THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 2, Issue of January 13, pp. 720–731, 1995
Printed in U.S.A.

Arrestin Interactions with G Protein-coupled Receptors

DIRECT BINDING STUDIES OF WILD TYPE AND MUTANT ARRESTINS WITH RHODOPSIN, β_2-ADRENERGIC, AND m2 MUSCARINIC CHOLINERGIC RECEPTORS*

(Received for publication, July 8, 1994, and in revised form, November 14, 1994)

Vsevolod V. Gurevich‡, Stephane B. Dion‡, James J. Onorato§, Judith Ptasienski¶, Chong M. Kim‡, Rachel Sterne-Marr‡, M. Marlene Hosey¶, and Jeffrey L. Benovic‡‖

From the ¶Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611, the §Department of Medicine, University of Wisconsin, Madison, Wisconsin 53792, and the ‡Department of Pharmacology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Arrestins play an important role in quenching signal transduction initiated by G protein-coupled receptors. To explore the specificity of arrestin-receptor interaction, we have characterized the ability of various wild-type arrestins to bind to rhodopsin, the β_2-adrenergic receptor (β_2AR), and the m2 muscarinic cholinergic receptor (m2 mAChR). Visual arrestin was found to be the most selective arrestin since it discriminated best between the three different receptors tested (highest binding to rhodopsin) as well as between the phosphorylation and activation state of the receptor (>10-fold higher binding to the phosphorylated light-activated form of rhodopsin compared to any other form of rhodopsin). While β-arrestin and arrestin 3 were also found to preferentially bind to the phosphorylated activated form of a given receptor, they only modestly discriminated among the three receptors tested. To explore the structural characteristics important in arrestin function, we constructed a series of truncated and chimeric arrestins. Analysis of the binding characteristics of the various mutant arrestins suggests a common molecular mechanism involved in determining receptor binding selectivity. Structural elements that contribute to arrestin binding include: 1) a C-terminal acidic region that serves a regulatory role in controlling arrestin binding selectivity toward the phosphorylated and activated form of a receptor, without directly participating in receptor interaction; 2) a basic N-terminal domain that directly participates in receptor interaction and appears to serve a regulatory role via intramolecular interaction with the C-terminal acidic region; and 3) two centrally localized domains that are directly involved in determining receptor binding specificity and selectivity. A comparative structure-function model of all arrestins and a kinetic model of β-arrestin and arrestin 3 interaction with receptors are proposed.

Many cells have the ability to rapidly regulate their responsiveness to external stimuli. This phenomenon, often termed desensitization, has been extensively studied in signaling pathways initiated by G protein-coupled receptors. Two of the best studied G protein-coupled receptors are rhodopsin, which mediates phototransduction in retinal rod cells, and the β_2-adrenergic receptor (β_2AR)[1], which stimulates cAMP production in many tissues. The molecular events underlying desensitization of rhodopsin and the β_2AR include a rapid activation-dependent phosphorylation of the receptor mediated by the specific G protein-coupled receptor kinases rhodopsin kinase (1) and the β-adrenergic receptor kinase (βARK) (2), respectively. Receptor phosphorylation then promotes the binding of another protein to the receptor. This protein has been termed S-antigen or arrestin in the phototransduction system while in the β_2AR system a related protein termed β-arrestin has been implicated. The binding of arrestin to the phosphorylated receptor appears to play the primary role in quenching signal transduction via its apparent ability to directly decrease receptor/G protein coupling (3–6).

Recent studies suggest a significant diversity in the arrestin gene family with four mammalian arrestins having been identified to date (5, 7–12). The mammalian arrestins include visual arrestin, which is predominantly localized in rods and appears to play a primary role in quenching phototransduction (13, 14); β-arrestin, which may play a role in desensitization of the β_2AR (5, 6); arrestin 3 (also termed β-arrestin 2), a recently identified member of the family that may interact with the β_2AR and possibly odorant receptors (8–10, 15); and a recently cloned retinal-specific arrestin termed X-arrestin (11) or C-arrestin (12). Recent studies suggest that at least three of the mammalian arrestins may also be represented by multiple polypeptide variants (10, 16, 17). These variants include a form of β-arrestin, βarrS, that lacks 8 amino acids found in the originally identified bovine brain β-arrestin (βarr) (5, 10, 16). Interestingly, the 8 amino acids that distinguish these two forms of β-arrestin are encoded by exon 13 in the human visual arrestin gene (18). While a similar variant may also exist for visual arrestin (16), a variant that has an alanine in place of the C-terminal 35 amino acids has also been identified (17). In addition, a form of arrestin 3 (arr3L) that contains an 11-amino

* This research was supported in part by Grants GM44944 (to J. L. B.), GM47417 (to J. L. B.), GM47120 (to J. J. O.), and HL31601 (to M. M. H.) from the National Institutes of Health, a grant-in-aid from the American Heart Association (to M. M. H.), and a grant from the National Kidney Foundation of Wisconsin (to J. J. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ Established Investigator of the American Heart Association. To whom correspondence should be addressed to: Thomas Jefferson University, 233 South 10th St., Philadelphia, PA 19107. Tel.: 215-955-4607; Fax: 215-923-1098.

[1] The abbreviations used are: β_2AR, β_2-adrenergic receptor; arrestin, a generic term that includes all arrestins; arr, visual arrestin; βarr, β-arrestin; arr3, arrestin 3; βARK, β-adrenergic receptor kinase; G protein, guanine nucleotide binding protein; m2 mAChR, m2 muscarinic cholinergic receptor; mAChR*, carbachol-activated mAChR; P-mAChR, phosphorylated mAChR; P-mAChR*, phosphorylated carbachol-activated mAChR; β_2AR*, isoproterenol-activated β_2AR; P-β_2AR, phosphorylated β_2AR; P-β_2AR*, isoproterenol-activated phosphorylated β_2AR; Rh, dark rhodopsin; Rh*, light-activated rhodopsin; P-Rh, phosphorylated rhodopsin; P-Rh*, phosphorylated light-activated rhodopsin; S, short; L, long.

acid insert between residues 361 and 362 has also been identified (10). While the predominant form of βarr varies between tissues and arr3S is the predominant form of arr3 in most tissues, little is presently known about the functional significance of these polypeptide variants.

The tremendous diversity of the G protein-coupled receptors, as compared to the more limited number of G protein-coupled receptor kinases and arrestins, raises obvious questions concerning the receptor specificity of the various G protein-coupled receptor kinases and arrestins. Here we have compared the ability of visual arrestin, βarr, βarrS, arr3L, and arr3S to bind to four different functional forms (phosphorylated, phosphorylated and agonist activated, unphosphorylated, and unphosphorylated and agonist activated) of three functionally distinct receptors: rhodopsin, the β_2AR, and the m2 muscarinic cholinergic receptor (m2 mAChR). We have utilized these receptors since they each have been demonstrated to undergo an activation-dependent phosphorylation both *in vitro* and in intact cells (2, 19–23). Moreover, more recent studies suggest that each of these receptors is also able to interact with arrestins *in vitro* (4–6, 9, 24).

The recent progress in elucidating the molecular mechanisms involved in visual arrestin binding to phosphorylated light-activated rhodopsin (P-Rh*) (25–27), as well as the cloning of an increasing number of arrestins, raises several important questions concerning arrestin function. First, do other members of the arrestin family use the same sequential multisite binding mechanism as does visual arrestin? In addition, what domains of the arrestin molecule determine its receptor specificity and what structural elements of arrestins and receptors are involved? In an attempt to address these questions we have utilized two strategies of mutagenesis. First, several truncated forms of βarr and arr3, analogous to the previously characterized truncated forms of visual arrestin (arr) (25, 26), were produced and functionally characterized. This approach allowed us to establish a similar localization of the major functional domains in all arrestin proteins and suggested that the molecular mechanisms involved in their binding may also be similar. These findings set the stage for the construction of a series of arr/βarr chimeras. In constructing the chimeric arrestins we attempted to divide the arrestins into the four functional domains previously identified in visual arrestin (25, 26). These studies have enabled us to obtain further insight into the mechanism of arrestin-receptor interaction and the structural basis of arrestin specificity.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP, [^{35}S]dATP, and [^{3}H]leucine were purchased from DuPont NEN. All restriction enzymes were purchased from either Boehringer Mannheim or Promega. Sepharose 2B, Sephadex G-25, and all other chemicals were from Sigma. Rabbit reticulocyte lysate and SP6 RNA polymerase were prepared as described previously (25, 26). 11-*cis*-Retinal was generously supplied by Dr. R. K. Crouch, National Institutes of Health. Other reagents were from sources previously described (25, 26).

Plasmid Constructions—A bovine visual arrestin cDNA was generously supplied by Dr. T. Shinohara (7). An arrestin construct containing the wild type N terminus of arrestin (pARR) was subcloned into the vector pG2S6-I as described previously (24). The bovine β-arrestin, β-arrestin S, arrestin 3L, and arrestin 3S cDNAs (10) were excised with *Nco*I and *Hin*dIII and subcloned into *Nco*I/*Hin*dIII digested pG2S6-I (resulting plasmids were termed pBARR, pBARRS, pARR3L, and pARR3S, respectively). Plasmids were linearized with *Hin*dIII before *in vitro* transcription to obtain full-length mRNAs. Plasmids encoding chimeric arrestins were constructed as follows. pBBBA and pAAAB were obtained by exchange of 350- and 377-bp *Bst*XI-*Hin*dIII fragments of pARR and pBARR, respectively. pBAAA and pABBB were constructed by exchange of 1225- and 1274-bp *Bam*HI/*Hin*dIII fragments of pARR and pBARR, respectively. pBAAB and pABBA were constructed by subcloning 1252- and 1247-bp *Bam*HI/*Hin*dIII fragments of pAAAB and pBBBA, respectively, into *Bam*HI/*Hin*dIII digested pBARR and pARR, respectively. pBBAA and pAABB were constructed by exchange of 730-and 786-bp *Bgl*II/*Hin*dIII fragments of pARR and pBARR, respectively. pABAA and pBABB were obtained by subcloning 1218- and 1300-bp *Bam*HI/*Hin*dIII fragments of BBAA and AABB, respectively, into *Bam*HI/*Hin*dIII-digested pARR and pBARR, respectively. pBBAB and pAABA were constructed by subcloning 377- and 350-bp *Bst*XI/*Hin*dIII fragments of pBARR and pARR, respectively, into *Bst*XI/*Hin*dIII-digested pBBAA and pAABB, respectively. The sequences of all constructs were confirmed by DNA sequencing (the structures of the resulting proteins are schematically shown in Fig. 4). Plasmids were linearized with *Hin*dIII before *in vitro* transcription to obtain full-length mRNAs. To obtain truncated mRNAs, pARR and pBAAA were linearized with *Stu*I to obtain mRNAs encoding arr(1–191) and BAAA(1–187); pBARR and pABBB were linearized with *Hin*dII to obtain mRNAs encoding βarr(1–217) and ABBB(1–221); pBARR was linearized with either *Bgl*II or *Asp*HI to obtain mRNAs encoding βarr(1–206) and βarr(1–367), respectively; while pARR3S was linearized with either *Pvu*II or *Sfu*I to obtain mRNAs encoding arr3(1–183) and arr3S(1–375), respectively. *In vitro* transcription and translation was carried out as described previously (25, 26).

Receptor Preparations—Urea-treated rod outer segment membranes were prepared, phosphorylated with βARK, and regenerated with 11-*cis*-retinal as described (26). The stoichiometry of phosphorylation for the rhodopsin preparation used in these studies was 2.4 mol of phosphate/mol of rhodopsin. The human m2 mAChR was expressed in Sf9 cells, purified by affinity chromatography, and reconstituted into chick heart phospholipid vesicles as described previously (23). The reconstituted m2 mAChRs were phosphorylated with βARK to a stoichiometry of 2.8–3.7 mol of phosphate/mol of m2 mAChR as described previously (24). Both human and hamster β_2AR were expressed in Sf9 cells, purified by affinity chromatography, reconstituted into soybean phosphatidylcholine vesicles, and phosphorylated by βARK to stoichiometries of 2.4–4.5 mol of phosphate/mol of β_2AR as described previously (28). Activation of the receptors was achieved with room light for rhodopsin, 100 μM (−)-isoproterenol for the β_2AR, and 100 μM carbachol for the m2 mAChR.

Arrestin Binding to Receptors—The basis of the arrestin/receptor binding assay was to assess a change in the mobility of an arrestin on a gel filtration column upon binding to the receptor. To study binding to rhodopsin, the *in vitro* translated [^{3}H]arrestins were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 100 mM potassium acetate with 7.5 pmol of the various functional forms of rhodopsin in a volume of 50 μl for 5 min at 37 °C either in the dark or in room light. The samples were then cooled on ice and under dim red light were loaded onto a 2-ml Sepharose 2B column equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA (buffer A). Bound arrestin eluted with the rod outer segments in the void volume (between 0.5 and 1.1 ml). To study binding of the arrestins to the m2 mAChR or β_2AR, 200 fmol of the receptor (phosphorylated or nonphosphorylated) were incubated for 50 min at 30 °C in a 50-μl reaction with 50 fmol of the respective [^{3}H]arrestin and 100 μM of either the respective antagonist (atropine or alprenolol) or agonist (carbachol or isoproterenol) in 50 mM Tris-HCl, pH 7.5, 50 mM potassium acetate, 0.5 mM $MgCl_2$ (and 0.2 mM dithiothreitol for the mAChR). The samples were then cooled on ice and loaded onto a 2-ml Sepharose 2B column equilibrated with buffer A. The columns were washed with 0.5 ml of buffer A and the phospholipid vesicles containing the receptor and bound arrestin were then eluted with 0.6 ml of buffer A. When saturation isotherms were prepared, samples contained 50 fmol of phosphorylated m2 mAChR or β_2AR, 100 μM of the appropriate ligand, and various concentrations of the arrestins. Nonspecific binding was determined in the presence of 0.3–1 μg of control liposomes and did not exceed 15% of the total binding. For all of the arrestin binding experiments the nonspecific binding was subtracted.

RESULTS

Interaction of Arrestins with Rhodopsin, m2 Muscarinic Cholinergic, and β_2-Adrenergic Receptors—In an effort to further characterize the interaction of arrestins with G protein-coupled receptors, we have studied the binding of *in vitro* translated arrestin, β-arrestin (short and long forms), and arrestin 3 (short and long forms) to various functional forms of rhodopsin (Rh), the β_2AR, and the m2 mAChR. These functional forms include the phosphorylated receptors (P-Rh, P-β_2AR,

P-mAChR), phosphorylated and activated[2] receptors (P-Rh*, P-β_2AR*, P-mAChR*), non-activated receptors (Rh, β_2AR, mAChR), and activated receptors (Rh*, β_2AR*, mAChR*).

As previously demonstrated, visual arrestin preferentially binds to P-Rh* with a P-Rh*/P-Rh binding ratio of ~12 (Fig. 1*A*). Arr3S, arr3L, βarr, and βarrS demonstrate a 2–3-fold lower level of binding to P-Rh* compared to arr. In addition, β-arrestin and arrestin 3 also bind to rhodopsin less selectively compared to arrestin with P-Rh*/P-Rh binding ratios from 1.3 for arr3L to 2.1 for βarr. The selectivity profiles of βarr and arr3 binding to the m2 mAChR qualitatively resemble those seen with rhodopsin (Fig. 1*B*). All of the arrestins bind preferentially to P-mAChR* with arr3S binding being ~1.5-fold higher than arr3L, βarr, and βarrL, and ~19-fold higher than arr. The P-mAChR*/P-mAChR binding ratio is 1.6–1.9 for all arrestins, similar to that observed for βarr and arr3 binding to rhodopsin.

Since no direct binding studies of arrestin interaction with the β_2AR have been reported, we initially attempted to optimize the conditions to study βarr and arr3 binding to the β_2AR. Binding to the β_2AR was found to reach an apparent equilibrium following a 30-min incubation at 30 °C, which was maintained for at least another 30 min. The minimum phosphorylation level necessary for βarr and arr3S to recognize the phosphorylated form of the β_2AR was 2–3 mol of P_i/mol of β_2AR, while additional phosphorylation up to 10–11 mol/mol did not increase βarr or arr3S binding (not shown). The selectivity profiles of βarr, arr3, and arr binding to the β_2AR are very similar to those observed with the m2 mAChR (Fig. 1*C*). Arr3S demonstrates the highest binding, followed by arr3L, βarr, and βarrS. Arr binding to the β_2AR was 4–9-fold lower than the other arrestins. The discrimination of βarr and arr3 between the P-β_2AR* and P-β_2AR is also very similar to that observed for the m2 mAChR (1.5–1.8-fold).

Previously, we demonstrated that a conventional Scatchard analysis could be used to study βarr and arr binding to the P-mAChR* (24). More extensive analysis demonstrates that arr3S and arr3L (K_d = 0.35–0.38 nM), and βarr and βarrS (K_d = 0.5–0.6 nM) have similar affinities for the human P-m2 mAChR*, while arr (K_d = 7.2 nM) has a substantially lower affinity (Table I). The number of binding sites, determined in the presence of 50 fmol of P-mAChR*, was also substantially different among the arrestins with a B_{max} of 15–19 fmol for arr3S, arr3L, βarrS, and βarr and only ~7 fmol for arr. A similar analysis of arrestin binding to the human P-β_2AR* demonstrates that βarr and βarrS have the highest affinity (K_d = 0.14–0.19 nM), followed by arr3S and arr3L (K_d = 0.33–0.36 nM), and arr (K_d = 2.1 nM) (Table I). Thus, the affinity of βarr and βarrS for the P-β_2AR* is 3–4 times higher than for the P-mAChR*, while the affinity of arr3L and arr3S for both receptors is comparable. The number of binding sites, determined in the presence of 50 fmol of P-β_2AR*, was highest for arr3S (17 fmol), followed by arr3L (11 fmol), βarrS (7.1 fmol), arr (5.5 fmol), and βarr (4.1 fmol).

A prominent difference between the binding of visual arrestin and the non-visual arrestins is the substantially greater selectivity of visual arrestin for the phosphorylated and activated form of the receptor. While β-arrestin and arrestin 3 preferentially bind to the phosphorylated form of a given receptor, they appear to be less dependent on the activation state of the receptor. To further analyze this phenomenon we studied the binding of βarr and arr3S to the P-mAChR and P-β_2AR in the presence of either agonist or antagonist. These studies revealed that the agonist promotes a 2–3-fold increase in B_{max} for βarr and arr3S compared to the antagonist with no appreciable difference in the K_d (Table I). These results suggest that the agonist predominantly alters the number of phosphorylated receptors capable of interacting with a given arrestin, presumably by converting more receptors to an "active" conformation. However, it is clear that not all of the receptors are active even in the presence of agonist since the B_{max} is always

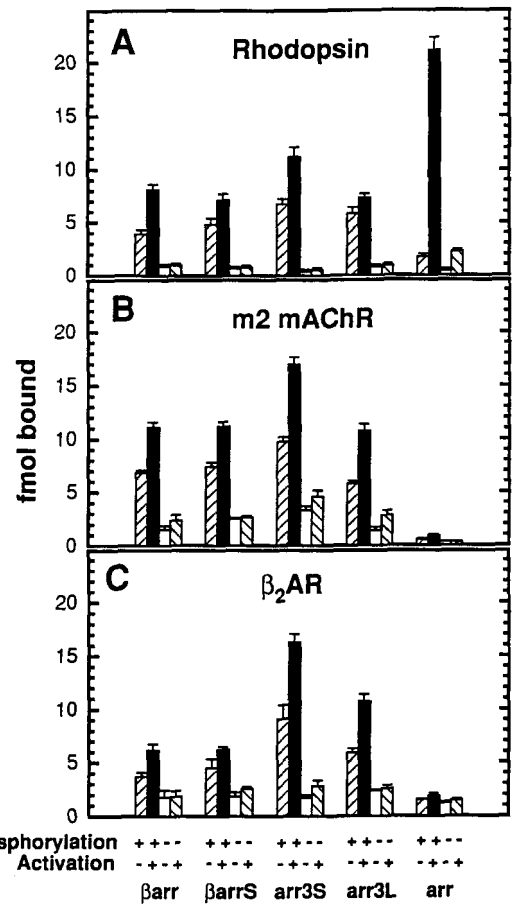

FIG. 1. **Interaction of *in vitro* translated arr, β-arr, β-arrS, arr3L, and arr3S with rhodopsin A.** 50 fmol of the various *in vitro* translated arrestins (1068–1205 dpm/fmol) were incubated with 7.5 pmol of the various functional forms of rhodopsin in 50 mM Tris-HCl, pH 7.5, 100 mM potassium acetate, 0.5 mM $MgCl_2$, 1.5 mM dithiothreitol in 50 μl for 5 min at 37 °C either in the dark or with illumination (room light). The samples were then cooled on ice before separation of bound and free arrestins on a 2-ml Sepharose 2B column as described under "Experimental Procedures." *B*, interaction of *in vitro* translated arrestins with the human m2 mAChR. 50 fmol of the various arrestins were incubated with 200 fmol of the different functional forms of the m2 mAChR and either 100 μM atropine or carbachol in 50 mM Tris-HCl, pH 7.5, 50 mM potassium acetate, 0.5 mM $MgCl_2$, 0.2 mM dithiothreitol in 50 μl at 30 °C for 50 min, cooled on ice, and chromatographed as described under "Experimental Procedures." The phosphorylation stoichiometry of the m2 mAChR used in these studies was 3.7 mol of phosphate/mol of m2 mAChR. *C*, interaction of *in vitro* translated arrestins with the human β_2AR. 50 fmol of the various arrestins were incubated with 200 fmol of the different functional forms of the β_2AR and 100 μM alprenolol or (−)-isoproterenol in 50 mM Tris-HCl, pH 7.5, 50 mM potassium acetate, 0.5 mM $MgCl_2$ in 50 μl at 30 °C for 35 min, cooled on ice and chromatographed as described under "Experimental Procedures." The phosphorylation stoichiometry of the β_2AR used in these studies was 2.4 mol of phosphate/mol of β_2AR. The specific binding (mean ± S.D. from two to four independent experiments performed in duplicate) is shown.

[2] We use the term *activated* to refer to the agonist-occupied receptor while *non-activated* refers to the antagonist-occupied receptor. However, this does not necessarily imply that all of the receptors are in a completely activated or non-activated conformation. We use the term *specificity* to refer to the ability of a given arrestin to bind to different receptors, while the term *selectivity* refers to the ability of an arrestin to discriminate among the different functional forms of a given receptor.

TABLE I
Receptor binding characteristics of wild type arrestins

Arrestin	P-m2 mAChR*		P-β_2AR*[a]	
	K_d	B_{max}	K_d	B_{max}
	nM	*fmol*[b]	*nM*	*fmol*[b]
βArr	0.48 ± 0.09	16.27 ± 1.62	0.14 ± 0.04	4.08 ± 0.26
βArr[c]	0.51 ±0.08	5.02 ± 0.82	0.18 ± 0.03	1.60 ± 0.16
βArrS	0.61 ± 0.11	15.02 ± 1.59	0.19 ± 0.04	7.10 ± 1.14
Arr3L	0.38 ± 0.04	15.95 ± 1.22	0.36 ± 0.06	11.11 ± 0.47
Arr3S	0.35 ± 0.03	18.87 ± 0.71	0.33 ± 0.08	17.12 ± 1.24
Arr3S[c]	0.48 ± 0.11	9.05 ± 0.66	0.35 ± 0.04	6.38 ± 0.95
Arr	7.20 ± 1.19	6.96 ± 0.98	2.07 ± 0.08	5.48 ± 1.08

[a] Purified human β_2AR was used in these experiments.
[b] In the presence of 50 fmol of respective receptor.
[c] In the presence of 100 μM antagonist (alprenolol or atropine) in the binding assay for human P-β_2AR or human P-m2 mAChR, respectively.

lower than the number of receptor molecules in the assay. The differences in B_{max} may reflect the ability of a given arrestin to promote the formation of and/or stabilize an agonist-phosphoreceptor-arrestin complex, analogous to the agonist-receptor-G protein ternary complex (29).

The relatively low B_{max} observed for arrestin binding (Table I) prompted us to search for experimental conditions that favored the formation of a high affinity agonist-receptor-arrestin complex. To this end we studied the effects of G protein βγ subunits on βarr and arr3S binding. For these studies the β_2AR and m2 mAChR were initially phosphorylated to a stoichiometry of 3–4 mol/mol in the absence of βγ subunits. When purified bovine brain βγ subunits (at a 1:1 ratio with receptor) were included in the binding assay they significantly increased the B_{max} of both βarr and arr3S binding to the P-mAChR* (31 and 38%, respectively), without effecting the K_d. Similar experiments with the P-β_2AR* revealed that βγ subunits had no effect on either the K_d or B_{max} of βarr or arr3S binding. Thus, it appears that βγ subunits do not directly interact with arrestins since the effects observed are similar for both βarr and arr3S and are strictly receptor-dependent. Conceivably, βγ subunits are able to bind to the P-m2 mAChR* shifting the equilibrium toward the high-affinity arrestin-phosphoreceptor complex.

In an attempt to resolve different interactions involved in arrestin-receptor binding we compared the salt sensitivity of βarr and arr3 binding to P-β_2AR, P-β_2AR*, P-mAChR, and P-mAChR* (Fig. 2, *upper panel*). The binding of βarr and arr3S to P-β_2AR* is modestly stimulated at physiological salt concentrations followed by slight inhibition at higher ionic strength. This resembles the effects of ionic strength on visual arrestin binding to P-Rh* (26), and suggests that both ionic (inhibited by salt) and hydrophobic (stimulated by salt) interactions are involved in βarr and arr3S binding to the P-β_2AR*. Physiological salt concentrations also stimulate βarr and arr3S binding to the P-mAChR*, while high salt significantly inhibits binding (Fig. 2, *upper panel*). This again suggests a role for both ionic and hydrophobic interactions, although both effects are more pronounced compared to the β_2AR. Thus, the contribution of ionic and hydrophobic interactions involved in arrestin binding appears to be determined in part by the receptor. However, these data demonstrate that both βarr and arr3 possess the sites necessary for high affinity interaction with either the P-β_2AR* or P-mAChR*. Previous studies have demonstrated that visual arrestin binding to dark P-Rh is predominantly mediated by ionic interactions since it is very sensitive to salt inhibition (26). In contrast, when the effect of receptor activation was tested on βarr and arr3S binding to the P-β_2AR, no appreciable difference in salt sensitivity was observed between P-β_2AR and P-β_2AR* (Fig. 2, *upper panel*). A similar salt sensitivity was also observed for βarr and arr3S binding to the P-mAChR* and P-mAChR (Fig. 2, *upper panel*). These results

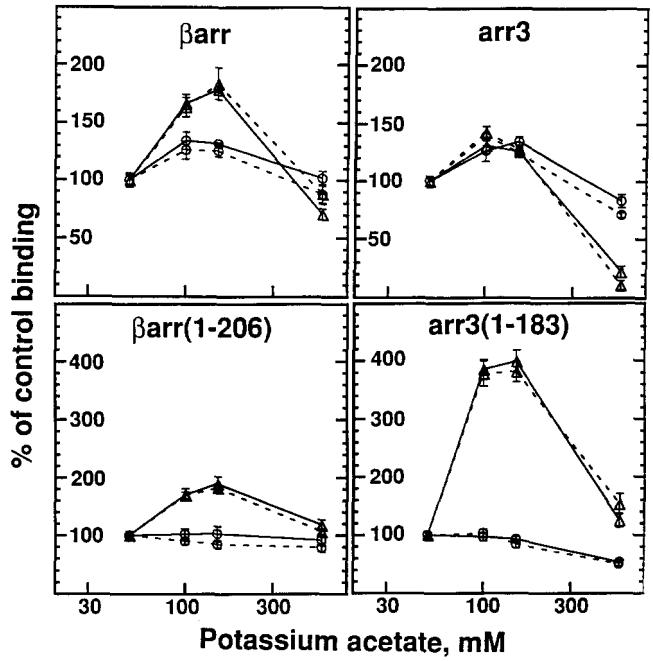

FIG. 2. **Salt effects on βarr, arr3S, βarr(1–206), and arr3(1–183) binding to hamster P-β_2AR (circles) and human P-m2 mAChR (triangles) in the presence of 100 μM of the agonists isoproterenol or carbachol (*solid lines*) or the antagonists atropine or alprenolol (*dotted lines*).** All assay conditions were as in the legend to Fig. 1 except for the potassium acetate concentration which varied from 50 to 500 mM. The nonspecific binding was determined at all salt concentrations (it decreased with an increase in salt) and subtracted. The specific binding (mean ± S.D. from two to three independent experiments performed in duplicate) is shown as the percentage of control binding determined at 50 mM potassium acetate.

suggest that similar interactions mediate the binding of both arrestins to the phosphorylated β_2AR and m2 mAChR regardless of the activation state of the receptor. This result is in agreement with the similar affinities of βarr and arr3 for the phosphorylated and phosphorylated/activated forms of the β_2AR and m2 mAChR (Table I).

Truncation Mutagenesis of β-Arrestin and Arrestin 3—Previous structure-function studies have suggested that there are at least four functional domains within the visual arrestin molecule (25, 26). These four domains include an N-terminal regulatory region; an N-terminal binding domain containing activation- and phosphorylation-recognition sites (residues 1–191); a C-terminal binding domain that interacts only when the activation- and phosphorylation-recognition sites are engaged (residues 191–365); and a C-terminal regulatory region that maintains the overall conformation of arrestin, possibly via direct interaction with the N-terminal regulatory region (27).

In order to initially probe the structural domains involved in βarr and arr3 interaction with G protein-coupled receptors, we utilized truncation mutagenesis, an approach previously used to study visual arrestin interaction with rhodopsin (25, 26). The truncated proteins produced in this study were βarr(1–367) and arr3S(1–375), which lack the putative regulatory C-terminal region (~40 amino acids) identified in arr (24–26), and βarr(1–217), βarr(1–206), and arr3(1–183), which lack the C-terminal half of the molecule. Previously we demonstrated that similarly truncated arr(1–191) largely retains its phosphorylation- and activation-recognition sites, two primary regions involved in arr interaction with rhodopsin (25, 26, 30).

Deletion of the C terminus of βarr to produce βarr(1–367) increases binding to all functional forms of rhodopsin, the m2 mAChR, and the β_2AR, with the predominant effect observed

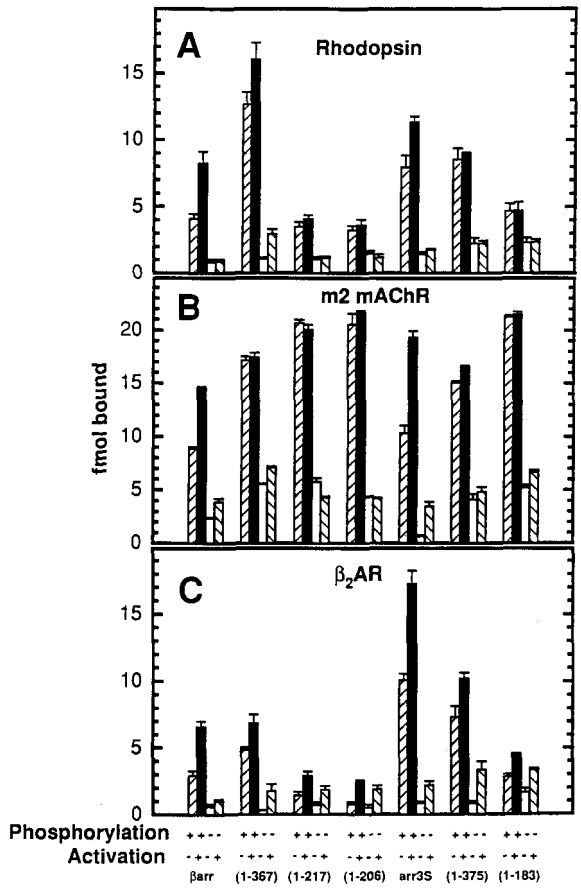

FIG. 3. **Interaction of full-length and truncated arrestins with rhodopsin (*A*), human m2 mAChR (*B*), and hamster β_2AR (*C*).** 50 fmol of βarr, βarr(1–367), βarr(1–217), βarr(1–206), arr3S, arr3(1–375), or arr3(1–183) (1006–1485 dpm/fmol) were incubated with receptor and then treated as described under "Experimental Procedures." The specific binding (mean ± S.D. from two to four independent experiments performed in duplicate) is shown.

on the phosphorylated and the activated forms of the receptor (Fig. 3). The analogous deletion in arr3S to generate arr3S(1–375) modestly increases binding to the activated form of the various receptors while decreasing binding to the phosphorylated/activated receptors. Thus, a major effect of C-terminal truncation of βarr and arr3S is to reduce the selectivity of the arrestin for the phosphorylated/activated form of the receptor. These results are very similar to the effects of C-terminal truncation of arr (25, 26), suggesting that the C terminus of βarr, arr3S, and arr plays a similar functional role.

The deletion of the entire C-terminal half of βarr and arr3S substantially decreases the binding to rhodopsin and the β_2AR, while increasing the binding to the m2 mAChR (Fig. 3). In general, the interaction of βarr(1–217), βarr(1–206), and arr3(1–183) with all three receptors demonstrate a uniformly lower selectivity compared to the wild type arrestins. However, it is noteworthy that for interaction with rhodopsin and the m2 mAChR, it is the activation-recognition of the truncated βarr and arr3 that is predominantly impaired, while for interaction with the β_2AR it is the phosphorylation-recognition that is primarily impaired.

The deletion of the C-terminal ~40 residues (βarr(1–367) and arr3S(1–375)) appears to decrease the affinity of βarr and arr3S for binding to human P-mAChR* and hamster P-β_2AR* (Table II). Since this region is likely not involved directly in receptor interaction (24–26), these data underscore the importance of this domain in keeping the proper functional conformation of βarr and arr3S. The deletion of the entire C-terminal

TABLE II
Receptor binding characteristics of truncated arrestins

Arrestin	P-m2 mAChR*		P-β_2AR[a]	
	K_d	B_{max}	K_d	B_{max}
	nM	*fmol*[b]	*nM*	*fmol*[b]
βArr	0.48 ± 0.09[c]	16.27 ± 1.62[c]	0.06 ± 0.01	3.41 ± 0.05
βArr(1–367)	0.96 ± 0.16	44.71 ± 4.59	0.23 ± 0.03	4.64 ± 0.48
βArr(1–217)	1.91 ± 0.21	37.89 ± 2.70	0.69 ± 0.07	3.95 ± 0.76
βArr(1–206)	1.94 ± 0.22	42.97 ± 5.53	0.71 ± 0.08	4.29 ± 0.12
Arr3S	0.35 ± 0.03[c]	18.87 ± 0.71[c]	0.23 ± 0.05	14.98 ± 1.45
Arr3(1–375)	0.91 ± 0.18	19.95 ± 3.47	0.36 ± 0.08	4.94 ± 0.49
Arr3(1–183)	2.01 ± 0.28	34.02 ± 2.96	0.80 ± 0.07	4.33 ± 0.37

[a] Purified hamster β_2AR was used in these experiments.
[b] In the presence of 50 fmol of respective receptor.
[c] Values are from Table I.

half (βarr(1–217), βarr(1–206), and arr3(1–183)) further impairs the affinity of both βarr and arr3 for P-mAChR* and P-β_2AR* (Table II). In contrast, the effect of C-terminal truncations of βarr and arr3S on the B_{max} for binding to P-mAChR* and P-β_2AR* is dramatically different between the receptors. Truncations of βarr modestly increase the B_{max} for the P-β_2AR* while truncations of arr3S significantly decrease the B_{max} (Table II). By comparison, all of the C-terminal truncations of βarr dramatically increase the B_{max} for the P-mAChR* while only arr3(1–183) was affected in a similar manner. It is noteworthy that the B_{max} for 4 out of the 5 truncated arrestins was increased to 70–90% of the level of P-mAChR* present in the assay (Table II). This demonstrates that most, if not all, of the receptors are accessible for binding arrestins and suggests that unavailability of receptors is not the reason for the relatively low B_{max} measured for full-length βarr and arr3 (Tables I and II).

In order to compare the interactions involved in the binding of truncated arrestins to phosphorylated receptors in the presence of agonists and antagonists, we studied the salt sensitivity of βarr(1–206) and arr3(1–183) binding to P-β_2AR and P-mAChR (Fig. 2, *lower panel*). In contrast to the binding of the corresponding full-length arrestins, the binding of βarr(1–206) and arr3(1–183) to the P-β_2AR* is not stimulated by salt, suggesting that a substantial portion of the hydrophobic interaction observed with βarr and arr3S is contributed by the C-terminal domain. In contrast, physiological salt concentrations stimulate the binding of both βarr(1–206) and arr3(1–183) to the P-mAChR* suggesting that the N-terminal domain significantly contributes to the hydrophobic interaction. Thus, the contribution of ionic and hydrophobic interactions involved in full-length and truncated arrestin binding appears to be determined in part by the receptor.

In contrast to arr-rhodopsin interactions (26), the salt sensitivity for βarr, arr3S, βarr(1–206), and arr3(1–183) binding to the activated or non-activated P-β_2AR and P-mAChR were found to be very similar (Fig. 2). These results suggest that similar interactions mediate the binding of all arrestins to the phosphorylated β_2AR and m2 mAChR regardless of the activation state of the receptor.

Structure-Function Analysis Using Arr/βArr Chimeras—Truncation mutagenesis of arr, βarr, and arr3 suggests that the localization of the major functional domains in all arrestins is similar. In an attempt to further elucidate the role of these various domains in arrestin function, we generated a series of 12 chimeric arrestins that were roughly divided into the four functional regions of arr and βarr (Fig. 4, *left panel*). The chimeras are designated using a four-letter description of their structure, where each letter describes the origin of the four respective regions in the chimeric molecule (A stands for arr, B for βarr). For example, the chimera ABBB contains the N-terminal regulatory region of arr and the N- and C-terminal

Arrestin	P-mAChR* Kd, nM	P-mAChR* Bmax, fmol	P-β2AR* Kd, nM	P-β2AR* Bmax, fmol
βarr	0.48±0.09	16.27±1.62	0.14±0.04	4.08±0.26
arr	7.20±1.19	6.96±0.98	2.07±0.08	5.48±1.08
BBBA	0.51±0.19	19.01±1.55	0.12±0.03	6.31±0.64
AAAB	12.20±2.10	7.63±1.52	ND	ND
ABBB	1.13±0.26	14.46±1.29	1.57±0.14	33.30±2.44
BAAA	7.78±1.57	10.04±1.41	ND	ND
ABBA	0.47±0.05	7.76±0.61	0.38±0.06	11.04±0.94
BAAB	6.25±1.47	4.95±0.85	ND	ND
BBAA	1.38±0.26	5.09±0.41	ND	ND
AABB	2.11±0.09	6.56±1.17	ND	ND
ABAA	3.10±0.81	2.28±0.32	ND	ND
BABB	1.25±0.29	4.28±0.49	ND	ND
BBAB	2.93±0.46	7.06±0.71	ND	ND
AABA	3.07±0.42	7.54±1.45	ND	ND

FIG. 4. **Structure and binding of chimeric arrestins to human P-mAChR* and P-β_2AR*.** The portion of the chimera derived from βarr is *shaded* in the schematic. The four structural elements of the chimeric arrestins are: 1) N-terminal region: residues 1–47 of arr or 1–43 of βarr (49% identity, 64% similarity); 2) N-terminal binding domain: residues 48–213 of arr or 44–207 of βarr (60%, 74%); 3) C-terminal binding domain: residues 214–345 of arr or 208–340 of βarr (60%, 77%); 4) regulatory C-terminal region: residues 346–404 of arr or 341–418 of βarr (32%, 39%). The average K_d and B_{max} from two to three experiments (± S.D.) is presented. *ND*, not determined.

binding and C-terminal regulatory regions of βarr.

We initially assessed chimera binding to rhodopsin. Analysis of wild type arrestin demonstrates a relatively low level of βarr binding to P-Rh* compared to arr. βArr also does not discriminate between Rh* and Rh (as compared to a 3–4-fold discrimination for arr). Moreover, βarr binds with low (~2-fold) selectivity for P-Rh* *versus* P-Rh (in contrast to an ~14-fold difference for arr). When chimeras that varied in their C-terminal regulatory region were compared, the only chimera virtually indistinguishable from arr was AAAB, which has the C-terminal regulatory region of βarr (Fig. 5*A*). These data further corroborate the hypothesis that this region is not directly involved in rhodopsin interaction (24–27) and demonstrate that the βarr C terminus is capable of fulfilling the functions of its arr counterpart. Chimera BBBA was also found to be very similar to βarr, although its binding to P-Rh and P-Rh* is somewhat higher. Both chimeras were found to bind to Rh* better than to Rh, while the P-Rh*/P-Rh binding ratio is βarr-like (2.3) for BBBA and arr-like (14.4) for AAAB.

The substitution of the N-terminal regulatory region of βarr into arr to generate the chimera BAAA significantly decreases binding to P-Rh* (compared to arr binding), but does not impair the preference for Rh* over Rh or the P-Rh*/P-Rh binding ratio (14.2). The symmetric substitution in βarr to generate the chimera ABBB substantially improves its binding to P-Rh* (compared to βarr) while the P-Rh*/P-Rh binding ratio remains βarr-like (1.5). Simultaneous exchange of both the N- and C-terminal regulatory regions yields the chimeras ABBA and BAAB which have comparable binding to P-Rh* (~50% of the arr binding level). The P-Rh*/P-Rh binding ratios of these chimeras appear to be determined by the origin of the central binding domains, being βarr-like (2.7) for ABBA and arr-like (15.2) for BAAB. These data suggest that while the N-terminal region has some direct participation in rhodopsin binding, the middle portion of the molecule determines the overall binding characteristics of the chimera.

We also constructed a series of 6 chimeras containing two central binding domains of different origin. The first pair of these (BBAA and AABB) demonstrates virtually no preference between Rh* and Rh and have P-Rh*/P-Rh binding ratios of 4.4 and 4.9, respectively (*i.e.* intermediate between βarr and arr). While the binding of AABB to P-Rh* is ~70% that of arr, BBAA binding to P-Rh* is only ~25% that of arr and is even lower than βarr binding. The chimeras ABAA and BABB demonstrate P-Rh*/P-Rh binding ratios of 5.6 and 5.5; however, their binding to P-Rh* was reduced compared to the binding of BBAA and AABB. Chimera AABA has a P-Rh*/P-Rh binding ratio of 3.7 while the symmetric chimera BBAB binds very poorly to P-Rh* and demonstrates no preference for P-Rh* over P-Rh. Thus, most of the chimeras that contain one binding domain from βarr and one from arr demonstrate a binding selectivity between that of βarr (~2-fold) and arr (~14-fold). The three chimeras with the structure *X*BA*X* (where *X* can be either A or B) demonstrate an unexpectedly low binding to P-Rh* (*i.e.* even lower than the corresponding arrestins with the structure *X*BB*X*) (compare BBAA with BBBA). In contrast, in all other chimeras a change from *XX*B*X* to *XX*A*X* enhances binding to P-Rh* as might be expected (compare AABB with AAAB).

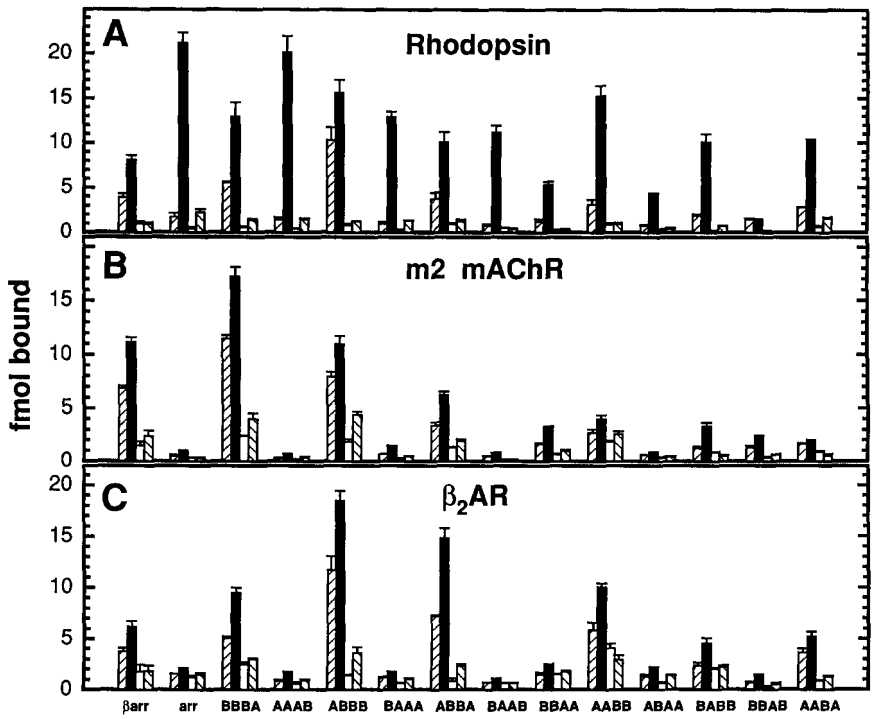

FIG. 5. **The binding of β-arr, arr, and 12 chimeric arrestins to rhodopsin (*A*), human m2 mAChR (*B*), and human β_2AR (*C*).** 50 fmol of the respective [^{3}H]arrestins (953–1177 dpm/fmol) were incubated with phosphorylated, phosphorylated activated, unphosphorylated, and unphosphorylated activated forms of the indicated receptors as described under "Experimental Procedures." The specific binding (mean ± S.D. from two to four independent experiments performed in duplicate) is shown.

When chimera binding to the m2 mAChR is compared, the most noticeable difference between βarr and arr is the extremely low level of arr binding to P-mAChR* (Fig. 5*B*). This is reflected in both a higher affinity of βarr for P-mAChR* (K_d = 0.48 nM) compared to arr (K_d = 7.2 nM) and a higher B_{max} for βarr (16.3 fmol) compared to arr (7.0 fmol) (Fig. 4). However, while the levels of βarr and arr binding to the m2 mAChR are very different, the selectivity of the binding (P-mAChR*/P-mAChR ratio) is similar. Substitution of the arr C terminus into βarr (BBBA) increases binding to all forms of the m2 mAChR without significantly affecting the affinity, while substitution of the N terminus (ABBB) has minimal effect on the binding but decreases the affinity ~2.4-fold (Figs. 4 and 5*B*). When both the N- and C-terminal regulatory domains are changed (ABBA) the binding is decreased ~2-fold without a change in affinity. The symmetric series of substitutions in arr (AAAB, BAAA, and BAAB) do not significantly improve arr binding to the m2 mAChR and AAAB actually binds with an affinity ~1.7-fold lower than that of arr. Overall, substitutions of either the C- or N-terminal regulatory regions lead to modest changes (≤2-fold) in affinities and B_{max} for binding to the P-mAChR*.

Chimeras BBAA and AABB demonstrate comparable binding to P-mAChR* (between that of βarr and arr), with BBAA being more selective than AABB (Fig. 5*B*). BBAA also has a modestly higher affinity for the P-mAChR* compared to AABB (K_d = 1.38 *versus* 2.11 nM, respectively) (Fig. 4). Chimera BABB is similar to BBAA in terms of selectivity profile and affinity (K_d = 1.25 nM) even though these proteins have the opposite binding domain combinations (Figs. 4 and 5*B*). In contrast, chimera ABAA, which has the same combination of binding domains as BBAA, demonstrates very poor selectivity, a low affinity (K_d = 3.1 nM) and a low B_{max} (2.3 fmol). Thus, the structure *X*BA*X* is as unfavorable for binding to the m2 mAChR as it is for binding to rhodopsin. The binding of chimera BBAB to the P-mAChR* most resembles the binding profile of BBAA although the affinity of BBAB is ~2-fold lower. While the chimera AABA most resembles AABB structurally, the B_{max} of AABA is significantly increased over that of AABB although there is a modest reduction in affinity (Fig. 4). Interestingly, AABA demonstrates almost as poor a discrimination between P-mAChR* and P-mAChR as did the symmetric chimera BBAB between P-Rh* and P-Rh (Fig. 5, *A* and *B*). Thus, both central binding domains play an important role in arrestin interaction with the m2 mAChR, in agreement with the rhodopsin binding studies.

In general, the binding of βarr and arr to the P-β_2AR* most resembles the P-mAChR* binding profiles (Fig. 5). This is seen in both the selectivity profile (P-β_2AR*/P-β_2AR binding ratio of ~2) and the ~15-fold higher affinity of βarr (K_d = 0.14 nM) compared to arr (K_d = 2.1 nM) (Fig. 4). However, in contrast to the m2 AChR binding studies, the B_{max} for arr and βarr binding to the P-β_2AR* are similar (Fig. 4). The chimeric arrestin studies demonstrate that substitution of the C terminus of βarr (BBBA) again modestly increases the B_{max} without changing the affinity (K_d = 0.12 nM) (Figs. 4 and 5*C*). Substitution of the arr C terminus (AAAB), N terminus (BAAA), or both (BAAB) again does not improve the binding to the β_2AR (Fig. 5*C*). However, substitution of the arr N terminus into βarr (ABBB) dramatically reduces the affinity (~11-fold) and increases the B_{max} (~8-fold) compared to βarr (Fig. 4). Substitution of both the N and C terminus of arr into βarr (ABBA) has a less dramatic reduction in affinity (~3-fold) and increase in B_{max} (~3-fold) (Fig. 4). These results suggest that the N terminus plays a direct role in βarr interaction with the β_2AR. An examination of the central binding domains reveals that, as expected, chimeras containing both βarr-derived binding domains bind better to the P-β_2AR* as compared to chimeras containing a combination of βarr- and arr-derived binding domains (Fig. 5*C*). The structural pattern *X*BA*X* appears to be even less favorable for binding to the β_2AR than it was for rhodopsin and the m2 mAChR, since BBAA, ABAA, and BBAB all demonstrate lower binding to the β_2AR than the corresponding symmetric chimeras AABB, BABB, and AABA.

In an attempt to more directly assess the role of the N-terminal half of arr and βarr in receptor interaction, we compared the binding of truncated arr(1–191), βarr(1–217), ABBB(1–221), and BAAA(1–187) (Fig. 6). In contrast to the corresponding full-length proteins (Fig. 5*A*), there is no dramatic difference between the ability of arr(1–191), βarr(1–217)

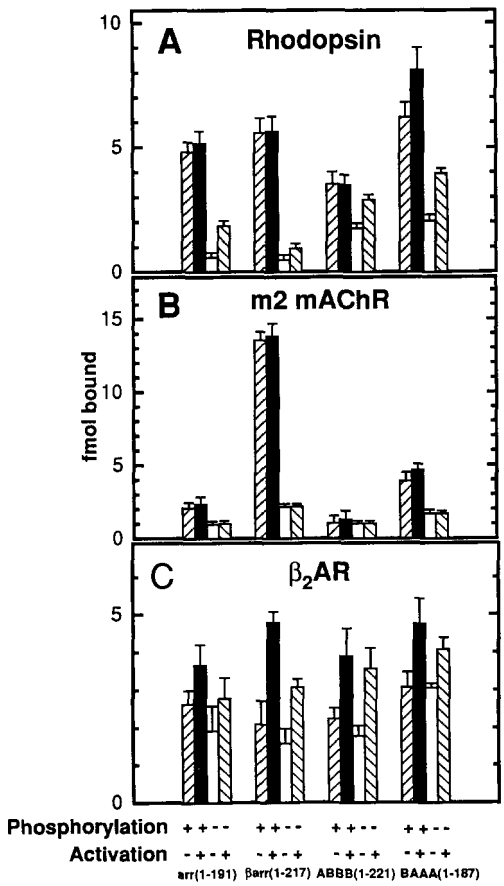

FIG. 6. **Truncated arr, βarr, and chimeric arrestin interaction with receptors.** 50 fmol of the indicated truncated arrestins were incubated with the indicated functional form of rhodopsin (*A*), human m2 mAChR (*B*), or human β_2AR (*C*), as described under "Experimental Procedures." The specific binding (mean ± S.D. from two to three independent experiments performed in duplicate) is shown.

and the truncated chimeras to bind to rhodopsin, although the chimeras appear to have a reduced preference for the phosphorylation state of the receptor (Fig. 6*A*). However, the binding levels of all truncated arrestins are lower than that of the corresponding full-length proteins (compare Figs. 5*A* and 6*A*), underscoring the important role of the C-terminal binding domain. Binding of the truncated arrestins to the m2 mAChR reveals that only βarr(1–217) demonstrates high binding and selectivity, while ABBB(1–221) and BAAA(1–187) do not significantly differ from arr(1–191). These data suggest that both the N terminus and N-terminal binding domain are required for high affinity interaction with the P-mAChR*. ABBB binding to the P-mAChR* is not significantly different from βarr (Fig. 5*B*), again underscoring the important role of the C-terminal binding domain. The binding of all four truncated proteins to the β_2AR is also surprisingly similar (Fig. 6*C*), unlike the binding of the full-length proteins (Fig. 5*C*). The selectivity profiles of both truncated chimeras resemble that of βarr(1–217), while arr(1–191) is less selective. Interestingly, while the binding of βarr(1–217) and ABBB(1–221) to the P-β_2AR* is lower than that of the full-length proteins, the binding of arr(1–191) and BAAA(1–187) is comparable to the full-length proteins (compare Figs. 5*C* and 6*C*). This suggests that the C-terminal binding domains of arrestin and BAAA do not significantly interact with the β_2AR. In general, both truncated chimeras and arr(1–191) demonstrate a greater specificity for rhodopsin and the β_2AR compared to the m2 mAChR. This may correlate with the localization of the relevant phosphorylation sites in these receptors and suggests that the specificity of wild type and chimeric arrestins is determined to a large extent by the C-terminal binding domain.

DISCUSSION

Rhodopsin, β_2AR, and m2 mAChR represent three distinct subfamilies of G protein-coupled receptors. Rhodopsin via its specific light-promoted interaction with the G protein transducin activates cGMP phosphodiesterase, the β_2AR via interaction with G_s activates adenylyl cyclase, and the m2 mAChR via its association with G_i inhibits adenylyl cyclase and activates K^+ channels. There are also distinct structural differences among these receptors (reviewed in Ref. 31) that likely play a role in arrestin binding. One distinction is the localization of relevant phosphorylation sites on the receptors. Another significant difference is the size and amino acid sequence of the third cytoplasmic loop and C-terminal domains. These domains have previously been implicated in receptor interaction with G proteins (32–38), G protein-coupled receptor kinases (39–44), and more recently in visual arrestin interaction with rhodopsin (30). Rhodopsin has a relatively short third cytoplasmic loop (29 residues) and a C terminus (39 residues) that contains all of the rhodopsin kinase phosphorylation sites. The third cytoplasmic loop of the β_2AR is intermediate in size (54 residues) and most (if not all) of the βARK phosphorylation sites are localized in the C terminus (84 residues). In contrast, the third cytoplasmic loop of the m2 mAChR is much longer (181 residues) and it is this loop, rather than the short C terminus (23 residues), that appears to contain all of the βARK phosphorylation sites (45).

Receptor Specificity of Arrestins

Arrestin was found to bind very specifically to phosphorylated/light-activated rhodopsin, with only weak binding to P-mAChR* and P-β_2AR* (Fig. 1). Interestingly, the affinity of arrestin for P-β_2AR* (K_d = 2.1 nM) is about 3.5-fold higher than its affinity for P-mAChR* (K_d = 7.2 nM), which correlates with the structural similarities between the β_2AR and rhodopsin. Both β-arrestin and arrestin 3 demonstrate very high affinities for the β_2AR and m2 mAChR (0.14–0.51 nM). However, β-arrestin has a 3–4-fold higher affinity for the P-β_2AR* *versus* the P-mAChR* suggesting that β-arrestin may be more specific for the β_2AR and other structurally related receptors. Arrestin 3 has a similar affinity for P-mAChR* and P-β_2AR* (0.33–0.38 nM) suggesting that arrestin 3 may be less specialized than arrestin and β-arrestin, perhaps serving as a generic arrestin in some non-visual cells. The most striking specificity observed is that of arr toward P-Rh*. This suggests that there are certain features shared by the β_2AR and m2 mAChR that preclude high affinity binding of visual arrestin. Since the cytoplasmic domains of both the β_2AR and m2 mAChR are substantially bulkier than the corresponding domains of rhodopsin, perhaps the receptor-binding pocket of visual arrestin is too small for the β_2AR and m2 mAChR. This hypothesis is further corroborated by the characteristics of arr/βarr chimeras (Figs. 4 and 5). Both the size of crucial arrestin binding cytoplasmic domains and the localization of relevant phosphorylation sites may also determine the relative specificity of β-arrestin toward the β_2AR, while arrestin 3 appears to be less sensitive to structural differences between receptors. In these studies we did not detect any striking functional differences between the variant forms of β-arrestin or arrestin 3, although both βarrS and arr3S appear to bind to a higher percentage of the P-β_2AR* compared to the longer polypeptide forms (Fig. 1, Table I).

Receptor Phosphorylation Levels Required for High-affinity Arrestin Binding

Receptor phosphorylation appears to be a prerequisite for arrestin interaction. Previous studies have demonstrated that only 2 mol of phosphate/mol of receptor are critical for high affinity arrestin binding to rhodopsin (26), the m2 mAChR (24), and the β_2AR (this study). Interestingly, 2 residues (serines 338 and 343) were recently identified as the major rhodopsin kinase phosphorylation sites in the rhodopsin C terminus (46). These data suggest that there may well be only a few basic residues within the phosphorylation-recognition domain of the arrestins that are involved in phosphoamino acid interaction. The extensive additional receptor phosphorylation demonstrated *in vitro* (each of these receptors can be phosphorylated by their respective receptor kinases to stoichiometries of 8–12 mol/mol) might conceivably serve as a backup mechanism *in vivo* to directly impair receptor/G protein coupling.

The Role of Arrestin Functional Regions in Receptor Interaction

The general molecular architecture of βarr and arr3 appears to be similar to that of arr (Figs. 2 and 3, Table I). Thus, the construction and characterization of chimeric arrestins served as a feasible approach to further elucidating the regions involved in determining receptor specificity. Moreover, the large number of chimeric arrestins studied enabled an assessment of the role of each individual region by comparison of the binding of several pairs of chimeras that differed only in a particular region.

Regulatory C-terminal Region—There are 5 pairs of chimeras that result from a substitution of this region of βarr into arr (arr to AAAB, BBAA to BBAB, BAAA to BAAB, ABBA to ABBB, and BBBA to βarr). The effects of this substitution do not appear to follow any particular pattern since this change can lead to a decrease (BBAA/BBAB and BBBA/βarr), an increase (ABBA/ABBB), or no change (arr/AAAB and BAAA/BAAB) in binding to P-Rh* (Figs. 4 and 5). The P-Rh*/P-Rh binding ratio also either does not change (arr/AAAB, BBBA/βarr, and BAAA/BAAB) or decreases (ABBA/ABBB and BBAA/BBAB). Similar non-discriminate differences in the binding patterns, affinities, and B_{max} of these chimeras for the P-mAChR* and P-β_2AR* are also observed. Thus, the effect of substitution of this region appears to be determined by the combination of the other functional domains, suggesting that the C-terminal regulatory region does not directly participate in receptor interaction. However, the C-terminal region clearly plays an important role in arrestin binding since partial deletion of this region dramatically changes the functional characteristics of both arr (25, 26) and βarr (Fig. 3, Table II). Moreover, the similar binding of βarr and BBBA on the one hand, and arr and AAAB on the other, suggests that the C terminus of βarr is capable of fulfilling the functions of its arr counterpart and vice versa. While the C terminus is the most divergent region in the arrestins (5, 7–12), these results suggest that the majority of the functionally important residues in this region are conserved between arr and βarr.

Regulatory N-terminal Region—There are also 5 pairs of chimeras that differ only in the origin of this region (arr to BAAA, AABB to BABB, ABAA to BBAA, ABBA to BBBA, and ABBB to βarr). Comparison of these chimeras reveals a more uniform pattern of changes. While binding to P-Rh* either decreases (arr/BAAA, AABB/BABB, and ABBB/βarr) or slightly increases (ABBA/BBBA and ABAA/BBAA), no changes in the P-Rh*/P-Rh and Rh*/Rh binding ratios are observed (Fig. 5*A*). Binding to the P-mAChR* either does not change (AABB/BABB and ABBB/βarr) or increases (arr/BAAA, ABAA/BBAA, and ABBA/BBBA) due to an increased affinity and/or B_{max} (Figs. 4 and 5*B*). While the affinity for P-β_2AR* increases for ABBA/BBBA and ABBB/βarr, the B_{max} decreases such that the binding at a fixed concentration (1 nM) also decreases or remains the same (ABAA/BBAA) (Figs. 4 and 5*C*). Although this pattern suggests that the N terminus is not a major direct participant in receptor binding, it appears to play some role in arrestin-receptor interaction.

N-terminal Binding Domain—Five pairs of chimeras differ only in the origin of this region (arr to ABAA, BAAA to BBAA, AABA to ABBA, BAAB to BBAB, and BABB to βarr). The functional consequences of this substitution are remarkably similar in all cases with decreased binding to P-Rh*, decreased P-Rh*/P-Rh and Rh*/Rh binding ratios, and increased binding to both P-mAChR* and P-β_2AR* due to an increased affinity (Figs. 4 and 5). However, no consistent effect of the origin of this region on the B_{max} was observed. Nevertheless, these results are consistent with this region being one of the most important domains of both arr and βarr involved in receptor interaction.

C-terminal Binding Domain—The effects of the substitution of this domain can also be analyzed using 5 pairs of chimeric arrestins (arr to AABA, BBAA to BBBA, ABAA to ABBA, BAAB to BABB, and BBAB to βarr). This change results in either no change in the binding (BAAB/BABB), decreased binding (arr/AABA), or increased binding (BBAA/BBBA, ABAA/ABBA, and BBAB/βarr) to P-Rh*. It should be noted that the increased binding occurs only when the first chimera has an apparently unfavorable *X*BA*X* structure. In contrast, other functional consequences are exceptionally uniform.

Intramolecular Interactions in Arrestins—The proposed multistep mechanism of arr interaction with rhodopsin (26, 27), which involves the mobilization of an additional binding site in response to the simultaneous occupancy of the phosphorylation- and activation-recognition sites, suggests the participation of intramolecular interactions in arrestin. Based on comparisons of the effects of C- and N-terminal deletions in arr (26) and the analysis of polyanion inhibition of arrestin-rhodopsin interaction (27), we previously proposed that the regulatory C terminus interacts with the basic N-terminal region of arr (residues 1–29). Thus, one might expect that the functional effect of the substitution of either of these regions would depend on whether a "matching" (*i.e.* from the same arrestin) pair of these regions is created or destroyed. When chimeras with N- and C-terminal substitutions are analyzed from this perspective, in all but one case where the substitution destroys the match the binding to P-Rh* increases, while substitutions creating the match decrease binding. Similarly, matched N and C termini decrease the B_{max} while mismatched termini increase the B_{max} for binding to the P-mAChR* and P-β_2AR*. These results are consistent with an interaction between the N- and C termini controlling the chimeras transition from a "low" to a "high" affinity binding conformation, as previously proposed for visual arrestin (26, 27).

The low level of binding of all *X*BA*X* chimeras suggests that it is this particular structure per se, rather than the origin of the binding domains, that makes these arrestins bind poorly. In fact, all *X*BA*X* chimeras have even lower binding than βarr(1–217), which lacks the C-terminal half of the molecule (Figs. 3–5, Table II). This demonstrates that the C-terminal binding domain in *X*BA*X* not only does not contribute to the binding, but actually seems to inhibit receptor interaction. These results suggest that there may be an obligatory interaction between the N- and C-terminal binding domains in arrestin, which is dramatically impaired in chimeras with the *X*BA*X* structure but not impaired in *X*AB*X* chimeras. The reason for

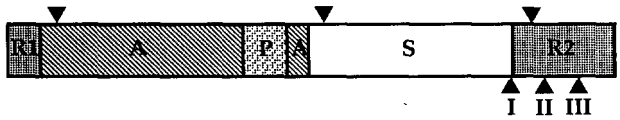

FIG. 7. **Molecular architecture of arrestins.** The putative major functional regions in the arrestins are shown with different fill patterns and are designated as follows: *R1*, N-terminal regulatory region (25–29 residues); *R2*, C-terminal regulatory region (60–80 residues); *A*, activation-recognition region; *P*, phosphorylation-recognition region (residues 163–182 in arr, residues 157–177 in βarr and arr3); *S*, region containing secondary binding site(s) (equivalent to the hydrophobic booster site in visual arrestin (26)). *Arrowheads above* show the borders between regions used in the chimera constructions (residue numbers are indicated in the legend to Fig. 4). *Arrowheads below* show the position where differences between polypeptide forms of arr (I and II), βarr (I), and arr3 (III) were found. See text for details.

this apparent lack of symmetry may be that a portion of one binding domain (the key) must fit into a pocket in the other binding domain (the keyhole). In this scenario a "small key/big keyhole" mismatch is less detrimental for interaction than a "big key/small keyhole" mismatch.

Molecular Architecture of Arrestins

The results of previous structure-function studies of arr (24–27, 30) and βarr (24, 27), the data on truncated and chimeric arrestins presented in this study (Figs. 3–6, Table II), and the comparison of the primary structures of the arrestins enables identification of the major functional elements within the arrestin molecule (Fig. 7). The very N-terminal region of 25–29 amino acids (R1) of all arrestins is highly positively charged (+8 in arr, +6 in βarr and arr3), and is most likely involved in interaction with the highly negatively charged C-terminal domain (R2). The next region of ~130 residues contains a domain involved in recognition of the activation state of the receptor (activation-recognition) (A). This appears to involve a multisite interaction between the arrestin and receptor that at least in the case of arr binding to P-Rh* and Rh* involves the first and third cytoplasmic loops of the receptor (30). The next segment of all arrestins contains a positively charged stretch of ~20 residues (net charge of +8 in arr and +7 in βarr and arr3) that appears to serve as a major phosphorylation-recognition site (P). The next 10–30-residue segment in visual arrestin may also participate in activation-recognition (A) (26). The next 120 to 150 residue stretch on the C-terminal half of the molecule contains secondary binding sites (S) that are involved in receptor interaction in response to the occupancy of the primary phosphorylation- and activation-recognition binding sites. The C-terminal 60–85 residues serve as a regulatory region (R2) which appears to take part in intramolecular interaction with the N terminus (R1). This interaction serves to maintain the overall conformation of the arrestin molecule and controls its transition into the high-affinity binding state. Interestingly, the polypeptide variants that have been identified for arr, βarr, and arr3 are within the C-terminal regulatory region. These variants might be expected to differ primarily in their sensitivity to regulatory effects (such as potential interaction with non-receptor proteins, phosphorylation, or other post-translational modifications).

The Mechanism of Arrestin-Receptor Interaction

Our proposed kinetic model of non-visual arrestin interaction with receptors is based on several experimental findings (Fig. 8). These include the finding that: 1) wild type non-visual arrestins demonstrate very low binding to unphosphorylated receptors (Fig. 1); 2) the binding of arrestins to phosphorylated receptors is activation-dependent (Figs. 1, 3, 5, and 6); 3) the interactions involved in arrestin binding to phosphorylated and

a) General model of arrestin-receptor interaction in the presence of agonist

$$\begin{array}{ccccc} A + R + L & \leftrightarrow & A + RL & \leftrightarrow & ARL & \leftrightarrow & \mathbb{A}RL \\ & & \updownarrow & & \updownarrow & & \updownarrow \\ & & A + \mathbb{R}L & \leftrightarrow & A\mathbb{R}L & \leftrightarrow & \mathbb{A}\mathbb{R}L \end{array}$$

b) Model for C-terminally truncated arrestins [e.g. βarr(1-367)]

$$\begin{array}{ccccc} A + R + L & \leftrightarrow & A + RL & \leftrightarrow & \mathbb{A}RL \\ & & \updownarrow & & \updownarrow \\ & & A + \mathbb{R}L & \leftrightarrow & \mathbb{A}\mathbb{R}L \end{array}$$

c) Model for arrestins lacking secondary binding sites [e.g. βarr(1-217)]

$$\begin{array}{ccccc} A + R + L & \leftrightarrow & A + RL & \leftrightarrow & ARL \\ & & \updownarrow & & \updownarrow \\ & & A + \mathbb{R}L & \leftrightarrow & A\mathbb{R}L \end{array}$$

d) Model for arrestin-receptor interaction in the presence of antagonist

$$A + R + N \leftrightarrow A + RN \leftrightarrow ARN \leftrightarrow \mathbb{A}RN \leftrightarrow \mathbb{A}\mathbb{R}N$$

FIG. 8. *a*, general model of arrestin-phosphoreceptor interaction in the presence of agonist (*L*). Wild type non-visual arrestin (*A*) binds to receptor-agonist complex (*RL*) in which phosphoreceptor (*R*) is in the basal (*RL*) or activated (ℝL) conformation, to form ARL or A℟L. Arrestin can then undergo a transition into its high-affinity binding conformation (𝔸), giving rise to 𝔸RL and 𝔸℟L complexes. While all four arrestin-phosphoreceptor-agonist complexes are at equilibrium, the 𝔸℟L complex has considerably higher affinity for arrestin and therefore represents the experimentally measured B_{max}. *b*, C-terminal truncated arrestin binding to receptor. C-terminally truncated arrestins such as βarr(1–367) and arr3(1–375) more readily assume the high-affinity binding conformation (𝔸) effectively eliminating one of the limiting steps leading to 𝔸℟L complex formation. *c*, binding of arrestins lacking their secondary binding sites. Arrestins lacking the C-terminal half of the molecule such as βarr(1–217), βarr(1–206), and arr3(1–183) also lack the secondary binding sites mobilized during the A → 𝔸 transition. *d*, arrestin binding to receptor in the presence of antagonist (N). The same model for full-length arrestin binding in the presence of an antagonist (N), which does not induce receptor activation. In this case the 𝔸℟N complex can be formed only via 𝔸RN due to the ability of non-visual arrestins to assume a high-affinity binding conformation (𝔸) while interacting with non-activated receptors (R). The resulting percentage of receptors in an 𝔸℟N complex (B_{max}) would be lower than an 𝔸℟L complex. See text for details.

phosphorylated-activated receptors are the same (no change in affinity or salt sensitivity) (Fig. 2 and Table I); 4) the B_{max} for βarr and arr3 binding to a given receptor is different and is always lower than the number of receptors available (Table I, Fig. 4, Ref. 24); 5) abolition of the strict control of arrestin binding by mutagenesis increases the B_{max} (Table II, Figs. 3 and 4); 6) receptor transition into an active state involves its conformational rearrangement (48–50); and 7) high-affinity arrestin binding to phosphorylated-activated receptors requires a conformational transition in the arrestin molecule (26, 47).

In the presence of agonist there is an equilibrium between free receptor (R) (here and below the receptor is presumed to be phosphorylated since this is a prerequisite for high affinity arrestin binding), free agonist (L), receptor-agonist complex (RL), and active receptor-agonist complex (ℝL). While a high concentration of agonist converts virtually all receptors into

RL, the number of $\mathbb{R}$L complexes depends on the equilibrium between RL and $\mathbb{R}$L. Conceivably, arrestin (A) can bind to both forms of the receptor forming either an ARL or A$\mathbb{R}$L complex. Bound arrestin then undergoes a conformational transition from a low to a high affinity state (A $\rightarrow$ $\mathbb{A}$) giving rise to $\mathbb{A}$RL and $\mathbb{A}\mathbb{R}$L complexes, which are again at equilibrium. The linear Scatchard plots obtained for the binding of all arrestins to the β_2AR and m2 mAChR indicate that one of these 4 arrestin-receptor-agonist complexes has a significantly higher affinity than the others. Therefore it is the formation of this particular complex, which is most likely $\mathbb{A}\mathbb{R}$L, that we are measuring. By increasing the arrestin concentration we can drive all of the receptor-agonist complexes to bind arrestin, however, the actual number of $\mathbb{A}\mathbb{R}$L complexes (the experimental B_{max}) still depends on the equilibrium between ARL, A$\mathbb{R}$L, $\mathbb{A}$RL, and $\mathbb{A}\mathbb{R}$L (Fig. 8). The RL to $\mathbb{R}$L equilibrium depends on the intrinsic characteristics of agonist binding to a particular receptor, while the ARL to A$\mathbb{R}$L and $\mathbb{A}$RL to $\mathbb{A}\mathbb{R}$L equilibrium may be shifted compared to RL to $\mathbb{R}$L. The magnitude of this shift is arrestin-dependent and gives rise to the observed differences in B_{max} for different wild type and mutant arrestins.

The deletion of the C terminus of arrestin promotes its transition into the high-affinity binding conformation ($\mathbb{A}$) (25, 26), thus eliminating one of the limiting steps in the sequence of events leading to $\mathbb{A}\mathbb{R}$L and increasing the observed B_{max} (Table I). While deletion of the entire C-terminal half of the arrestin molecule produces an arrestin that cannot form a high affinity binding conformational state (27), it still appears capable of forming an A$\mathbb{R}$L complex that is stable enough for detection (25–27, Figs. 3 and 6). This truncation also shortens the chain of events leading to the high-affinity complex and therefore also increases the B_{max} (Table I). While antagonists (N) do not induce receptor activation, arrestins are still capable of assuming a high-affinity binding conformation ($\mathbb{A}$RN), albeit with a lower probability, and consequently induce a conformational change in the receptor to form $\mathbb{A}\mathbb{R}$N. Thus, while a lower percentage of the receptors will be present in the $\mathbb{A}\mathbb{R}$N complex at equilibrium (compared to $\mathbb{A}\mathbb{R}$L), the complex itself is essentially the same as in the presence of agonist (at least as far as arrestin-receptor interaction is concerned). This would translate into no change in the K_d but a lower B_{max} as was experimentally observed (Table I). Truncated arrestins would be expected to shift the equilibrium toward $\mathbb{A}\mathbb{R}$N in the presence of an antagonist more effectively than the full-length arrestins, again in agreement with the experimental data (Fig. 3).

The kinetic model of arrestin-receptor interaction suggests that analogous to visual arrestin, non-visual arrestins are selective toward the activated/phosphorylated form of receptors. The major difference between the visual and non-visual arrestins appears to be in their ability to induce an activation-like conformational change in the phosphorylated inactive receptor. Thus, βarr and arr3 appear to be able to promote formation of an $\mathbb{A}\mathbb{R}$ (L) complex even in the absence of ligand. The data with truncated arrestins (Figs. 3 and 6) suggest that both rhodopsin and the β_2AR are rather rigid while the m2 mAChR appears to be more flexible, since even the shortest arrestin species are able to induce an active conformation in a large proportion of the P-mAChR* (Table I). Arr3S appears to possess the best ability to shift the equilibrium toward a high-affinity complex since it has the highest B_{max} with both P-β_2AR* and P-mAChR*, and is second only to visual arrestin in binding to P-Rh* (Fig. 1, Table I).

The model of arrestin-receptor interaction is analogous to the current model of G protein-receptor interaction in several important aspects (48–50). Both arrestins and G proteins preferentially bind to agonist-activated receptors, although both are capable of interacting with the receptor in its basal state (49, 50). In the presence of a saturating concentration of agonist, only a certain percentage of the receptors are able to form a high-affinity complex with either an arrestin (Table I) or G protein (48, 49). Since the same functional state of the receptor is capable of interacting with both G protein and arrestin, this suggests that direct competition between the two is the most plausible mechanism of arrestin action. Since receptor associated with G protein has a higher affinity for agonists than the receptor alone (48, 49), the $\mathbb{A}\mathbb{R}$L complex might also be expected to have a higher affinity for agonists. In the visual system, where all-*trans*-retinal bound to metarhodopsin II is analogous to the agonist-occupied activated form of the receptor, transducin interaction stabilizes the metarhodopsin II state. This is fully analogous to the stabilization of the receptor-agonist complex by other G proteins. Interestingly, visual arrestin was also shown to stabilize the metarhodopsin II state of phosphorylated rhodopsin (47). The affinity of P-β_2AR and P-mAChR for agonists in the presence and absence of non-visual arrestins remains to be determined.

The other implication of these data and the model of arrestin-phosphoreceptor interaction is a very slow dissociation of arrestin. While dissociation of visual arrestin from P-Rh* is facilitated by the decay of phosphometarhodopsin II to phosphoopsin (26), dissociation of the agonist from $\mathbb{A}\mathbb{R}$L only modestly facilitates arrestin dissociation (Table I). Thus, either non-visual arrestin dissociation is indeed a relatively slow process in the cell or it might be facilitated by either receptor sequestration or modification (*e.g.* phosphorylation) of the bound arrestin. In any event the arrestins are likely to stay bound to the phosphoreceptor at least until agonist dissociation, thus ensuring high fidelity of the signal termination.

Conclusions

This series of studies utilizing various truncated and chimeric arrestins demonstrates that the relatively high degree of amino acid homology among the arrestins translates into a similar localization of their functional domains. The mechanisms ensuring the selectivity of visual arrestin (26, 27) appear to be operational in the case of non-visual arrestins as well. Two centrally localized domains are primarily involved in receptor interaction and therefore are major determinants of arrestin specificity. Both domains seem to play comparable roles in arrestin interaction with receptors. These data corroborate the hypothesis (26, 27) that there is an interaction between N- and C-terminal regulatory regions in arrestins. The significant interchangeability of the arr and βarr regulatory regions suggests that future mutagenesis studies should target conserved residues within these domains.

Acknowledgments—We are grateful to Dr. T. Shinohara for providing the visual arrestin cDNA and R. Bodine and X. Cheng for technical assistance.

REFERENCES

1. Shichi, H., and Somers, R. L. (1978) *J. Biol. Chem.* **253,** 7040–7046
2. Benovic, J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2797–2801
3. Bennett, N., and Sitaramayya, A. (1988) *Biochemistry* **27,** 1710–1715
4. Wilden, U., Hall, S. W., and Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1174–1178
5. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* **248,** 1547–1550
6. Lohse, M. J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J.-P., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 8558–8564
7. Shinohara, T., Dietzschold, B., Craft, C. M., Wistow, G., Early, J. J., Donoso, L. A., Horwitz, J., and Tao, R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6975–6979
8. Rapoport, B., Kaufman, K. D., and Chazenbalk, G. D. (1992) *Mol. Cell. Endocrinol.* **84,** R39-R43
9. Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.*

267, 17882–17890
10. Sterne-Marr, R., Gurevich, V. V., Goldsmith, P., Bodine, R. C., Sanders, C., Donoso, L. A., and Benovic, J. L. (1993) *J. Biol. Chem.* **268,** 15640–15648
11. Murakami, A., Yajima, T., Sakuma, H., McLaren, M. J., and Inana, G. (1993) *FEBS Lett.* **334,** 203–209
12. Craft, C. M., Whitmore, D. H., and Wiechmann, A. F. (1994) *J. Biol. Chem.* **269,** 4613–4619
13. Kuhn, H. (1978) *Biochemistry* **17,** 4389–4395
14. Kuhn, H., Hall, S. W., and Wilden, U. (1984) *FEBS Lett.* **176,** 473–478
15. Dawson, T. M., Arriza, J. L., Jaworsky, D. E., Borisy, F. F., Attramadal, H., Lefkowitz, R. J., and Ronnett, G. V. (1993) *Science* **259,** 825–829
16. Parruti, G., Peracchia, F., Sallese, M., Ambrosini, G., Masini, M., Rotilio, D., and DeBlasi, A. (1993) *J. Biol. Chem.* **268,** 9753–9761
17. Smith, W. C., Milam, A. H., Dugger, D., Arendt, A., Hargrave, P. A., and Palczewski, K. (1994) *J. Biol. Chem.* **269,** 15407–15410
18. Yamaki, K., Tsuda, M., Kikuchi, T., Chen, K. H., Huang, K. P., and Shinohara, T. (1990) *J. Biol. Chem.* **265,** 20757–20762
19. Kuhn, H., and Dreyer, W. J. (1972) *FEBS Lett.* **20,** 1–6
20. Bownds, D., Dawes, J., Miller, J., and Stahlman, M. (1972) *Nat. New Biol.* **237,** 125–127
21. Kwatra, M. M., Leung, E., Maan, A., McMahon, K. K., Ptasienski, J., Green, R. D., and Hosey, M. M. (1987) *J. Biol. Chem.* **262,** 16314–16321
22. Kwatra, M. M., Benovic, J. L., Caron, M. G., Lefkowitz, R. J., and Hosey, M. M. (1989) *Biochemistry* **28,** 4543–4547
23. Richardson, R. M., Kim, C., Benovic, J. L., and Hosey, M. M. (1993) *J. Biol. Chem.* **268,** 13650–13656
24. Gurevich, V. V., Richardson, R. M., Kim, C. M., Hosey, M. M., and Benovic, J. L. (1993) *J. Biol. Chem.* **268,** 16879–16882
25. Gurevich, V. V., and Benovic, J. L. (1992) *J. Biol. Chem.* **267,** 21919–21923
26. Gurevich, V. V., and Benovic, J. L. (1993) *J. Biol. Chem.* **268,** 11628–11638
27. Gurevich, V. V., Chen, C.-Y., Kim, C. M., and Benovic, J. L. (1994) *J. Biol. Chem.* **269,** 8721–8727
28. Kim, C. M., Dion, S. B., Onorato, J. J., and Benovic, J. L. (1993) *Receptor* **3,** 39–55
29. Lefkowitz, R. J., Caron, M. G., Michel, T., and Stadel, J. M. (1982) *Fed. Proc.* **41,** 2664–2670
30. Krupnick, J. G., Gurevich, V. V., Schepers, T., Hamm, H. E., and Benovic, J. L. (1994) *J. Biol. Chem.* **269,** 3226–3232
31. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C. (1992) *DNA & Cell Biol.* **11,** 1–20
32. Franke, R. R., Sakmar, T. P., Graham, R. M., and Khorana, H. G. (1992) *J. Biol. Chem.* **267,** 14767–14774
33. Franke, R. R., Sakmar, T. P., Oprian, D. D., and Khorana, H. G. (1988) *J. Biol. Chem.* **263,** 2119–2122
34. Franke, R. R., Konig, B., Sakmar, T. P., Khorana, H. G., and Hofmann, K. P. (1990) *Science* **250,** 123–125
35. Munch, G., Dees, C., Hekman, M., and Palm, D. (1991) *Eur. J. Biochem.* **198,** 357–364
36. Strader, C. D., Dixon, R. A. F., Cheung, A. H., Candelore, M., Blake, A. D., and Sigal, I. S. (1987) *J. Biol. Chem.* **262,** 16439–16443
37. O'Dowd, B. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G., and Lefkowitz, R. J. (1988) *J. Biol. Chem.* **263,** 15985–15992
38. Wong, S. K. F., Parker, E. M., and Ross, E. M. (1990) *J. Biol. Chem.* **265,** 6219–6224
39. Palczewski, K., Buczylko, J., Kaplan, M. W., Polans, A. S., and Crabb, J. W. (1991) *J. Biol. Chem.* **266,** 12949–12955
40. Fowles, C., Sharma, R., and Akhtar, M. (1988) *FEBS Lett.* **238,** 56–60
41. Chen, C.-Y., Dion, S. B., Kim, C. M., and Benovic, J. L. (1993) *J. Biol. Chem.* **268,** 7825–7831
42. Kelleher, D. J., and Johnson, G. L. (1990) *J. Biol. Chem.* **265,** 2632–2639
43. Benovic, J. L., Onorato, J., Lohse, M. J., Dohlman, H. G., Staniszewski, C., Caron, M. G., and Lefkowitz, R. J. (1990) *Br. J. Clin. Pharmacol.* **30,** 3S-12S
44. Haga, K., Kameyama, K., and Haga, T. (1994) *J. Biol. Chem.* **269,** 12594–12599
45. Nakata, H., Haga, K., Kameyama, K., and Haga, T. (1994) *Eur. J. Biochem.* **220,** 29–36
46. Papac, D. I., Oatis, J. E., Jr., Crouch, R. K., and Knapp, D. R. (1993) *Biochemistry* **32,** 5930–5934
47. Schleicher, A., Kuhn, H., and Hofmann, K. P. (1989) *Biochemistry* **28,** 1770–1775
48. Lefkowitz, R. J., Caron, M. G., Michel, T., and Stadel, J. M. (1982) *Federation Proc.* **41,** 2664–2670
49. Limbird, L. E., and Lefkowitz, R. J. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 228–232
50. Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268,** 4625–4636