained receptor/β-arrestin interactions. We show in real time and in live human embryonic kidney (HEK-293) cells that a β-arrestin-2-green fluorescent protein conjugate internalizes into endocytic vesicles with agonist-activated neurotensin-1 receptor, oxytocin receptor, angiotensin II type 1A receptor, and substance P receptor. Using receptor mutagenesis, we demonstrate that the ability of β-arrestin to remain associated with these receptors is mediated by specific clusters of serine and threonine residues located in the receptor carboxyl-terminal domain and serve as primary sites of agonist-dependent receptor phosphorylation. In addition, we identify a β-arrestin mutant with enhanced affinity for the agonist-activated β2-adrenergic receptor that traffics into endocytic vesicles with receptors that lack serine/threonine clusters and normally dissociate from wild-type β-arrestin at the plasma membrane. By identifying receptor and β-arrestin residues critical for the formation of stable receptor-β-arrestin complexes, these studies provide novel targets for regulating GPCR responsiveness and treating diseases resulting from abnormal GPCR/β-arrestin interactions.

G protein-coupled receptors (GPCRs) comprise a large gene family of more than 1000 members that regulate a diverse array of physiological functions such as phototransduction, olfaction, neurotransmission, vascular tone, cardiac output, digestion, and pain. The signaling of GPCRs is intimately controlled by a family of intracellular proteins termed arrestins that includes visual arrestin, β-arrestin-1, and β-arrestin-2 (1, 2). Arrestins bind agonist-activated GPCRs at the plasma membrane that have been phosphorylated by G protein-coupled receptor kinases (GRKs) on serine and threonine residues located in the third intracellular loop or carboxyl-terminal tail (3–5). The association of a single arrestin with a GRK-phosphorylated receptor uncouples the receptor from its cognate G protein, resulting in the termination of GPCR signaling, a process termed desensitization (3–7). The nonvisual arrestins, β-arrestin-1 and β-arrestin-2, then target desensitized receptors to clathrin-coated pits for endocytosis by functioning as adaptor proteins that link the receptor to components of the endocytic machinery such as AP-2 and clathrin (8–11). The internalized receptors are dephosphorylated in endosomes and recycled back to the cell surface fully resensitized (12–14).

The association of β-arrestins with agonist-activated receptors at the plasma membrane is a feature common to almost all GPCRs (15, 16). However, the fate of these receptor-β-arrestin complexes differs markedly among receptors (14, 16). β-Arrestins dissociate from some receptors, such as the β2-adrenergic receptor (β2AR), at or near the plasma membrane and are excluded from receptor-containing endocytic vesicles. In contrast, β-arrestins remain associated with other receptors, such as the vasopressin V2 receptor (V2R), and traffic with them into endocytic vesicles. The stability of the receptor-β-arrestin complex appears to regulate the rate of receptor resensitization (14). Receptors that dissociate from β-arrestin at or near the plasma membrane are rapidly dephosphorylated and recycled, whereas receptors that remain associated with β-arrestin are slowly dephosphorylated and recycled.

The ability of the V2R to form a stable complex with β-arrestin is mediated by a specific cluster of GRK-phosphorylated serine residues in the receptor carboxyl-terminal tail (14). Whether a similar motif is necessary for other GPCRs to remain associated with β-arrestin is unknown. Identifying such residues is a critical goal of current research not only because the strength of the receptor/β-arrestin interaction controls GPCR responsiveness but also because the inappropriate formation of these complexes may underlie the pathology of several GPCR-based diseases. For example, a nonsignaling V2R

V2R, vasopressin V2 receptor; NTR-1, neurotensin-1 receptor; OTR, oxytocin receptor; AT1AR, angiotensin II type 1A receptor; SFR, substance P receptor; GFP, green fluorescent protein; YFP, yellow fluorescent protein; HA, hemagglutinin; HEP, human embryonic kidney.

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1 The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; β2AR, β2-adrenergic receptor; HA, hemagglutinin; HEP, human embryonic kidney.
mutant associated with nephrogenic diabetes insipidus is complexed with β-arrestin in endocytic vesicles in the absence of agonist (17). The normal plasma membrane localization and the ability of this constitutively desensitized receptor to signal can be restored by eliminating the cluster of serine residues that promotes the high affinity V2R/β-arrestin interaction (17). Moreover, several recent studies report that the continued association between rhodopsin and visual arrestin may underlie some forms of retinal degeneration (18–20).

In the following study, we investigate the molecular determinants underlying the formation of stable GPCR-β-arrestin complexes that persist inside the cell. We demonstrate in real time and in live cells that the ability of β-arrestin to remain associated with a variety of GPCRs is mediated by specific clusters of phosphorylated serine and threonine residues strategically positioned within the receptor carboxyl-terminal tail. The requirement for these phosphorylated serine/threonine clusters can be bypassed, however, by mutations in β-arrestin that enhance its affinity for agonist-activated GPCRs. Identification of the molecular determinants underlying the formation of stable receptor-β-arrestin complexes provides novel targets for manipulating the affinity of these two proteins and regulating GPCR responsiveness.

**EXPERIMENTAL PROCEDURES**

**Materials**—Neurotensin was purchased from Peninsula Laboratories. Oxytocin, angiotensin II, substance P, and isotretinoin were obtained from Sigma. The anti-hemagglutinin (HA) 12C5 mouse monoclonal antibody and the rhodamine-conjugated anti-HA 12C5 mouse monoclonal antibody were purchased from Roche Molecular Biochemicals. [32P]Orthophosphate was purchased from PerkinElmer Life Sciences.

**Plasmid DNA**—Construction of plasmids containing the hemagglutinin epitope-tagged rat NTR, rat angiotensin II type 1A receptor (AT1AR), and human β2AR have been described previously (21–23). β-Arrestin-2 with GFP conjugated to its carboxyl terminus (βarr2-GFP) and β-arrestin 1 with yellow fluorescent protein (YFP) conjugated to its amino terminus (YFP-βarr1) have been described previously (15, 16). All other constructs were generated by polymerase chain reaction following standard protocols. The YFP-βarr1-383T truncation mutant was constructed by inserting the nine-amino acid sequence (YPYDVPDYA) recognized by the anti-HA 12CA5 mouse monoclonal antibody immediately after the amino-terminal initiator methionine of the human OTR cDNA. The OTR-AAA-2 mutant was generated by replacing nucleotides TAG encoding a stop codon in OTR with alanine residues. The HA-tagged human oxytocin receptor (OTR) was constructed by inserting the nine-amino acid sequence (YPYDVPDYA) recognized by the anti-HA 12CA5 mouse monoclonal antibody and the rhodamine-conjugated anti-HA 12CA5 mouse monoclonal antibody were purchased from Roche Molecular Biochemicals. [32P]Orthophosphate was purchased from PerkinElmer Life Sciences.

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RESULTS

Trafficking of β-Arrestin with the Agonist-activated NTR-1, OTR, AT1AR, and SPR into Endocytic Vesicles—We employed a functional β-arrestin-2-green fluorescent protein conjugate (βarr2-GFP) (15) to visualize the association of β-arrestin with the agonist-occupied NTR-1, OTR, AT1AR, and SPR in live HEK-293 cells. In the absence of agonist, β-arrestin was uniformly distributed in the cytoplasm of cells, as indicated by the homogeneous βarr2-GFP fluorescence (Fig. 1, 0 min). The addition of agonist promoted the rapid redistribution of β-arrestin from the cytoplasm to each of the receptors at the plasma membrane (Fig. 1, 2 min). The punctate pattern of βarr2-GFP fluorescence at the plasma membrane reflects its localization with receptors in clathrin-coated pits (9, 15, 21). With a longer agonist exposure, βarr2-GFP was observed to redistribute from the plasma membrane to endocytic vesicles in each of the receptor-expressing cells (Fig. 1, 30 min). These vesicles, which were first detected within 3–5 min of agonist addition, grew in size and number and were still observed after 1 h of agonist treatment.

To determine whether β-arrestin colocalized with the receptor in these endocytic vesicles, we examined the distribution of receptor and the distribution of βarr2-GFP in the same living HEK-293 cells before and after agonist treatment. In the absence of agonist, receptor immunofluorescence was present at the plasma membrane and βarr2-GFP fluorescence was uniformly distributed in the cytoplasm, and no colocalization was observed (data not shown). However, after a 30-min treatment with agonist, receptor immunofluorescence (red) and βarr2-GFP fluorescence (green) showed extensive colocalization (yellow) in endocytic vesicles (Fig. 2).

These results demonstrate that β-arrestin binds the agonist-activated NTR-1, OTR, AT1AR, and SPR at the plasma membrane and traffics with them into endocytic vesicles.

Molecular Determinants Underlying the Trafficking of β-Arrestin with the NTR-1, OTR, and AT1AR into Endocytic Vesicles—The ability of the V2R to form a stable complex with β-arrestin that is preserved in endocytic vesicles is mediated by a specific cluster of three serine residues that resides in the receptor carboxyl-terminal tail and is phosphorylated in response to agonist (14). The carboxyl-terminal tails of the NTR-1, OTR, and AT1AR are very similar in size to the V2R tail and contain multiple clusters of potential phosphate acceptor sites (Fig. 3A). Clusters were defined as serine/threonine residues occupying three out of three, three out of four, or four out of five consecutive positions. Based on this criterion, the NTR-1 contains two clusters (SMSS and STS), the OTR contains three clusters (TSAS, SSS, and SSS), and the AT1AR contains three clusters (SSLST, STLS, and SSS).

To determine whether these clusters are important for the formation of stable receptor-β-arrestin complexes that persist inside the cell, we selectively mutated each cluster to alanine residues (Fig. 3A). The resulting receptor mutants were expressed in HEK-293 cells and found to undergo agonist-dependent sequestration at levels comparable to that observed for their wild-type counterparts (data not shown). We next assessed the distribution of the βarr2-GFP fusion protein in cells expressing each receptor mutant before and after a 30-min treatment with agonist. For the NTR-1, mutation of the proximal SMSS cluster to alanine residues (NTR1-AMAA) did not affect the ability of βarr2-GFP to traffic with this receptor into endocytic vesicles (Fig. 4A). In marked contrast, however, mutation of the more distal STS cluster to alanine residues...
(NTR1-AAA) essentially abolished the ability of βarr2-GFP to internalize with the receptor (Fig. 4A). βarr2-GFP still translocated to the NTR1-AAA mutant at the plasma membrane upon agonist addition; however, even with a prolonged agonist incubation, β-arrestin did not redistribute with the receptor into endocytic vesicles but rather remained at the plasma membrane in clathrin coated pits (Fig. 4A). For the OTR, mutation of the TSAS cluster to alanine residues (OTR-AAAA) did not prevent β-arrestin from trafficking with this receptor into endocytic vesicles (Fig. 4B). However, when either one of the two serine triplets was mutated to alanine residues (OTR-AAA-1 and OTR-AAA-2), the ability of βarr2-GFP to internalize with the receptor was eliminated (Fig. 4B). Two adjacent clusters of serine and threonine residues were also found to mediate the ability of the AT1AR to form a stable complex with β-arrestin. As shown in Fig. 4C, mutation of the SSLST or STLS cluster to alanine residues (AT1AR-AALAA and AT1AR-AALA) essentially abolished the ability of βarr2-GFP to traffic with the AT1AR into endocytic vesicles, whereas mutation of the more distal SSS cluster to alanine residues (AT1AR-AAA) had no effect. These results demonstrate that specific clusters of serine and threonine residues located in the receptor carboxyl-terminal tail promote the formation of high affinity receptor-β-arrestin complexes that remain together inside the cell after receptor endocytosis. In the absence of this motif, receptor-β-arrestin complexes are presumably less stable and dissociate at or near the plasma membrane, and β-arrestin is excluded from receptor containing endocytic vesicles.

We next investigated whether the identified serine/threonine
clusters necessary for the formation of stable receptor-β-arrestin complexes were actually phosphorylated in response to agonist. For these experiments, whole cell phosphorylations were performed on HEK-293 cells expressing wild-type or mutant receptors. Compared with their wild-type counterparts, the agonist-induced phosphorylation of the NTR1-AAA mutant was reduced by 95% (Fig. 5A), the agonist-induced phosphorylation of the OTR-AAA-1 and OTR-AAA-2 mutants was each reduced by 32% (Fig. 5B), and the agonist-induced phosphorylation of the AT1AR-AALAA and AT1AR-AALA mutants was each reduced by 67% (Fig. 5C). These results show that the clusters of serine and threonine residues necessary for the continued association of β-arrestin with the NTR-1, OTR, and AT1AR are important sites of agonist-dependent receptor phosphorylation. In addition, because clusters not essential for the high affinity interaction may also be phosphorylated (NTR-AMAA and AT1AR-AAA mutants), these results suggest that the phosphorylated clusters responsible for the stable receptor-β-arrestin complex must be properly positioned within the receptor carboxyl-terminal tail.

Molecular Determinants Underlying the Trafficking of β-Arrestin with the SPR into Endocytic Vesicles—The SPR carboxyl-terminal tail is much longer than the tails of the V2R, NTR-1, OTR, and AT1AR and contains four clusters of serine and threonine residues (TTIST, TPSS, SSRS, and SFSS) (Fig. 3B). To determine if one or more of these clusters mediates the ability of the SPR to form a stable complex with β-arrestin, we initially truncated the receptor at position 383 (SPR-383X) to eliminate all four clusters (Fig. 3B). The agonist-activated SPR-383X mutant recruited βarr2-GFP into endocytic vesicles just as well as the wild-type SPR (Fig. 6, compare upper left and upper middle panels). In contrast, the ability of the SPR-355X mutant to recruit βarr2-GFP into endocytic vesicles was severely compromised (Fig. 6, compare upper left and upper right panels). A small amount of β-arrestin internalized with the SPR-355X mutant; however, much more β-arrestin was now found at the plasma membrane in clathrin-coated pits and in a uniform distribution back in the cytoplasm. These results suggested that the TTIST and/or TPSS clusters play an important role in promoting a high affinity SPR-β-arrestin complex; therefore, we selectively mutated each of these clusters to alanine residues (SPR-AAIAA and SPR-APAA) (Fig. 3B). Trafficking of βarr2-GFP with the SPR-APAA mutant was indistinguishable from its trafficking with the wild-type SPR (Fig. 6, compare upper left and lower middle panels). In contrast, the ability of β-arrestin to traffic with the SPR-AAIAA mutant into endocytic vesicles was impaired, as indicated by the reduced amount of β-arrestin in endocytic vesicles and the increased amount at the plasma membrane (Fig. 6, compare upper left and lower left images). Thus, it appears that the TTIST cluster is important for the formation of high affinity SPR-β-arrestin complexes that persist inside the cell after receptor endocytosis.

Our finding that βarr2-GFP still traffics, albeit weakly, with the SPR-355X mutant into endocytic vesicles suggests that other receptor residues may also be involved in promoting a stable SPR-β-arrestin complex. To investigate the contribution
made by the remaining serine and threonine residues in the tail of the SPR-355X mutant, we truncated the receptor at position 325 (SPR-325X) (Fig. 3B). This receptor mutant is missing almost the entire carboxyl-terminal tail and is devoid of serine and threonine residues downstream of the NPXYXY motif. However, as shown in the lower right image of Fig. 6, the agonist-activated SPR-325X mutant still recruited a small amount of βarr2-GFP into endocytic vesicles. These data suggest that residues outside the SPR carboxyl-terminal tail are responsible for the residual amount of β-arrestin recruited into endocytic vesicles with the SPR-355X and SPR-AAIAA mutants (Fig. 6).

We next evaluated the phosphorylation status of the wild-type and mutant SPR in transfected HEK-293 cells. In response to agonist, the wild-type SPR and SPR-383X truncation mutant were phosphorylated to similar levels, whereas no phosphorylation was observed for the SPR-355X truncation mutant (Fig. 7). This result suggests that the principal site of agonist-dependent receptor phosphorylation is the TTST and/or TPSS cluster (Fig. 3B). Mutation of these clusters to alanine residues (SPR-AAIAA and SPR-APAA), however, did not appreciably reduce the level of SPR phosphorylation (Fig. 7). The SPR is phosphorylated in an agonist-dependent manner by GRKs rather than second messenger kinases (27), and the abundance of serine and threonine residues (13 of the last 24 amino acids) downstream of these two clusters may allow GRK phosphorylation to take place even in the absence of the preferred site (28, 29).

Trafficking of a β-Arrestin 1 Truncation Mutant with GPCRs Lacking Serine and Threonine Clusters into Endocytic Vesicles—If the importance of serine/threonine clusters in the formation of stable GPCR/β-arrestin complexes is simply to enhance the affinity of the receptor/β-arrestin interaction, then it might be possible to enhance the affinity of this interaction in some other way. Removal of the last 36 amino acids of β-arrestin-1 produces a truncated protein (βarrestin-1–383T) that desensitizes the β2AR more rapidly than the wild-type β-arrestin-1 (30). This difference in desensitization kinetics suggests that the mutant β-arrestin-1 has higher affinity for the β2AR than does wild-type β-arrestin-1. We measured the relative affinity of these two proteins for the β2AR by fusing the YFP to the amino terminus of β-arrestin-1 (YFP-βarr1) and β-arrestin-1–383T (YFP-βarr1–383T). We then compared the ability of these YFP-tagged β-arrestins to bind the agonist-activated β2AR in real time and in live cells. As shown in Fig. 8A, both β-arrestins redistributed from the cytoplasm to the receptor at the plasma membrane upon agonist addition. However, as indicated by the increase in fluorescence at the plasma membrane, YFP-βarr1–383T translocated faster and to a greater extent than YFP-βarr1. In fact, the robust translocation of β-arrestin-1–383T cleared out the cytoplasm and revealed the pool of YFP-βarr1–383T left behind in the nucleus.

Differences in the ability of β-arrestin-1 and β-arrestin-1–383T to translocate to the β2AR were quantitated by measuring the time-dependent loss of β-arrestin fluorescence from the cytoplasm after treatment with agonist. Analysis of these data revealed three major differences in the translocation profiles (Fig. 8B). First, β-arrestin-1–383T began translocating sooner than β-arrestin-1. The delay in time between agonist addition (arrow) and the initial loss of β-arrestin from the cytoplasm was less than 1 s for YFP-βarr1–383T and ~12 s for YFP-βarr1. Second, β-arrestin-1–383T translocated faster than β-arrestin-1. The half-life of β-arrestin depletion from the cytoplasm was 27.6 ± 0.9 s for YFP-βarr1–383T and 41 ± 1.9 s for YFP-βarr1. Third, β-arrestin-1–383T translocated to a much greater extent than β-arrestin-1. The fraction of cytoplasmic β-arrestin that translocated to the plasma membrane was 73.4 ± 0.4% for YFP-βarr1–383T and only 31.6 ± 0.3% for YFP-βarr1. These results demonstrate that the β-arrestin-1–383T truncation mutant has a much higher affinity for the β2AR than the wild-type β-arrestin-1.

The agonist-activated β2AR, which lacks serine/threonine clusters in its carboxyl-terminal tail, dissociates from β-arrestin at the plasma membrane and internalizes without β-arrestin into endocytic vesicles (14). However, this receptor can be made to form a stable complex with β-arrestin that persists inside the cell after receptor endocytosis by adding a serine cluster to its carboxyl-terminal tail (14). If the molecular mechanism underlying the formation of stable receptor-β-arrestin complexes is simply a high affinity receptor/β-arrestin interaction, then the increased affinity of the β-arrestin-1–383T trun-
consists of serine and threonine residues that are clustered persist inside the cell after GPCR internalization. This motif translocation to the second scan. Data represent the mean 5 time cells was measured from confocal images collected every 24.8 s (scan and trafficked with them into endocytic vesicles. n 5 than contrast, YFP-arr1 and YFP-arr1–383T trafficked with the receptor at the plasma membrane and was associated from the receptor at the plasma membrane and was distributed to the b2AR. b-Arrestin fluorescence in the cytoplasm of cells was measured from confocal images collected every 24.8 s (scan time = 3.9 s). The agonist was added (arrow) immediately after the second scan. Data represent the mean ± S.E. of three to four independent experiments (n = 22–26 cells) and were analyzed using a plateau with exponential decay nonlinear regression function in GraphPad Prism.

**DISCUSSION**

In the present study, we identify a conserved motif in the carboxyl-terminal tail of GPCRs that mediates the agonist-dependent formation of stable receptor-b-arrestin complexes that persist inside the cell after GPCR internalization. This motif consists of serine and threonine residues that are clustered together within the receptor carboxyl-terminal tail and serve as primary sites of agonist-dependent receptor phosphorylation. In the absence of this motif, the receptor/b-arrestin interaction is less stable, and the agonist-occupied receptors dissociate from b-arrestin at or near the plasma membrane. We also identify a mutation in b-arrestin that allows it to form stable complexes with agonist-activated GPCRs that lack serine/threonine clusters. These studies, by identifying receptor and b-arrestin residues critical for the formation of high affinity receptor-b-arrestin complexes, provide novel targets for regulating GPCR responsiveness and treating diseases resulting from abnormal GPCR/b-arrestin interactions.

The serine/threonine clusters mediating the formation of stable receptor-b-arrestin complexes show a significant degree of conservation in their relative position within the receptor carboxyl-terminal tail. For example, the clusters are 36, 45, 38/47, 25/32, and 50 amino acids downstream of the NPXY motif for the V2R, NTR-1, OTR, AT1AR, and SPR, respectively (Fig. 3). For the subgroup of receptors with carboxyl-terminal tails of similar length and containing putative sites of palmitoylation (V2R, NTR-1, and OTR), the conservation of the cluster location is even more remarkable. The serine/threonine clusters are 36, 45, and 38/47 amino acids downstream of the NPXXY motif; 19, 26, and 20/29 amino acids downstream of the putative sites of palmitoylation; and 7, 7, and 10/19 amino acids upstream from the end of the carboxyl-terminal tail (Fig. 3A). Because the cluster motif is essentially absent from the carboxyl-terminal tails of GPCRs that form unstable complexes with b-arrestin (Fig. 10A), it should be possible to predict whether a given receptor will bind b-arrestin with low or high affinity by simply analyzing the amino acid sequence of the receptor carboxyl-terminal tail. This in turn will provide valuable information as to how the cellular complement of arrestin...
isoforms might regulate the pattern of receptor desensitization, sequestration, and resensitization (16, 31).

Binding of β-arrestin to agonist-activated GPCRs at the plasma membrane is thought to involve multiple interactions (2, 32). A large region within the amino-terminal half of β-arrestin, termed the activation recognition domain, binds the second and/or third intracellular loops of the receptor (33). This is followed by the binding of a smaller, positively charged region in the central portion of β-arrestin, termed the phosphorylation recognition domain, to the GRK-phosphorylated receptor carboxyl-terminal tail (34). Phosphorylation of a cluster of serine and threonine residues would produce a localized concentration of negative charges, and the ionic interaction between the cluster of negative charges in the receptor carboxyl-terminal tail and the cluster of positive charges in the phosphorylation recognition domain of β-arrestin might be sufficient to stabilize the receptor-β-arrestin complex. Alternatively, engagement of the phosphorylated serine/threonine cluster may induce a conformational change in β-arrestin that allows a subsequent high affinity interaction between the receptor and β-arrestin to take place (35). In favor of the latter model is our finding that the β-arrestin-1–383T truncation mutant remains associated with GPCRs lacking serine/threonine clusters. Removal of the β-arrestin-1 carboxyl terminus may constitutively expose the high affinity receptor binding site and allow the mutant β-arrestin to bind GPCRs in the absence of phosphorylation (30).

The stability of the GPCR-β-arrestin complex has been shown to regulate the kinetics of receptor resensitization. For example, the ability of β-arrestin to remain associated with the V2R impairs the efficient recycling of this receptor back to the plasma membrane (14). In agreement with these findings, we show that the NTR-1 and SPR form stable complexes with β-arrestin, and both these receptors have been reported to recycle poorly (36, 37). However, the stability of the receptor-β-arrestin complex may also be involved in initiating and/or regulating other signaling pathways. Recent studies have demonstrated that β-arrestin-bound internalized receptors are important for GPCR-mediated activation of mitogen-activated protein kinases. β-Arrestin appears to function as a molecular scaffold that organizes and recruits components of the mitogen-activated protein kinase cascade following the agonist-activated GPCR (44). For example, stimulation of the AT1AR activates the c-Jun amino-terminal kinase 3 (JNK3), which colocalizes with the receptor and β-arrestin in endocytic vesicles (38). In addition, activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) by the proteinase-activated receptor 2 requires the association of receptor, β-arrestin, and ERK1/2 in endocytic vesicles (39). Proteinase-activated receptor 2 has been reported to form stable complexes with β-arrestin that persist inside the cell (39, 40), and its carboxy-terminal tail contains three serine/threonine clusters occupying positions very similar to those occupied by the clusters found in the tails of the V2R, NTR, and OTR (Fig. 10B, compare with Fig. 3A). For both the AT1AR and proteinase-activated receptor 2, the prolonged association between receptor, β-arrestin, and mitogen-activated protein kinase may ensure the proper localization, specificity, and/or duration of the mitogen-activated protein kinase response.

In summary, we have identified a motif in the carboxy-terminal tail of GPCRs that promotes the formation of stable receptor-β-arrestin complexes. In addition, we have shown that similar stable receptor-β-arrestin complexes can be achieved with GPCRs lacking this motif by mutations in β-arrestin that enhance its affinity for receptors. Understanding the molecular determinants underlying high affinity GPCR/β-arrestin interactions may be useful in a variety of pathophysiological conditions. For example, reducing the affinity of the receptor-β-arrestin complex may restore signaling to nonfunctional V2R mutants that are constitutively bound to β-arrestin and responsible for some forms of nephrogenic diabetes insipidus (17). Alternatively, increasing the affinity of the receptor/β-arrestin interaction may be an effective treatment for certain forms of hyperthyroidism and familial precocious puberty that result from constitutively active GPCRs (41, 42). Finally, modulating the stability of GPCR-β-arrestin complexes may enhance the efficacy of GPCR-acting drugs by altering the kinetics of receptor desensitization and/or resensitization (43).

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