Targeted Construction of Phosphorylation-independent β-Arrestin Mutants with Constitutive Activity in Cells*

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Arrestin proteins play a key role in the desensitization of G protein-coupled receptors (GPCRs). Recently we proposed a molecular mechanism whereby arrestin preferentially binds to the activated and phosphorylated form of its cognate GPCR. To test the model, we introduced two different types of mutations into β-arrestin that were expected to disrupt two crucial elements that make β-arrestin binding to receptors phosphorylation-dependent. We found that two β-arrestin mutants (Arg169Glu and Asp383Asp) (DOR) and restore the agonist-induced desensitization of a truncated DOR lacking the critical G protein-coupled receptor kinase (GRK) phosphorylation sites. The kinetics of the desensitization induced by phosphorylation-independent mutants in the absence of receptor phosphorylation appears identical to that induced by wild type β-arrestin + GRK3. Either of the mutations could have occurred naturally and made receptor kinases redundant, raising the question of why a more complex two-step mechanism (receptor phosphorylation followed by arrestin binding) is universally used.

The decrease of a response to a persistent stimulus (desensitization) is a widespread biological phenomenon. Signaling by diverse G protein-coupled receptors (GPCRs)† is believed to be terminated by a uniform two-step mechanism (1). According to the model, activated receptor is first phosphorylated by a G protein-coupled receptor kinase (GRK). An arrestin protein binds to the activated phosphoreceptor, thereby blocking G protein interaction. Arrestin-receptor complex is then internalized, whereupon receptor is either dephosphorylated and recycled back to the plasma membrane (resensitization) or sorted to lysosomes and destroyed (down-regulation). Thus, the formation of the arrestin-receptor complex appears to be the final step of desensitization and the first step of resensitization and/or receptor down-regulation, which puts it at the crucial cross-roads of the processes regulating cellular responsiveness. The tremendously diverse superfamily of G protein-coupled receptors with more than 1000 members is the largest known group of proteins that translate a wide variety of external stimuli into intracellular “language.” In contrast, the repertoire of receptor kinases and arrestins involved in the desensitization of these receptors is rather limited: only six GRKs and four arrestins have thus far been found in mammals (reviewed in Ref. 1). This suggests that at least some of the kinases and arrestins regulate numerous receptors. Thus, these proteins are attractive targets for research designed to delineate common molecular mechanisms underlying the regulation of GPCR signaling in cells (and to create fairly universal tools for the experimental and/or therapeutic intervention in the process).

EXPERIMENTAL PROCEDURES

Mutagenesis and Biochemical Characterization of β-Arrestins—Mutations Arg169Glu to Glu (CGG to GAG), Gln394Ter to Ter (CAA to TAA), and Asp383Glu (GAT to TAG) were introduced by polymerase chain reaction in β-arrestin construct pBARR (3), that was used for in vitro transcription and translation, as described (3). Nes1/HindIII 1404-base pair open reading frame was then subcloned into appropriately digested Escherichia coli expression vector pTReHiSb (Invitrogen). All β-arrestin species were expressed in the in vitro translation system and tested in the direct binding assay (3), overexpressed in E. coli, purified to apparent homogeneity (16), and characterized in the agonist affinity shift assay (7), essentially as described.

Direct Binding Assay—In vitro translated tritiated arrestins (50 fmol) were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl2, 1.5 mM dithiothreitol, 50 mM potassium acetate with 7.5 pmol of the various functional forms of rhodopsin or with P2AR or β2AR (100 fmol) in a final volume of 50 μl for 5 min at 37 °C in room light (rhodopsin) or for 60 min at 30 °C in the presence of 0.1 μM β-agonist isoproterenol. The samples were immediately cooled on ice and loaded onto 2 ml Sepharose 2B columns equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA. Bound arrestin eluted with receptor-containing membranes in the void volume (between 0.5 and 1.1 ml). Nonspecific binding determined in the presence of 0.3 μg of liposomes was subtracted.

Agonist Affinity Shift Assay—P2AR or β2AR (10–15 fmol) was incubated in 0.25 ml of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl (buffer A) containing 0.1 mg/ml bovine serum albumin 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 to 4 °C onto 2 ml of 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. Nonspecific binding was determined in the presence of 10 μM alprenolol. All experiments were repeated two to three times, and data are presented as means ± S.D.

Desensitization Studies in Xenopus Oocytes—Stage IV oocytes from αβ2AR, β2-adrenergic receptor; P-β2AR, phosphorylated β2AR; DOR, δ opioid receptor; Rh*, light-activated rhodopsin; P-Rh*, phosphorylated Rh*; HAC, high agonist affinity complex; Ter, stop codon.
mature female Xenopus laevis frogs were harvested, defolliculated, and cultured as described previously (8). cRNA was prepared for oocyte injection from cDNA template using Ambion message machine kit (Ambion, TX) according to manufacturer’s protocol. cDNAs (GenBank accession numbers in parentheses) for rat GRK3 (AA144586), human β-AR (A052644), mouse δ opioid receptor (L06322), and rat G protein-gated inwardly rectifying potassium channel subunits Kir3.1 (U01071) and Kir3.4 (X83584) were amplified and linearized prior to cRNA synthesis. cDNAs for all forms of β-arrestin were first amplified by polymerase chain reaction using oligonucleotides designed to add a T7 promoter upstream and a 45-base poly(A) tail downstream of the translation counter. *, p < 0.01, Student’s t-test, compared with the binding of corresponding wild type arrestin.

RESULTS AND DISCUSSION

Recently we have proposed a molecular mechanism that explains an amazing selectivity of arrestins for the activated phosphorylated forms of GPCRs (2, 3). According to previous in vitro studies (2, 3) arrestins have two primary binding sites: an activation-recognition site that recognizes the agonist-activated conformation, and a secondary site that interacts with GRK-phosphorylated elements of the receptor. A potent secondary receptor-binding site is mobilized when an arrestin encounters activated receptor. A possible molecular mechanism that explains an amazing selectivity of arrestins for the activated receptor alone, and only a fraction of the receptors forms such a high-affinity complex (HAC) even at saturating concentrations of arrestin. The maximum percentage of the receptor in HAC gives a good estimate of the propensity of a given arrestin protein to bind tightly to the receptor (arrestin competency) (7). Consistent with the direct binding data (Fig. 1A),

![Fig. 1. Direct binding assay](image1)

**Fig. 1. Direct binding assay.** A, 100 fmol of βAR-phosphorylated (P-βAR, 2.7 ± 0.2 mol phosphate/mol receptor) or unphosphorylated purified β-AR reconstituted into liposomes was incubated in a 50 μl reaction with 50 fmol of the indicated form of tritiated arrestin (specific activities: 140–160 dpm/fmol) in the presence of 100 μM agonist isoproterenol in 50 mM Tris-HCl, pH 7.5, 50 mM potassium acetate, 0.5 mM MgCl₂ for 45 min at 37 °C. The samples were then cooled on ice and loaded at 4 °C on to 2-ml Sepharose 2B columns, equilibrated with 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, containing 0.1 mg/ml bovine serum albumin in the presence of 65–75 fmol of βAR-antagonist [125I]iodopindolol (IPN) (NEN Life Science Products) and the indicated concentrations of βAR-agonist isoproterenol for 60 min at 22 °C in the absence (●) or presence of 1 μM β-arrestin (●), β-arrestin-(Arg⁶⁹ → Glu) (▲), β-arrestin-(1–382) (●), or β-arrestin-(1–393) (●). Samples were then cooled on ice and loaded onto 2-mL Sephadex G-50 columns at 4 °C. Receptor-containing liposomes with bound radioligand were eluted with the same buffer (between 0.6 and 1.5 mL), and the radioactivity was quantitated. For the unphosphorylated β-AR competition curves in the absence of arrestins and in the presence of wild type and β-arrestin-(1–393) are monophasic (analysed using Prism 2.0 for Power Macintosh). In the presence of β-arrestin-(Arg⁶⁹ → Glu) and β-arrestin-(1–382) the curves are biphasic, suggesting the presence of high- and low-affinity sites. With P-β-AR competition curves generated in the presence of all forms of β-arrestin are biphasic, while the curve in the absence of arrestins is monophasic.

**Multisite binding (2, 3), and the recent crystal structure of visual arrestin (4) set the stage for the targeted construction of arrestin mutants in which one of the triggers is constitutively pulled by an appropriate mutation. In order to test the validity of the model we constructed three β-arrestin mutants: 1) Arg⁶⁹ → Glu, that reverses the charge of the putative phosphorylation-sensitive trigger (Arg⁶⁹ → Glu) is homologous to the Arg⁶⁹ → Glu mutation in visual arrestin, that makes its binding to rhodopsin phosphorylation-independent (5); 2) Glu¹³⁴ → Ter; and 3) Asp¹³⁵ → Ter, that delete a part or all of the regulatory arrestin COOH terminus, which keeps arrestin in a basal conformation and suppresses an untimely mobilization of the secondary binding site (6). First, we tested the ability of these mutants to interact with purified β-AR reconstituted into phospholipid vesicles by performing direct binding studies (3) and agonist affinity shift assays (7) in vitro. Wild type β-arrestin and β-arrestin-(1–393) bind poorly to activated unphosphorylated receptor (Fig. 1A). In contrast, β-arrestin-(Arg⁶⁹ → Glu) and β-arrestin-(1–382) demonstrate significantly higher binding to activated unphosphorylated receptor (Fig. 1A). Wild type β-arrestin and all three mutants readily bind to activated and phosphorylated β-AR (Fig. 1A). Thus, β-arrestin-(Arg⁶⁹ → Glu) and β-arrestin-(1–382) bind to activated β-AR in a phosphorylation-independent fashion.

Recently (7) we found that arrestin-receptor complex is similar to G protein-receptor complex in two respects: agonists have higher affinity for arrestin-receptor complex than for receptor alone, and only a fraction of the receptors forms such a high-affinity complex (HAC) even at saturating concentrations of arrestin. The maximum percentage of the receptor in HAC gives a good estimate of the propensity of a given arrestin protein to bind tightly to the receptor (arrestin competency) (7). Consistent with the direct binding data (Fig. 1A),
β-arrestin-(Arg169 → Glu) and β-arrestin-(1–382) induced the formation of HAC by unphosphorylated β2AR (22 ± 4% in both cases) (Fig. 2A). In contrast, all forms of β-arrestin induced the formation of HAC by phosphorylated β2AR (P-β2AR) (Fig. 2B). The percentage of HAC formed by P-β2AR in the presence of saturating (1 μM) concentration of β-arrestin, β-arrestin-(Arg169 → Glu), β-arrestin-(1–382), and β-arrestin-(1–393) was 31 ± 6, 52 ± 3, 41 ± 3, and 20 ± 4%, respectively (Fig. 2B). In summary, in both in vitro assays β-arrestin-(Arg169 → Glu) and β-arrestin-(1–382) demonstrate constitutive activity (phosphorylation-independent receptor binding).

Next we tested whether these β-arrestin species can functionally desensitize unphosphorylated β2AR in living cells. To this end GPCRs were expressed in Xenopus oocytes and the activation of coexpressed G protein-gated inwardly rectifying K⁺ channel Kir3 was used as a measure of receptor function. Under these conditions, the application of receptor agonists produced a large increase in inwardly rectifying potassium conductance (8). Undetectable levels of endogenous arrestins and GRKs are expressed in these cells (data not shown). As a result, only a very slow response desensitization was evident during prolonged agonist treatment when β2AR (or another GPCR) is expressed alone. The rate of desensitization was not significantly increased when the receptor was coexpressed with either GRK alone or arrestin alone. However, a dramatic increase in desensitization rate was observed when both GRK and arrestin were coexpressed with a receptor (8, 9). To compare the relative activity of different forms of β-arrestin, we expressed β2AR with or without GRK3 (also called β-adrenergic receptor kinase 2 or βARK2) in the presence or absence of different forms of β-arrestin. As shown on Fig. 3 (A and C), both wild type and β-arrestin-(1–393) facilitated β2AR desensitization only when βARK2 was present. In contrast, β-arrestin-(Arg169 → Glu) and β-arrestin-(1–382) in the absence of βARK2 produced high rates of desensitization similar to that produced by wild type β-arrestin in the presence of βARK2, suggesting that these mutants did induce phosphorylation-independent desensitization of β2AR in the cell.
In order to test whether the constitutively active forms of β-arrestin retain the characteristic broad receptor specificity of wild type nonvisual arrestins (9, 10), we performed similar series of experiments with β opioid receptor (DOR) (Fig. 3, B and D), which was previously shown to be desensitized following agonist activation in oocytes coexpressing wild type β-arrestin and βARK2 (8). Again, the constitutively active mutants induced DOR desensitization, even in the absence of βARK2, suggesting that these mutations do not appreciably change receptor specificity of β-arrestin (or, rather, lack thereof). It should be noted that wild type visual and β-arrestin, visual arrestin mutant (Arg175 → Glu) and β-arrestin mutants (Arg169 → Glu), (1–382), and (1–393), readily bind to activated phosphorylated forms of both rhodopsin and β2AR (Fig. 1). Visual arrestin mutant (Arg175 → Glu) also binds to unphosphorylated activated rhodopsin, while β-arrestin mutants (Arg169 → Glu) and (1–382) bind to unphosphorylated activated β2AR. However, phosphorylation-independent visual arrestin mutant does not bind to unphosphorylated activated β2AR and phosphorylation-independent β-arrestin mutants do not bind to unphosphorylated activated rhodopsin (Fig. 1). Thus, the preference of β-arrestin for β2AR over rhodopsin and that of visual arrestin for rhodopsin over β2AR (3) appears, if anything, enhanced by these mutations.

Interestingly, in the presence of βARK2 both of the phosphorylation-independent β-arrestin mutants induced a more rapid receptor desensitization than wild type β-arrestin (Fig. 3), although the expression levels of all forms of β-arrestin in oocytes were virtually the same (0.72 ± 0.34, 0.90 ± 0.27, 0.85 ± 0.32, and 1.44 ± 0.87 ng/µg of total protein for wild type, (Arg169 → Glu), (1–382), and (1–393) forms, respectively). Apparently, faster desensitization in the presence of βARK2 reflects stronger binding of the mutants to phosphorylated receptor (Figs. 1, 2). Because the peak agonist-induced β2AR and DOR responses were not significantly different in oocytes expressing constitutively active β-arrestins (compared with oocytes expressing no β-arrestin or wild type β-arrestin; data not shown), the mutants do not appear to be prebound to the receptor before agonist application.

Our previous studies demonstrated that the crucial GRK phosphorylation sites are localized on the carboxyl-terminal part of DOR, and that the truncation of the receptor yielding DOR-(1–339) blocked homologous desensitization mediated by β-arrestin + βARK2 (8). We tested whether constitutively active β-arrestin mutants can rescue the desensitization of DOR-(1–339). Both phosphorylation-independent β-arrestin mutants induced the desensitization of truncated DOR with virtually the same kinetics as evident for the full-length DOR (Fig. 4). These data suggest that constitutively active β-arrestins are equally capable of tight binding to (and blocking the signaling of) a receptor without phosphates on the COOH terminus and without the COOH terminus itself. An important implication of this finding is that the major role of the GRK-phosphorylated elements of the receptor is to pull the phosphorylation-sensitive trigger on the arrestin molecule; they do not appear to be required for tight arrestin binding to the receptor per se.

Thus, the binding of β-arrestin-(Arg169 → Glu) and β-arrestin-(1–382) to unphosphorylated receptor detectable in both in vitro assays (Figs. 1 and 2) translates into the ability of these mutants to induce phosphorylation-independent receptor desensitization in the living cell (Figs. 3 and 4). Taken together, the data corroborate the model of sequential multisite arrestin-receptor interaction (2, 3) and open an enticing prospect of targeted construction of mutant arrestins with different special functional characteristics. Recent studies suggest that arrestin binding targets the receptors for internalization (10, 11), apparently by virtue of the ability of nonvisual arrestins to interact with clathrin (12), which is unaffected by the mutations introduced in this study (data not shown). β-Arrestin mutants capable of tight phosphorylation-independent binding to the receptor may change the pattern of intracellular receptor trafficking.

Phosphorylation-independent arrestins are likely to prove valuable tools for the experimental manipulation of the efficacy of signaling by different GPCRs. Uncontrolled signaling by various naturally occurring mutant forms of G protein-coupled receptors has been linked to a wide variety of pathological conditions in humans, from stationary night blindness and retinitis pigmentosa (Refs. 13 and 14 and references therein) to Jansen-type metaphyseal chondrodysplasia (15), autosomal dominant hypocalcemia (17, 18), autosomal dominant hyperthyroidism (19, 20), and numerous forms of cancer (reviewed in Ref. 21). Arrestin mutants with an enhanced ability to block this excessive signaling appear promising tools for the gene therapy of these disorders.

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6834 Constitutevly Active β-Arrestin Mutants