A β-Arrestin Binding Determinant Common to the Second Intracellular Loops of Rhodopsin Family G Protein-coupled Receptors* 

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β-Arrestins have been shown to inhibit competitively G protein-dependent signaling and to mediate endocytosis for many of the hundreds of nonvisual rhodopsin family G protein-coupled receptors (GPCR). An open question of fundamental importance concerning the regulation of signal transduction of several hundred rhodopsin-like GPCRs is how these receptors of limited sequence homology, when considered in toto, can all recruit and activate the two highly conserved β-arrestin proteins as part of their signaling/desensitization process. Although the serine and threonine residues that form GPCR kinase phosphorylation sites are common β-arrestin-associated receptor determinants regulating receptor desensitization and internalization, the agonist-activated conformation of a GPCR probably reveals the most fundamental determinant mediating the GPCR and arrestin interaction. Here we identified a β-arrestin binding determinant common to the rhodopsin family GPCRs formed from the proximal 10 residues of the second intracellular loop. We demonstrated by both gain and loss of function studies for the serotonin 2C, β2-adrenergic, α2-adrenergic, and neuropeptide Y type 2 receptors that the highly conserved amino acids, proline and alanine, naturally occurring in rhodopsin family receptors six residues distal to the highly conserved second loop DRY motif regulate β-arrestin binding and β-arrestin-mediated internalization. In particular, as demonstrated for the β2AR, this occurs independently of changes in GPCR kinase phosphorylation. These results suggest that a GPCR conformation directed by the second intracellular loop, likely using the loop itself as a binding patch, may function as a switch for transitioning β-arrestin from its inactive form to its active receptor-binding state.

In the eye the light-induced signaling mediated through the G protein transducin is competitively blocked by the binding of visual arrestin to rhodopsin. An analogous paradigm is repeated outside the visual system to terminate G protein-mediated signaling for rhodopsin family GPCRs, except visual arrestin is replaced by β-arrestins. Variations in β-arrestin affinity for individual receptors and between different GPCRs primarily rest on the following two factors: the agonist-induced conformation of the receptor, and the ability of G protein-coupled receptor kinases (GRK) to phosphorylate serine and threonine residues on the C-tail and third intracellular loop of a receptor (1–5).

Receptor agonist-induced phosphorylation has long been demonstrated to be of great importance for β-arrestin binding, being initially described for visual arrestin binding of the phosphorylated MII state of light-activated rhodopsin (6–8). More recently, the formation of stable β-arrestin complexes with agonist-activated GPCRs has been shown to require phosphorylation of serine and threonine clusters located in the C-terminal tails of the receptor (9). It has been proposed that in addition to phosphorylation, high affinity arrestin/receptor binding also requires receptor determinants that are exposed only in the active receptor conformation (9, 10). Supporting this alternative are observations that agonist-activated GPCRs bind β-arrestins even in the absence of GRK phosphorylation (2). This phosphorylation-independent binding suggests that determinants, resulting from conserved primary amino acid sequences or protein secondary structural motifs, exist in all GPCRs to regulate receptor/arrestin association. However, the receptor regions that would comprise these arrestin-binding motifs have not been thoroughly defined, perhaps as a result of the sequence variability occurring throughout the entire GPCR family and the absence of crystal structure data other than for rhodopsin.

GPCRs are structurally similar in their seven transmembrane architecture and share behaviors that originate from commonly occurring stretches of amino acids. The (E/D)RY motif, one of the most conserved of these sequences in the large family of rhodopsin-like GPCRs, is found at the cytoplasmic/intracellular loop junction of transmembrane III. The DRY motif presumably mediates interactions with both G proteins and arrestins and serves to maintain the receptor transmembranes in an inactive conformation in the absence of ligand (11–15). Scattered residues on the first two rhodopsin intracellular loops have been identified as contributing to visual arrestin binding exclusive of the phosphorylated rhodopsin C-tail (4, 16, 17). In particular, a proline residue in the rhodopsin second intracellular loop distal to the ERY motif is involved (4, 17). In addition, computational modeling of molecular docking between rhodopsin and G proteins highlights that this proline and other residues of the second loop may directly engage transducin (18). Although many of the biochemical behaviors observed for rhodopsin in the visual system generalize to the larger subfamily of class I rhodopsin-like GPCRs, significant differences remain. For example, regulatory behavior in nonvisual cell systems that does not normally apply to rhodopsin includes β-arrestin, clathrin-mediated receptor endocytosis (19, 20). Thus, in the absence of appropriate crystallographic data, the extent to which a rhodopsin paradigm applies to nonvisual GPCRs is unclear (21, 22).

In this study we used several GPCRs to investigate the ability of nat-
urally occurring proline and alanine residues, which are present 6 amino acids downstream of the DRY motif, to modulate β-arrestin/receptor interactions. β-Arrestin translocation and receptor endocytosis were used as a direct read-out to assess the effects of mutation at this position on β-arrestin/receptor association for serotonin 2C, β2-adrenergic, α2-adrenergic, and neuropeptide Y2 receptors. Our data combined with sequence analysis of over 175 human rhodopsin familyGPCRs suggest that in these receptors a contiguous 10-amino acid region beginning with the DRY motif forms a phosphorylation-independent structural determinant for binding β-arrestin.

EXPERIMENTAL PROCEDURES

Materials—[3H]Adenine for measurement of cAMP generation and 125I-cyanopindolol for receptor binding were purchased from PerkinElmer Life Sciences, and [3H]CGP-12177 was from Amersham Biosciences. Isoproterenol, propranolol, neuropeptide Y, and SB206553 were from Sigma. Norepinephrine was from Bioanalytical Systems (West Lafayette, IN). The anti-phospho-βAR (Ser-355/Ser-356) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids—FLAG- and GPP-tagged 5-HT2c nonedited receptors were described previously (21). NPY2R receptor was cloned by total RNA extraction from mouse brain using cDNA synthesis kit from New England Biolabs. β2AR, NPY2R, and α2AR refer to receptors with a hemagglutinin tag at the N terminus. Receptor cDNA containing mutations for the β2AR-P138A, 5HT2cR-P159A, and NPY2R-H159P were generated by standard PCR methods using a proofreading polymerase (Pfu; Stratagene). β-Arrestin2-GFP was made as described (23).

Antagonist Binding, Agonist Binding, cAMP Assays, and GTPγS Binding—These procedures in which receptor expression levels were closely matched between experimental groups have been described previously (5, 22). Measurement of receptor surface expression by ELISA was performed under nonpermeabilized conditions (25).

Microscopy and β-Arrestin Localization—Confocal microscopy of HEK-293 cells containing either β-arrestin2-GFP and one of the β2AR, α2AR, or NPY2R receptor variants or the 5HT2cR-GFP wild type or mutant was performed as described (23) using a Zeiss LSM 510.

RESULTS

Occurrence of Conserved Residues in the Second Intracellular Loop of Class I GPCRs—Examination of the human GPCR data base (www.gpcr.org) demonstrated 360 class I rhodopsin-like entries of which 244 contained glutamic/aspartic residues followed by arginine residues (E/D) at the DRY motif. 175 entries were randomly selected from a list of predominantly deorphanized class I GPCRs and were evaluated for the frequency of residue occurrence at positions 1–23 of the second loop, with the lead DRY motif residue defined as position 1. The second loop amino acids were then grouped according to their charge or hydrophobic potential.

Fig. 1A shows these relative frequencies determined for receptors where the lead second loop residue is an aspartic acid. (Note, where the lead residue was glutamic acid, a similar frequency result was obtained (data not shown).) The Arg/Lys/His group is the most probable to occur in this loop region, particularly at positions 2 and 8.

Fig. 1B presents a plot of the next most common group, the hydrophobic residues Iso/Leu/Val/Phe in red plotted among the basic group (blue). In Fig. 1B, the red and blue horizontal dashed lines show the frequency at which a member of the hydrophobic or basic group, respectively, would be expected to occur randomly. It is notable that the hydrophobic group members Iso/Leu/Val/Phe occur at position 6 98% of the time, a level comparable with the 99% occurrence of arginine at position 2. It is also apparent from Fig. 1A that at position 8 a basic residue, and position 9 a proline/alanine combination.

FIGURE 1: Homology of the N-terminal portion of the second intracellular loop among various rhodopsin-like GPCRs. A, frequency of occurrence by position for 136 GPCRs beginning with the Asp of the E/DRY motif. B, frequency per position of the basic residues His, Lys, and Arg and hydrophobic residues Iso, Leu, Val, and Phe beginning with the DRY motif in 136 GPCRs. C, composite sequence representing the most probable substitutions and second most probable ones (above) generated from analysis of the second loop composition of 175 GPCRs.
motivated by the fact that alanine is the most likely natural replacement residue to occur at this position in GPCRs (Fig. 1A).

We first investigated whether substitution of Pro-9 in the most constitutively active 5HT2cR isoform (i.e. nonedited INI isoform) could affect the subcellular distribution of the receptor. Fig. 3A shows a typical intracellular vesicular pattern of the constitutively internalized GFP-tagged 5HT2cR in the absence of agonist, a pattern that is dependent on its interaction with β-arrestin (21). By contrast, the Ala-9 5HT2cR has a more pronounced plasma membrane presence. To quantify the extent to which the proline/alanine substitution modifies 5HT2cR distribution, we measured by ELISA the cell surface expression of either 5HT2cR-GFP or Gαq, with FLAG-5HT2cR or FLAG-5HT2c-P159A in transiently transfected HEK-293 cells. IB, immunoblot. D and F, quantification of β-arrestin2-GFP or Gαq, that was co-immunoprecipitated in three independent experiments each normalized by the amount of receptor expressed is presented. Bar, 10 μm.

FIGURE 2. Geometry of the rhodopsin second intracellular loop. A, a second intracellular loop of bovine rhodopsin shown with the alanine substitution at position 9. B, difference in degrees between the angles omega, psi, and phi of the C-α carbon from the 2.2-Å rhodopsin crystal structure and the resulting structure because of alanine substitution at position 9. C, putative arrestin-interacting face formed from residues 3, 4, and 7–10 and centered on proline 9 of the second intracellular loop.

FIGURE 3. Effects of proline substitution in the second intracellular loop of the constitutively active 5HT2cR. A, representative confocal images of HEK-293 cells transiently transfected with 4 μg of either 5HT2cR-GFP (top panel) or 5HT2cR-P159A-GFP (bottom panel) are shown. Pictures were taken in basal conditions. B, cell surface expression of FLAG-5HT2cR (●) or FLAG-5HT2c-P159A (□) was measured by ELISA before and after 15 and 30 min of inverse agonist treatment (SN206553 2.5 μM) at 37 °C. Data represent the mean of three independent experiments done in triplicate. C and E, co-immunoprecipitation (IP) of β-arrestin2 (Flag)-GFP or Gαq, with FLAG-5HT2cR or FLAG-5HT2c-P159A in transiently transfected HEK-293 cells. IB, immunoblot. D and F, quantification of β-arrestin2-GFP or Gαq, that was co-immunoprecipitated in three independent experiments each normalized by the amount of receptor expressed is presented. Bar, 10 μm.

stutively activated/desensitized receptors (24, 27). However, the functional dependence on β-arrestin binding from point substitutions beyond the DRY motif in residues 4–10 has received scant attention, although alanine substitution for Pro-9 in rhodopsin was demonstrated to decrease visual arrestin binding (4, 17). Because the crystal structure of rhodopsin in its inactive conformation is known, we computationally modeled through the ExPASy Proteomics Server software DeepView (28) if Ala-9 substitution could preserve a Pro-9 like conformation for the second loop. We used the recently published structure of rhodopsin (Protein Data Bank access code 1U19) (29), which has a completely resolved polypeptide chain and is also in agreement with the model of the rhodopsin oligomer (30–32).

Secondary Structure Resulting from Substitution of Proline 9 with Alanine Based on Rhodopsin Crystal Data—To assess local conformational changes in the second loop resulting from an alanine for proline substitution (Fig. 2A), the differences in the planar (ω) and rotational (ϕ and ψ) bond angles (in degrees) about the C-α carbon were calculated (Fig. 2B). Not only is the alanine-computed conformation of the second loop practically identical to the native one, perturbations in the bond angles quickly dampen within 1–2 residues of the proline (Fig. 2B). Moreover, this region formed with the side chains of residues 3–10 centered about the second loop Pro-9 and impairs the ability of 5-HT2cR isoforms to constitutively internalize (21). 15 min of SB206553 treatment resulted in an absolute increase of cell surface 5HT2cRs already resides at the plasma membrane in the absence of agonist and therefore interacts much less well with β-arrestins.

The basal intracellular pattern showing 5HT2cR in vesicles is a reflection of the ability of the receptor to interact constitutively with β-arres-
TABLE 1

|              | $K_d$ 125I-Cyp (pm) | $K_{high}$ for iso (nM) | $R$ in high affinity state (%) |
|--------------|---------------------|-------------------------|--------------------------------|
| Pro-9 $\beta_2$AR | 61 ± 13             | 4.2 ± 1.5               | 39 ± 2                         |
| Ala-9 $\beta_2$AR | 26 ± 3              | 34 ± 10                 | 16 ± 1                         |

| Maximum amount of GTPyS binding | GTPyS EC$_{50}$ (nM) | Maximum amount of cAMP | EC$_{50}$ for cAMP production (nM) |
|---------------------------------|-----------------------|------------------------|-----------------------------------|
| Pro-9 $\beta_2$AR               | 1.00 ± 0.05           | 1.00 ± 0.05            | 4.9 ± 1                           |
| Ala-9 $\beta_2$AR               | 0.83 ± 0.04           | 1.34 ± 0.07            | 4.4 ± 2                           |

Following these data provide strong evidence that the experimental substitution of Pro-9 may affect coupling between the receptor and its cognate G protein. In Table 1, the interaction between each receptor and the G protein was measured by receptor-stimulated binding of nonhydrolyzable GTP analogues (Table 1). A 1 log decrease in affinity for the Ala-9 $\beta_2$AR was observed (Table 1 and Fig. 4A).

The agonist and GTPyS binding data suggest that second messenger signaling should decrease at least mildly in the Ala-9 $\beta_2$AR. Most surprisingly, an opposite trend in signaling was observed. The Ala-9 $\beta_2$AR mutant had 34% greater cAMP accumulation than the wild type receptor (Table 1 and Fig. 4B). Because the DRY motif regulates GPCR desensitization through facilitation of GRK/β-arrestin binding (15, 24, 27), the increases in the magnitude of the ratio of cAMP signaling/G protein coupling (coupled as GTPyS binding) for the Ala-9 $\beta_2$AR relative to the Pro-9 $\beta_2$AR (ratios of 1.00 wild type and 1.61 Ala-9 $\beta_2$AR) may suggest a reduction in the Ala-9 $\beta_2$AR ability to desensitize. Therefore, we tested the extent of agonist-mediated β-arrestin2-GFP translocation to these receptors.

Upon stimulation, wild type $\beta_2$AR strongly recruits β-arrestin2 to the plasma membrane (Fig. 4C, top panels). Although β-arrestin2-GFP recruitment to the Ala-9 $\beta_2$AR approaches a qualitatively similar distribution to that of wild type receptor after 15 min with saturating concentrations of agonist (data not shown), at early time points (1–4 min) the wild type receptor demonstrates greater β-arrestin association (Fig. 4, C and D). This significant delay in redistribution of β-arrestin to the

*FIGURE 4. Effects of proline substitution in the second intracellular loop of the $\beta_2$AR.* A and B. G protein coupling and cAMP signaling of the wild type and second loop substitution mutant $\beta_2$AR ($\beta_2$AR-AF) containing membranes prepared from HEK-293 cells transfected with cDNA for the bovine $\beta_2$AR, were exposed to increasing concentrations of isoproterenol and evaluated for GTPyS binding. C. HEK-293 cells transiently transfected with $\beta_2$AR-AF ($\beta_2$AR-AF) were exposed to increasing concentrations of isoproterenol for 10 min at 37 °C and were presented relative to the plateau response of the wild type receptor being defined as 1.00 and presented as mean ± S.D. Data are representative of four independent experiments. C and D. $\beta$-arrestin2 (β-arrestin2) translocation to the $\beta_2$AR and Pro-9 mutant $\beta_2$AR. HEK-293 cells were transfected with 2.5 µg of cDNA for the $\beta_2$AR (top panels) or $\beta_2$AR-AF (bottom panels) in addition to 1 µg of cDNA for β-arrestin2-GFP. $\beta$-arrestin2-GFP translocation was assessed at 37 °C before and after 20 µM isoproterenol, D. cytosolic β-arrestin fluorescence of cells expressing either the $\beta_2$AR or $\beta_2$AR-AF ($\beta_2$AR-AF) was measured as a mean density per pixel. Data were collected in four independent experiments from three fixed squares of a same area in 3-5 cells per condition. E. agonist-induced desensitization and sequestration of the Pro-9 $\beta_2$AR (Pro-9) and $\beta_2$AR-AF mutant. Immunoprecipitates of membranes from HEK-293 cells transfected with hemagglutinin-tagged receptor cDNA were subjected to SDS-PAGE and evaluated for incorporated [3H]CGP-12177 binding. Data were analyzed using the single value analysis of variance function of GraphPad Prism. Means were shown to be significantly different at $p < 0.05$. Results are representative of four independent experiments with data presented as mean ± S.E. F. HEK-293 cells transiently transfected with 1.5 µg of either $\beta_2$AR or $\beta_2$AR-AF were exposed or not to 10 µM of isoproterenol for 15 min at 37 °C. Cells were then homogenized and receptors were immunoprecipitated in the presence of phosphatase inhibitors. Representative immunoblots depict the total amount of receptor immunoprecipitated (bottom blot) and the amount of receptor phosphorylated on serines 355 and 356 (upper blot).
GPCR Second Loop and β-Arrestin Binding

Ala-9 β2AR in comparison with the wild type receptor is consistent with the observed enhancement in signaling.

Confidence for a putative arrestin-binding role of the β2AR second intracellular loop depends to a large degree on a lack of change in the GRK phosphorylation status of the mutant receptor as well as its inability to undergo downstream β-arrestin-directed behaviors. Fig. 4E depicts an autoradiograph of the basal and agonist-mediated phosphorylation of purified β2AR wild type and mutant receptors. Of the two major kinase families that regulate βAR phosphorylation in HEK cells, GRKs account for ~75% of the amount (39). Quantitative PhosphoImager analysis showed no significant differences in the agonist-mediated enhancement of phosphorylation among the wild type and mutant for the same amount of receptor. In addition we assessed that agonist-induced GRK-dependent phosphorylation of serines 355 and 356 in Pro-9 β2AR and in the Ala-9 mutant β2AR was preserved (Fig. 4F). These two series account for 48% of the GRK-dependent phosphorylation (49). The phosphorylation signal was identical between wild type and mutant receptors when normalized for total immunoprecipitated receptor (data not shown). Together these results suggest that GRK-mediated phosphorylation remains intact in the Ala-9 β2AR.

The magnitude of GPCR internalization also provides a quantitative reflection of receptor/β-arrestin association and was assessed in HEK-293 cells (Fig. 4E). Consistent with the previous β-arrestin2-GFP translocation results, a 22 ± 3% decrease in sequestration of the Ala-9 β2AR was observed in comparison to Pro-9 β2AR. Moreover, the additional mutation of the following hydrophobic residue at position 10 to alanine, which may be part of a common G protein binding determinant among receptors (40) and which is subject to editing in the 5HT2c (20), further decreases the endocytosis as well as substantially reducing β-arrestin translocation (data not shown).

Effects of Proline 9 Insertion in the Second Intracellular Loop of α2a-Adrenergic and NPY2 Receptors—With agonist exposure the α2aAR has a much weaker endocytic response than either the α2bAR or α2cAR (41, 42). Similarly, the NPY2R internalizes poorly compared with the NPY1 and -4 receptors (43, 44). Both the α2bAR and NPY2R lack proline at position 9, possessing instead alanine and histidine. We therefore investigated these two receptors to determine whether substitution of an alanine by a proline at position 9 would result in greater agonist-mediated recruitment of β-arrestin2 and a more robust internalization of these receptors. Fig. 5A shows the cellular distribution of β-arrestin2-GFP after 15 min of agonist treatment in the presence of either wild type or Pro-9 α2bARs. In contrast to the wild type, the Pro-9 α2bAR recruits a significantly greater amount of β-arrestin2 to the plasma membrane. Following 15 and 30 min of norepinephrine stimulation, quantification of receptor cell surface expression demonstrates that Pro-9 α2bAR internalizes 3.6-fold more than the wild type Ala-9 α2bAR (Fig. 5B).

We also assessed after 15 min of NPY stimulation the β-arrestin2-GFP distribution in cells expressing equivalent amounts of either the wild type His-9 or Pro-9 NPY2R (Fig. 5C). His-9 NPY2R by contrast to Pro-9 NPY2R only weakly recruits β-arrestin2 from the cytosol. Quantification by ELISA measurement of surface receptor expression (Fig. 5D) verifies a greater magnitude in endocytosis for the Pro-9 NPY2R variant. With 15 min of NPY stimulation, no wild type NPY2R internalization is measurable, but by contrast 17 ± 3% of the Pro-9 NPY2R internalizes. After 30 min, 14 ± 4% of His-9 NPY2R and 45 ± 4% of Pro-9 NPY2R internalize, a 3.3-fold augmentation of endocytosis.

Taken together, the loss and gain of function data for the four receptors indicate that a proline at second intracellular loop position 9 favors ligand-activated receptor recruitment of β-arrestin. Moreover, this property, as illustrated by the β2AR, occurs without changes in the extent of GRK phosphorylation of the receptor.

DISCUSSION

An important unrealized goal for understanding GPCR signal transduction lies in identifying the pieces comprising a common determinant for ligand-mediated interaction between the β-arrestin and each of the hundreds of GPCRs outside the visual system. A similar statement also applies to G proteins and GPCRs, and indeed a role for second loop hydrophobic residue 10 as a general site for G protein coupling has been proposed (40). We might expect that this β-arrestin determinant should be positioned independent of the variable GRK phosphorylation sites in receptor third loops and tails, compete with a general G protein binding determinant for receptor, and recognize, dock, and activate arrestin for every agonist-bound GPCR. Although candidate motifs from sequence and structural considerations alone have not been forthcoming, a clue may lie in the common behaviors receptor-β-arrestin complexes undergo upon ligand activation. The translocation of the receptor-β-arrestin complexes to coated pits is one behavior that relies on recognition and docking. If function recapitulates structure, then observing how conserved substitutions in analogous receptor segments affect β-arrestin recruitment and receptor endocytosis may serve to identify a portion of the common arrestin-binding determinant.

The DRY motif is a highly invariant segment in several hundred rho-
GPCR Second Loop and β-Arrestin Binding

We present evidence that the first 10 amino acids of the second intracellular loop beginning at the DRY motif are highly conserved when grouped according to type, charge, or hydrophobic nature. This conservation in the composition of such a large cohort of GPCRs suggests this loop region regulates a property common to all these receptors. Our data from several rhodopsin family receptors demonstrate functional gains and losses in endocytosis and arrestin binding occurring with substitution of second intracellular loop residue 9. Alanine or histidine replacement by proline in the β2AR and NPY2R, respectively, enhances these functions, whereas replacement by alanine at position 9 in the 5HT2CR, and the β2AR decreases both arrestin translocation and endocytosis.

We have demonstrated previously that mutation of second loop arginine 2 to histidine facilitates the agonist-independent association of several GPCRs with arrestins (15). Therefore, at least three proximate second loop positions 2 and 9 and presumably 10 (4), regulate a β-arrestin/GPCR interaction. Arginine 2 of the DRY motif and the conserved hydrophobic second loop residue 6 are buried within the receptor. Functionally, residues 1, 2, and 6 may serve to reorient the relative position of transmembrane domain III upon agonist interaction with its ligand-binding residues (12). In contrast, proline/alanine 9 locally centers an externally accessible patch of the receptor defined by the DRY motif tyrosine 3 and residues at positions 4, 7, 8, and 10. Our biochemical data taken together with sequence analysis data and the crystallographic findings in rhodopsin suggest that the proximal 10 residues in the second intracellular loop of a rhodopsin family GPCR provide both switching residues 1, 2, 5, and 6 and the binding residues 3, 4, and 7–10 to regulate ligand-activated binding with β-arrestin.

The substitution of Pro-9 by Ala-9 has a large functional effect on β-arrestin regulation for a variety of receptors despite a minimal and very localized change in the second loop conformation as calculated from the inactive structure of rhodopsin. Two distinct explanations accounting for decreases in β-arrestin binding subsequent to the proline mutation are as follows: 1) either residues immediately adjacent to Pro-9 and/or Pro-9 itself directly participate in β-arrestin binding, or 2) the Pro-9 mutation supports very different perturbations in the active conformations of the second loop.

In the nonactive Ala-9-substituted receptor, conformational states are constrained by the geometry and tethering imposed by transmembrane orientations. Although Ala-9 substitution does not appear to disrupt the general form of the more rigid, proline-based loop secondary structure, it should produce an increase in loop entropy, and therefore more local flexibility. By using rhodopsin as a template, computational modeling indicates that Ala-9 substitution can maintain a proline-like nonhelical conformation in the center of the loop while only minimally perturbing orientation of adjacent residues. However, agonist stimulation-induced movement of transmembrane helix VI relative to helix III for both rhodopsin and β2AR (45–47) could add another level of local flexibility in the vicinity of the Pro-9 residue. Thus, it would not be surprising that the gain of endocytic function observed with the Ala-9 to Pro-9 substitution could result from a constraint on the geometry of nearby loop residues, suggesting a direct involvement of these residues in arrestin binding.

This prediction is supported by the additional mutation of phenylalanine 10 to alanine in the β2AR (see also Ref. 40 for the effect on G protein signaling), which substantially prevents β-arrestin translocation to the agonist-activated receptor. Consequently, it appears that the profound effects of Pro-9 to Ala-9 mutation on β-arrestin binding might be mainly due to a direct involvement of residues surrounding Pro-9, perhaps the Pro itself. Nevertheless, we cannot rule out that the mutated i2 loop could be subjected to a greater conformational perturbation in the active versus inactive receptor state. Identifying the sites governing β-arrestin binding to receptors has more than a theoretical interest. This is no more evident than in the regulation of RNA-edited 5HT2c receptors, whose differing endocytic behaviors could be understood on the basis of discrete changes in the proximal portion of their second loops. Our data suggest that the conserved first half of the second intracellular loop contributes greatly to the formation of a receptor-β-arrestin complex that is dependent on agonist occupancy, whereas the more variable second half, although not directly involved in arrestin binding, may indirectly regulate arrestin interactions through phosphorylation and dephosphorylation of serine, threonine, or tyrosine residues (26, 48). The results presented here also support the idea that structural elements that are not subject to phosphorylation are exposed during receptor activation and contribute in forming a high affinity complex between GPCR and β-arrestin.

We propose a model in which the 10-amino acid segment of the proximal second intracellular loop provides binding determinants for arrestin recognition in addition to structural determinants for a transmembrane switch that mediates transition of the receptor from an inactive to an active state. Presumably, the general structural analogy between GPCRs suggests that nonidentical sequences underlying the same function are located relatively similar to the corresponding transmembrane domains. The existence of a common loop determinant linked to a ligand-dependent transmembrane molecular switch would offer a simple but elegant means to regulate signal transduction. Our findings suggest that the critical transmembrane position of the DRY motif and the seven subsequent residues that follow provide this mechanism for rhodopsin-like GPCRs.

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FEBRUARY 3, 2006 • VOLUME 281 • NUMBER 5
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