Agonist-Receptor-Arrestin, an Alternative Ternary Complex with High Agonist Affinity*

(Received for publication, July 14, 1997, and in revised form, September 4, 1997)

Vsevolod V. Gurevich‡§, Robin Pals-Rylaarsdam¶¶, Jeffrey L. Benovic**‡‡, M. Marlene Hosey¶¶, and James J. Onorato¶¶

From the ‡Sun Health Research Institute, Sun City, Arizona 85372, the §Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, Illinois 60611, the ¶¶Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, and the **Department of Medicine, University of Wisconsin, Madison, Wisconsin 53706

The rapid decrease of a response to a persistent stimulus, often termed desensitization, is a widespread biological phenomenon. Signal transduction by numerous G protein-coupled receptors appears to be terminated by a strikingly uniform two-step mechanism, most extensively characterized for the β2-adrenergic receptor (β2AR), m2 muscarinic cholinergic receptor (m2 mAChR), and rhodopsin. The model predicts that activated receptor is initially phosphorylated and then tightly binds an arrestin protein that effectively blocks further G protein interaction. Here we report that complexes of β2AR-arrestin and m2 mAChR-arrestin have a higher affinity for agonists (but not antagonists) than do receptors not complexed with arrestin. The percentage of phosphorylated β2AR in this high affinity state in the presence of full agonists varied with different arrestins and was enhanced by selective mutations in arrestins. The percentage of high affinity sites also was proportional to the intrinsic activity of an agonist, and the coefficient of proportionality varies for different arrestin proteins. Certain mutant arrestins can form these high affinity complexes with unphosphorylated receptors. Mutations that enhance formation of the agonist-receptor-arrestin complexes should provide useful tools for manipulating both the efficiency of signaling and rate and specificity of receptor internalization.

Agonist binding activates G protein1-coupled receptors and initiates two intimately intertwined cascades of events, resulting in signal transduction and signal termination (desensitization). The receptor-arrestin complex initially interacts with G protein(s) to form a transient agonist-receptor-G protein ternary complex that is the first intermediate in transmembrane signaling (1, 2). This ternary complex has a higher affinity for agonists than receptor alone (1, 2). Formation of this complex promotes GDP release from the G protein, which is followed by rapid GTP binding and dissociation of the active Gα-GTP and Gβγ subunits. The agonist-occupied receptors are then phosphorylated by G protein-coupled receptor kinases, resulting in arrestin binding and consequent disruption of receptor-G protein interaction (3). Recent studies suggest that arrestin binding also targets the receptors for internalization (4, 5), apparently by virtue of the ability of non-visual arrestins to interact with clathrin (6), a process that appears to be a prerequisite for resensitization (3). Thus, the formation of the arrestin-receptor complex is not only the final step of signal termination but also an initial step of subsequent resensitization, representing a critical juncture in the signaling process. Because of this the arrestin-receptor complex appears to be a tempting target for a more detailed characterization.

EXPERIMENTAL PROCEDURES

Agonist Binding Activity in Escherichia coli and Purification—Bovine arrestin cDNAs were subcloned using the Nool and HindIII sites of pTREB (Invitrogen). BL-21 cells transformed with the pTREB-arrestin constructs were grown at 30 °C in LB containing 0.1 mg/ml ampicillin to an A600 of 0.2–0.4, induced with 30 μM isopropyl-β-D-thiogalactopyranoside, and grown for an additional 4–6 h. Cells were harvested by centrifugation and lysed, and arrestins were purified by sequential heparin-Sepharose (6) and Q-Sepharose chromatography as described (6–8), adjusting salt gradients for elution of different arrestin proteins.

Receptor Purification, Reconstitution, and Phosphorylation—Hamster β2-adrenergic receptor (β2AR) and human m2 muscarinic cholinergic receptor (m2 mAChR) were expressed in Sf9 cells and purified by affinity chromatography as described (9). Purified β2AR (90–120 pmol) was mixed with 0.6–0.8 mg of sonicated bovine phosphatidyldcholine in 0.4 ml of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl (buffer A) containing 2 mg/ml BSA and incubated on ice for 7 min. Samples were loaded onto Extran-Gel columns (Pierce) at 4 °C, previously equilibrated with 3 ml of buffer A containing 2 mg/ml BSA and 2 ml of buffer A containing 20 mM MgCl2, and then eluted with 1.7 ml of the latter buffer. 50% polyethylene glycol 8000 (0.6 ml) was added to the eluant, mixed, and incubated for 7 min at 22 °C. Samples were diluted with 30 ml of iced-cold buffer A, and liposomes with recombinant receptor were pelleted by centrifugation at 35,000 1 g for 90 min. Pellets were resuspended in 0.4 ml of 20 mM Tris-HCl, pH 7.4, 2 mM EDTA. The m2 mAChR was reconstituted and phosphorylated, as described (10). Receptors were phosphorylated in the presence of respective agonists by purified β2-adrenergic receptor kinase to a stoichiometry of 2.7  0.2 mol/mol (Pβ2AR) or 8  0.2 mol/mol (P-m2 mAChR), as described (7, 9, 10). To remove the agonist, Pβ2AR was washed with 20 mM Tris-HCl, pH 7.4, 2 mM EDTA three times by centrifugation as above, whereas P-m2 mAChR was gel-filtered on a 2-ml Sephadex G-50 column. Control receptors were similarly prepared but in the absence of kinase.

Receptor Binding Assays—4-Pβ2AR or 4-Pm2 mAChR (10–15 fmol/assay) were incubated with 0.25 ml of buffer A containing 0.1 mg/ml BSA in the presence of 65–75 fmol of [125I]iodopindolol (NEN Life Science Products) and the indicated concentrations of arrestins and agonists for 60 min at 22 °C. Samples were then cooled on ice and loaded at 4 °C onto 2-ml Sephadex G-50 columns. Receptor-containing liposomes with bound radioligand were eluted with buffer A (between 0.6 and 1.5 ml), and radioactivity was quantitated in a liquid scintillation counter.

muscarinic cholinergic receptor; BSA, bovine serum albumin; ISO, isoprenaline.
mACHr and m2mACHr (10) (50 fmol/assay) were incubated in 0.5 ml of the same buffer ABSA in the presence of 250 fmoles of [3H]quinuclidinyl benzilate (Amersham Corp.) with the indicated concentrations of arrestins and agonists for 60 min at 22°C. 150 µl of ice-cold 30% (w/v) polyethylene glycol were subsequently added to each assay tube, and the samples were incubated on ice for 10 min and then filtered through GF/F filters (presoaked for 1 h in 1% polyethyleneimine to reduce non-specific binding). The radioactivity retained on filters was then determined in the presence of 10 µM alprenolol (β2AR) or atropine (m2 mACHr). All binding experiments were repeated 2–4 times, and data are presented as means ± S.D.

RESULTS AND DISCUSSION

Our initial studies examined ligand binding characteristics of phosphorylated β2AR in the absence or presence of arrestins (Fig. 1). Strikingly, both β-arrestin and arrestin3, but not visual arrestin, induced a pronounced leftward shift of the isoproterenol (ISO) competition curve (Fig. 1A). We next tested the effects of varying the arrestin concentration on ligand binding to P-β2AR in the presence of either 30 or 100 nM isoproterenol. This yielded EC50 values of 19.4 ± 7.4 and 5.0 ± 1.8 nM for β-arrestin and arrestin3, respectively, values well within the physiologically relevant range (6, 11).

Similar curve shifts were observed for the β-agonists epinephrine and norepinephrine (Fig. 1, B and C), whereas affinities for the antagonists alprenolol (IC50=3.47 ± 0.14 nM) and propranolol (IC50=-1.19 ± 0.06 nM) were unchanged by arrestins. As shown in Fig. 1, the agonist competition curves in the presence of β-arrestin or arrestin3 are shallower than control curves performed in the absence of arrestins. Analysis of these curves reveals the presence of two distinct sites that differ in their agonist affinity (Fig. 1). β-arrestin and arrestin3 induce very similar high and low affinity sites for isoproterenol with IC50 values of 26.7 ± 9.1 and 871 ± 71 nM, respectively, the latter value being very similar to the affinity for the receptor in the absence of arrestin (IC50 of 656 ± 127 nM). β-arrestin and arrestin3 also promote similar high and low affinity binding sites for epinephrine and norepinephrine with IC50 values of 90.3 ± 21.8 µM and 3.84 ± 0.69 µM for epinephrine and 1.20 ± 0.40 and 43.1 ± 13.4 µM for norepinephrine, respectively (Fig. 1). However, the percentage of high affinity sites is clearly dependent upon the particular arrestin present: 31.5 ± 1.4% with β-arrestin and 56.9 ± 1.0% with arrestin3. Thus, it appears that arrestin3 more efficiently shifts the equilibrium of the receptor between the two distinct functional states than does β-arrestin. Although neither arrestin3 induces 100% of the receptors into a high affinity state, these results are in agreement with direct binding studies where arrestin3 was found to bind better than β-arrestin to the P-β2AR (9).

Arrestins bind preferentially to phosphorylated receptors (9, 11). To further explore this relationship, we tested the phosphorylation dependence of the high affinity agonist binding to ascertain whether the increased affinity directly reflects arrestin-receptor interaction. β-Arrestin did not increase the affinity of unphosphorylated β2AR for ISO, whereas arrestin3 evoked a marginal shift of the curve (Fig. 1D), apparently reflecting the stronger propensity of arrestin3 to interact with unphosphorylated receptor (9). A more systematic study using β2AR phosphorylated by the β-adrenergic receptor kinase to various stoichiometries (0.6–3.4 mol/mol) revealed no effect of β-arrestin on the ISO competition curve at stoichiometries of 0.6 or 1.4 mol/mol, a marginal effect at 1.9 mol/mol, and significant and virtually indistinguishable effects at 2.5–3.4 mol/mol. These data are consistent with our earlier observation that two phosphates/receptor are necessary and sufficient for high affinity arrestin interaction (9) and provide additional evidence that it is the arrestin-receptor complex that demonstrates higher affinity for agonists. Collectively, all these data strongly suggest that the receptor complex with high agonist affinity is the same arrestin-receptor complex previously characterized by direct binding studies (9).

Additional analysis of the agonist-arrestin-arrestin ternary complex reveals that it is insensitive to physiological concentrations of MgCl2 (1 mM), CaCl2 (up to 11.6 µM free ± 1 µM calmodulin), ATP, or GTP (0.1 mM ± 1 mM MgCl2), or 100 nM G protein βγ subunits. Although both β-arrestin and arrestin3 bind with high affinity to clathrin (6) without influencing clathrin lattice formation (12), the addition of 50 nM purified clathrin had no appreciable effect on the ISO competition curve shift induced by either 10 or 50 nM of the non-visual arrestins (not shown). Thus, clathrin interaction with arrestins does not interfere with arrestin binding to P-β2AR, suggesting that different regions of the arrestins are involved in these two interactions.

Various arrestin mutations were previously shown to change the propensity of arrestins to form a high affinity complex with receptors (9, 11) and to affect the selectivity of the arrestins (8, 13, 14). For example, replacing the N-terminal ~45 amino acids of β-arrestin with the corresponding region of visual arrestin (yielding the chimera ABBB) was found to dramatically increase binding to P-β2AR (9). When this chimera was purified and tested on the ISO competition curves for P-β2AR and β2AR, it induced a more significant change in the curve than did wild type β-arrestin, converting 45.4 ± 6.6% of P-β2AR into a high affinity state (Fig. 2A), with no appreciable effect on β2AR binding (Fig. 2B). We previously demonstrated that a single point mutation within the phosphorylation-recognition region of visual arrestin (R175E) yields a phosphorylation-independent arrestin (i.e. an arrestin capable of high affinity binding to both phosphorylated and unphosphorylated light-activated rhodopsin) (8, 14). Therefore, a similar form of β-arrestin (β-arrestin-R169E) was constructed and assessed for its ability to induce high affinity agonist binding to P-β2AR and β2AR. Interestingly, this mutant not only promotes high affinity agonist binding to β2AR (21.9 ± 3.9% high affinity sites) but also induces a more significant shift in the ISO competition curve of P-β2AR (52.1 ± 2.3% high affinity sites) than does β-arrestin (31.4 ± 5.6%) (Fig. 2). Since previous studies demonstrated that C-terminal arrestin truncation also enhances phosphorylation-independent receptor binding (9, 11), we tested a C-terminal truncation of arrestin3. Purified arrestin3- (1–393) promotes significant high affinity agonist binding to
both P-β2AR (82.5 ± 4.6%) and β2AR (65.6 ± 4.7%) (Fig. 2). Notably, the EC50 values of both arrestin3-(1–393) (2.98 ± 0.27 nm) and ABBB (1.91 ± 0.75 nm) for inducing high affinity agonist binding are comparable with the Kf values previously determined in direct binding assays (9), whereas the EC50 values for β-arrestin and arrestin3 are substantially higher. These differences may be attributed to the kinetically complex multistep nature of arrestin binding to receptor (9, 11), which appears to be simplified for conformationally unrestrained arrestin mutants (9).

All non-visual arrestins tested exhibited a profound effect on agonist affinity and no effect on antagonist binding, suggesting that the effect may correlate with the intrinsic activity of the ligand. To test this hypothesis we used two partial agonists: salbutamol, with relatively high (0.65) intrinsic activity, and dobutamine, with relatively low (0.25) intrinsic activity. In conjunction with these ligands we used three arrestin proteins with relatively low, medium, and high propensity to induce the high agonist affinity state of P-β2AR, i.e. β-arrestin, arrestin3, and arrestin3-(1–393). These arrestins induced leftward shifts of the competition curves of both partial agonists, with the effect on salbutamol being less profound than that on full agonists, although stronger than that on dobutamine (not shown). The competition curves in the presence of arrestins were shallow, and analysis of these curves again revealed the presence of high and low affinity sites. The values observed for the high affinity sites for salbutamol and dobutamine were 53 ± 9 and 640 ± 240 nm, respectively, in the presence of all three arrestins tested. The affinities of low affinity sites were 3.7 ± 1.1 and 15 ± 3 μM for salbutamol and dobutamine, respectively, i.e. remarkably close to the affinities observed in the absence of arrestins (3.1 ± 0.2 and 14.5 ± 3.1 μM, respectively). The percentage of high affinity sites was clearly dependent on the nature of both the arrestin and the ligand. In the presence of β-arrestin, arrestin3, and arrestin3-(1–393) the percentage of high affinity sites for salbutamol was 20 ± 2, 39 ± 2, and 42 ± 2%, respectively, whereas in the case of dobutamine, the percentage was 9 ± 1, 29 ± 6, and 32 ± 3%. As shown in Fig. 3A, the percentage of high affinity sites in the presence of a given arrestin appears to be roughly proportional to the intrinsic activity of the ligand. Most interestingly, the coefficient varies with different arrestin proteins from 0.32 for β-arrestin through 0.59 for arrestin3 to 0.76 for arrestin3-(1–393). These results suggest that various arrestins each possess a characteristic propensity to form an arrestin-receptor complex with high agonist affinity. We propose to term this propensity of an arrestin its competency. Practically speaking, the percentage of high affinity sites formed in the presence of a full agonist appears to give a fairly accurate estimate of the competency of a given arrestin (compare Figs. 1, 2, and 3).

To test whether the high agonist affinity of the arrestin-receptor complexes is observed with other G protein-coupled receptors, we performed similar experiments with purified, reconstituted m2 mAChR. Both wild type β-arrestin and arrestin3 promote a >10-fold increase in agonist affinity for phosphorylated m2 mAChR (Fig. 4A), whereas there was no significant shift in carbachol affinity for the unphosphorylated m2 mAChR (Fig. 4B). In addition, in contrast to the β2AR, there was no significant change in the slope of the curve, suggesting that most of the phosphorylated m2 mAChR forms solely a high agonist affinity complex with either arrestin. This may be attributable to the high stoichiometry of phosphorylation of the P-m2 mAChR preparation (8 mol/mol) or to inherent differences between the two receptors. Interestingly, β-arrestin(R169E) had virtually the same effect as wild type β-arrestin on the P-m2 mAChR, whereas arrestin3-(1–393) induced larger shifts on P-m2 mAChR and exhibited significant phosphorylation-independent interaction with mAChR (Fig. 4C, D).

In many respects the receptor-arrestin complex behaves similarly to the previously identified receptor-G protein complex (1, 2, 15). Both complexes promote high affinity agonist binding to receptors. The percentage of receptor in the high affinity state appears to be proportional to the intrinsic activity of the ligand in both cases. The increase in agonist affinity due to the formation of either of these complexes is 10–100-fold. A major

---

**Fig. 2. Effects of mutant and chimeric arrestins on P-β2AR (A) or β2AR (B) affinity for isoproterenol.** Isoproterenol competition curves were obtained in the absence (○) or presence of 1 μM of the chimera ABBB (▼), 1 μM β-arrestin-R169E (▲), or 300 nM truncated arrestin3-(1–393) (●). The parameters of the competition curves (two-state model) were as follows: A, Kc = 660 ± 30 nM (○), Kc = 920 ± 150 nM, Kf = 19 ± 8 nM, H = 45 ± 7% (▼), Kf = 830 ± 110 nM, Kc = 26 ± 10 nM, H = 52 ± 3% (▲), and Kf = 850 ± 110 nM, Kc = 26 ± 5 nM, H = 83 ± 5% (●); B, Kc = 430 ± 130 nM (○), Kc = 450 ± 50 nM (▼), Kc = 760 ± 100 nM, Kf = 65 ± 18 nM, H = 22 ± 4% (▲), and Kf = 760 ± 100 nM, Kc = 17 ± 4 nM, H = 66 ± 5% (●), where Kc is an apparent Kc of low affinity sites, Kf is an apparent Kf of high affinity sites, and H is the percentage of high affinity sites. IPIN, iodopindolol.

---

**Fig. 3. Structure and functional characteristics of arrestin proteins.** A, dependence of the percentage of high affinity sites on the intrinsic activity of ligands in the presence of 1 μM β-arrestin (▲), 300 nM arrestin3 (■), or 300 nM truncated arrestin3-(1–393) (●). Ligands with intrinsic activities of 0 (propranolol, alpenrolodol), 0.25 (dobutamine), 0.65 (salbutamol), and 1 (isoproterenol, epinephrine, and norepinephrine) were used. The equation H = aI, where H is the percentage of high affinity sites and I is an intrinsic activity, yields the best fit. Coefficients “a” (competency) for β-arrestin (▲), arrestin3 (■), and truncated arrestin3-(1–393) (●) were 0.32, 0.59, and 0.76, respectively. B, structure, phosphorylation dependence, and competency of arrestin proteins used in this study. The origin of the sequence of corresponding arrestins is coded by a filled pattern; the check mark denotes the position of a point mutation, whereas relative length shows the extent of truncation. Competency values for β-arrestin, arrestin3, and arrestin3-(1–393) are from the graph on panel A, whereas the competency of the other three arrestins was estimated by the percentage of high affinity sites with full agonists.
difference between receptor-arrestin complex and receptor-G protein complex lies in the sensitivity of the agonist-receptor-G protein ternary complex to GTP, which promotes G protein dissociation, whereas the agonist-receptor-arrestin complex is relatively stable and insensitive to nucleotides, ions, and even whole cell lysate (not shown). Since the agonist affinity of two structurally and functionally diverse receptors, the β2AR and m2 mACHR, increases upon arrestin binding, it is tempting to speculate that this is a universal phenomenon among G protein-coupled receptors. The ability of visual arrestin to stabilize the active conformation of rhodopsin (16) supports this hypothesis. The formation of the P-β2AR-β-arrestin complex promotes internalization via clathrin-coated pits (7), thus, whereas β-arrestin and arrestin3 appear to be functionally similar in a number of assays in vitro and in transfected cells (4, 5, 7, 9, 17, 18), we observed a significant difference in their competency, i.e. the ability to form a high affinity agonist-receptor-arrestin complex with β2AR. Interestingly, arrestin3 also has a 6-fold higher affinity for binding to clathrin cages (7). Thus, whereas β-arrestin and arrestin3 may similarly desensitize the β2AR they may differ in their ability to promote receptor internalization and subsequent resensitization. The diverse characteristics of the various arrestin mutants (the ability to form tight complexes with phosphorylated and unphosphorylated receptors more effectively, the relative specificity of β-arrestin(R169E) toward P-β2AR and β2AR, and more promiscuous nature of truncated arrestin3) makes these proteins useful tools for experimental manipulation of the duration and extent of receptor signaling and internalization in cells.

The relative ability of a given arrestin to form arrestin-receptor complexes with high agonist affinity appears to be its intrinsic characteristic, which we termed “competency.” Conceivably, arrestin competency may reflect its affinity for a given receptor, the propensity of an arrestin to undergo a transition into the active receptor-binding state, or both. We found no apparent relationship between the EC50 values of different arrestins and their competency with P-β2AR. However, mutations that increased competency appear to reduce arrestin selectivity (Figs. 2 and 4), suggesting that competency predominately reflects the ease with which an arrestin undergoes a transition into its active state. The apparent similarity of the rank order of competency of different arrestin proteins with the P-β2AR and P-m2 mACHR (Figs. 1, 2, and 4) supports this hypothesis. Competency has no analogous concept for G proteins, perhaps because the high affinity state of any receptor has not been systematically studied in the presence of more than one type of G protein. Intuitively the most related concept to arrestin competency in receptor-G protein interactions appears to be coupling efficiency. Our data suggest that certain mutations can substantially increase arrestin competency, and we believe that additional mutants with even higher competency can be engineered. Arrestin competency in vitro may correlate with its ability to desensitize and/or promote internalization of a given receptor in vivo, but this hypothesis needs to be experimentally tested.

Acknowledgments—We thank Dr. L. A. Donoso for the arrestin monoclonal antibody 4F11, Dr. D. B. Goodman and Dr. J. H. Keen for purified clathrin, Dr. S. Kennedy for purified G protein β2s subunits, Dr. J. Hirsch for help in protein characterization by mass spectrometry and N-terminal sequencing, J. Ptasienski for the purification of m2 mACHR, and Dr. R. B. Penn for critical reading of the manuscript.

REFERENCES
Agonist-Receptor-Arrestin, an Alternative Ternary Complex with High Agonist Affinity
Vsevolod V. Gurevich, Robin Pals-Rylaarsdam, Jeffrey L. Benovic, M. Marlene Hosey and James J. Onorato

J. Biol. Chem. 1997, 272:28849-28852. doi: 10.1074/jbc.272.46.28849

Access the most updated version of this article at http://www.jbc.org/content/272/46/28849

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 18 references, 11 of which can be accessed free at http://www.jbc.org/content/272/46/28849.full.html#ref-list-1