The Active Conformation of β-Arrestin1

DIRECT EVIDENCE FOR THE PHOSPHATE SENSOR IN THE N-DOMAIN AND CONFORMATIONAL DIFFERENCES IN THE ACTIVE STATES OF β-ARRESTINS 1 AND -2*

Received for publication, December 14, 2006, and in revised form, April 25, 2007 Published, JBC Papers in Press, May 18, 2007, DOI 10.1074/jbc.M611483200

Kelly N. Nobles‡, Ziqiang Guan†, Kunhong Xiao§, Terrence G. Oas¶, and Robert J. Lefkowitz‡‡

From the Departments of ‡Biochemistry and ††Medicine and ‡‡Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

β-Arrestins are multifunctional adaptor proteins that regulate seven transmembrane-spanning receptor (7TMR) desensitization and internalization and also initiate alternative signaling pathways. Studies have shown that β-arrestins undergo a conformational change upon interaction with agonist-occupied, phosphorylated 7TMRs. Although conformational changes have been reported for visual arrestin and β-arrestin2/postsynaptic density-95 ( PSD-95), these studies are not representative of conformational changes in β-arrestin1. Accordingly, in this study, we determine conformational changes in β-arrestin1 using limited tryptic proteolysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis in the presence of a phosphopeptide derived from the C terminus of the V2 vasopressin receptor (V2Rpp) or the corresponding unphosphorylated peptide (V2Rnp). V2Rpp binds specifically to β-arrestin1 causing significant conformational changes, whereas V2Rnp does not alter the conformation of β-arrestin1. Upon V2Rpp binding, we show that the previously shielded Arg393 becomes accessible, which indicates release of the C terminus. Moreover, we show that Arg285 becomes more accessible, and this residue is located in a region of β-arrestin1 responsible for stabilization of its polar core. These two findings demonstrate "activation" of β-arrestin1, and we also show a functional consequence of the release of the C terminus of β-arrestin1 by enhanced clathrin binding. In addition, we show marked protection of the N-domain of β-arrestin1 in the presence of V2Rpp, which is consistent with previous studies suggesting the N-domain is responsible for recognizing phosphates in 7TMRs. A striking difference in conformational changes is observed in β-arrestin1 when compared with β-arrestin2, namely the flexibility of the interdomain hinge region. This study represents the first direct evidence that the “receptor-bound” conformations of β-arrestins 1 and 2 are different.

Seven transmembrane-spanning receptors (7TMRs), G-protein-coupled receptors, constitute the largest family of cell-surface receptors. Although individual properties of 7TMRs show some variation, the overall structural and regulatory features appear to be conserved. Fundamental to the regulation of 7TMRs are the families of G-protein-coupled receptor kinases (GRKs) and β-arrestins, which facilitate receptor desensitization, clathrin-mediated internalization, and scaffolding of additional signaling processes to 7TMRs (1–4). GRKs are a distinct class of kinases that phosphorylate serine and threonine residues on the C terminus of agonist-occupied 7TMRs. This phosphorylation event facilitates subsequent binding of β-arrestins to 7TMRs, which precludes further interaction of the receptor with G-proteins (desensitization) (2). The β-arrestins also recruit clathrin and other endocytic elements to 7TMRs, which initiate clathrin-mediated endocytosis of receptors (5). Recent evidence indicates that β-arrestins also function as adaptor proteins to recruit signaling molecules to activated, phosphorylated 7TMRs thereby initiating a wave of signaling that follows the initial G-protein-dependent signaling (6). Moreover, β-arrestins 1 and 2 may have different roles in modulating cellular processes, although extant evidence suggests that the two β-arrestin isoforms are structurally very similar. The roles of β-arrestins in 7TMR desensitization, internalization, and signaling are agonist-dependent, which demonstrates that β-arrestins must first interact with agonist-occupied, phosphorylated 7TMRs suggesting that the conformation of “receptor-bound” β-arrestin is required to elicit some of its cellular functions.

The arrestin gene family in mammals consists of four members as follows: arrestins 1 and 4 (visual arrestins) and arrestins 2 and 3, also known as β-arrestin1 and -2, respectively. Solved crystal structures of the basal state of visual arrestin1, visual arrestin4, and β-arrestin1 in conjunction with extensive biochemical and mutagenesis studies support the idea that a conformational change occurs in arrestins upon binding activated, phosphorylated 7TMRs (7–28). The basal conformations of arrestins are elongated two-domain (N- and C-domain) mole-

* This work was supported in part by Grants HL16037 and HL70631 (to R. J. L.) from the National Institutes of Health. 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.
1 Supported by LIPID MAPS Large Scale Collaborative Grant GM069338 from the National Institutes of Health.
2 To whom correspondence should be addressed: Howard Hughes Medical Institute, Duke University Medical Center, Box 3821, Durham, NC 27710. Tel: 919-684-2974; Fax: 919-684-8875; E-mail: lefko001@receptor-biol.duke.edu.

3 The abbreviations used are: 7TMR, seven transmembrane-spanning receptor; GRK, G-protein-coupled receptor kinase; V2R, human V2 vasopressin receptor; V2Rnp and V2Rpp, synthetic nonphosphopeptide and phosphopeptide, respectively; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; DTT, dithiothreitol; GST, glutathione S-transferase; GS, glutathione-Sepharsose; LC/ESI-MS, liquid chromatography electrospray ionization mass spectrometry; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; AT1aR, angiotensin 1A receptor.
The Active Conformation of β-Arrestin1

β-Arrestin1 and -2 are ubiquitously expressed, and their universal regulation of 7TMRs attests to their importance in modulating cellular function. Despite the ubiquitous expression of both β-arrestin1 and -2, recent evidence has suggested that the two isoforms are in fact not functionally redundant. For example, β-arrestin1 is responsible for scaffolding RhoA activation in conjunction with Goα11 and also IGF-1 activation of phosphatidylinositol 3-kinase (30, 31). β-Arrestin2 scaffolds the mitogen-activated protein kinase (MAPK) cascade to activate ERK1/2, c-Jun N-terminal kinase (JNK3), and in some cases, p38 (4, 6, 32–36). Our laboratory has previously reported conformational changes in β-arrestin2 with a phosphorylated peptide corresponding to the C terminus of the human V2 vasopressin receptor (V2Rpp) as assessed by limited tryptic proteolysis and MALDI-TOF analysis (29). Using this same approach, we now report that the conformation of β-arrestin1 is also altered upon binding V2Rpp, but in ways that suggest that its activated conformation is different from that of β-arrestin2.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—The synthesis of both V2Rnp and V2Rpp have been described elsewhere, and the sequence of both peptides is as follows with phosphorylation sites boldface and underlined: ARGRTPPSLGPQDESCITASSLAKDTS (29). Two other nonspecific synthetic peptides, a 28- and 30-mer, derived from GRK2 were used as controls and have been described previously (29).

Purification of Recombinant Rat β-Arrestin1—Wild type rat β-arrestin1 was subcloned into a pGEX4T1 vector and expressed as a GST fusion protein (GST-β-arrestin1). This construct was confirmed by DNA sequencing and transformed into Escherichia coli strain BL21(DE3) pLYsS. To overexpress GST-
**The Active Conformation of β-Arrestin1**

β-arrestin1, cultures were grown at 37 °C to an A_{600} of 0.8, and the cultures were then equilibrated to 17 °C. GST-β-arrestin1 expression was induced with 0.1 mM isopropyl 1-thio-β-d-galactopyranoside for 16 h, and cells were then harvested by centrifugation at 4,500 × g. The bacterial pellet was resuspended in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 2 mM DTT). The cleaved GST-β-arrestin1 bound was resuspended in 2 column volumes of CB1, and the GST fusion protein was cleaved with thrombin protease (Hematologic Technologies Inc.) at a mg/mg ratio of 1:1000 of thrombin: GST-β-arrestin1. The thrombin digestion was carried out at 4 °C for 16 h with gentle agitation of the GS resin, and the supernatant was collected followed by two additional washes of the GS resin with CB1. The washes and supernatant were pooled, and the NaCl concentration was diluted dropwise to 50 mM NaCl by the addition of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 2 mM DTT. The cleaved β-arrestin1, which contains eight additional amino acids at the N terminus after thrombin cleavage (Fig. 2), was then loaded onto a 5-ml HiTrap Mono-Q column (GE Healthcare) and then eluted with a binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 2 mM DTT), GST-arrestin2, a trypsin to arrestin2 ratio of 1:2,000 was used. After incubation with ligand, trypsin was added to the β-arrestin:ligand mixture, and the samples were incubated at 25 °C for the indicated time points. At each time point, 5 μl (5 μg) of β-arrestin1 or -2 were removed and transferred to a new microcentrifuge tube containing 5 μl of 2× SDS-PAGE buffer, and samples were analyzed on 4–20% SDS-PAGE (Invitrogen). For MALDI-TOF analysis, 5 μl of proteolysis sample were transferred to an empty microcentrifuge tube and flash-frozen in liquid N2.

**MALDI-TOF MS Analysis**—Spectra were collected in positive-ion mode on a Voyager DE Biospectrometry workstation (Applied Biosystems Inc.) in linear mode using a N2 laser (337 nm). The acceleration voltage, grid voltage, guide wire voltage, delay time, low mass gate, and laser intensity were set to 25 kV, 92.5%, 0.11%, 1200 ns, 10,000 m/z and 2500, respectively. Sixty laser shots were collected for each sample, and the spectra shown represent the sum of these 60 laser shots. Samples for MALDI-TOF MS analysis were thawed on ice and immediately diluted 25–50-fold in matrix solution (45% acetonitrile, 0.1% trifluoroacetic acid, and 5 mg/ml sinapinic acid) giving a final β-arrestin1 concentration of 2–4 μM before depositing 1 μl of the sample mixture onto the MALDI-TOF target plate (Applied Biosystems). Both internal and external standards were used to calibrate the data. For internal calibration, 1 μl of carbonic anhydrase (Sigma) was deposited on the MALDI-TOF target after the β-arrestin1 sample had dried. Aporphoggin and aldolase (Sigma) were used as external calibrants and were deposited on empty target spots. Samples were air-dried at room temperature prior to MALDI-TOF analysis. For each limited tryptic digestion, the mean mass and standard deviations were calculated from at least five independent experiments (Table 1). For low abundance peaks, samples were prepared by quenching the tryptic digest with 1 mM phenylmethylsulfonyl fluoride, and samples were then passed through a ZipTip (Millipore) according to the manufacturer’s instructions. Samples were eluted from the ZipTip with matrix solution, and 1 μl was directly deposited on the MALDI-TOF plate. Protein Prospector was used to determine all theoretical trypsin digestion fragments for rat β-arrestin1. The theoretical trypsin digest was compared with experimentally determined masses to assign candidate fragments for each β-arrestin1 fragment observed in the MALDI-TOF spectra (Table 1). The limited proteolytic fragments with only one possible theoretical candidate fragment from Protein Prospector were assigned directly. Fragments with more than one theoretical candidate were assigned by Western blot analysis with antibodies that recognize either the N or C terminus of β-arrestin1 and by determining more accurate masses by liquid chromatography electrospray ionization MS (LC/ESI-MS).

**LC/ESI-MS**—β-Arrestin1 samples were proteolyzed as described above either in the absence of ligand or in the presence of V_{12}Ralpha, V_{12}Rbeta, and flash-frozen in liquid N2 at various time points. Samples were analyzed on a Shimazu LC system (consisting of a solvent degasser, two LC-10A pumps, and an SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems/MDM-Sciex, Toronto, Canada) equipped with an electrospray source. LC, with a Vydvuc C4 reverse phase column (2.1 × 50 mm), was operated at a flow rate of 200 μl/min with a linear gradient as follows: 100% A was held isocratically for 2 min and then linearly increased to 60% B over 18 min and then increased to 100% B over 5 min. Mobile phase A consists of water:aceto-
nitrile (98:2 v/v) with 0.1% acetic acid. Mobile phase B consists of acetonitrile:water (90:10 v/v) with 0.1% acetic acid. The acquisition and deconvolution of ESI mass spectra were performed using the Analyst QS software.

**Clathrin Binding**—To determine the effects of ligand on β-arrestin1 binding to clathrin, 2.5 μM β-arrestin1 was incubated at 4 °C for 30 min in the absence or presence of a 5:1 molar ratio of ligand:β-arrestin1 in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl). The β-arrestin1 ligand mixture was diluted to 50 nM β-arrestin1 (500 μl) with binding buffer, and clathrin was subsequently added at a 1:1 molar ratio to the reaction mixture. The reactions were tumbled at 4 °C for 4 h, and then 7 μl of a clathrin monoclonal antibody (BD Transduction Laboratories) was added to the mixture and tumbled for an additional 1 h at 4 °C. 14 μl of protein A-agarose beads (Roche Applied Science) was then added to the mixture and tumbled for 1 h at 4 °C. The beads were centrifuged at 20,000 × g in a benchtop microcentrifuge and washed with 1 ml of binding buffer five times and resuspended in 20 μl of 2× SDS-PAGE loading buffer. β-Arrestin1 binding to clathrin was measured by Western blot analysis with an anti-β-arrestin1 antibody (A1CT). Samples were also subjected to SDS-PAGE and Western blot analysis with an anti-clathrin antibody (BD Transduction Laboratories) to normalize the amount of clathrin for each reaction.

**RESULTS**

Conformational Changes in β-Arrestin1 upon V2Rpp Binding—Recombinant β-arrestin1 was purified as described under “Experimental Procedures,” and the primary sequence is shown in Fig. 2. To study conformational changes in β-arrestin1 upon its association with a 7TMR, we employed an in vitro model system utilizing both a phosphopeptide (V2Rpp) and non-phosphopeptide (V2Rnp) corresponding to the C terminus of the human V2 vasopressin receptor (V2, R peptide sequences are given under “Experimental Procedures”). β-Arrestin1 was incubated in the absence or presence of ligand (a 5:1 molar ratio of ligand:β-arrestin1 was used in all experiments unless otherwise stated) and then subjected to limited tryptic proteolysis. The proteolysis patterns of β-arrestin1 alone or in the presence of V2Rnp are different from those in the absence of peptide or in the presence of V2Rpp. Panel III shows the tryptic fragments of β-arrestin1 in the presence of V2Rpp, and the apparent molecular weights of these fragments are indicated to the right of panel III. STD, standard. B, schematic representation of tryptic fragments for β-arrestin1. The control panel on the left is the pattern seen for limited tryptic digestion of β-arrestin1 alone or in the presence of V2Rnp. The V2Rpp panel is shown on the right.
The Active Conformation of β-Arrestin1

β-arrestin1 (Gly\(^8\)–Arg\(^{418}\)) has an apparent molecular mass of 47 kDa, and the addition of trypsin results in slow, continuous digestion generating fragments with apparent molecular masses of 47, 40, 32, 25, and 21 kDa. The addition of the V2Rpp resulted in a distinct digestion pattern with an accelerated proteolysis of the 40- and 32-kDa species as well as the full-length protein (Fig. 3A, panel III). This new digestion pattern also featured the appearance of new species with molecular weights of 44 and 45 kDa and the accumulation of the 21-kDa species over time. The V2Rpp pattern is depicted schematically in Fig. 3B. Proteolysis of β-arrestin1 was also conducted in the presence of two nonspecific peptides, a 28- and 30-mer, and β-arrestin1 digestion in the presence of these peptides resulted in the control pattern (data not shown).

Conformational Changes in β-Arrestin1 Require Phosphate Moieties—We have shown that the limited proteolytic digestion pattern of β-arrestin1 is unaltered by the addition of V2Rnp; the possibility exists that V2Rnp can indeed bind to β-arrestin1 with a lower affinity than V2Rpp, and binding of V2Rnp can also induce conformational changes in β-arrestin1 at a higher V2Rnp to β-arrestin1 ratio. We therefore titrated β-arrestin1 with increasing amounts of peptides and performed limited proteolysis to assess the effects of changes in peptide to β-arrestin1 ratios. V2Rnp does not alter the digestion pattern of β-arrestin1 even at a peptide to β-arrestin1 molar ratio of 20:1, whereas V2Rpp alters the conformation of β-arrestin1 even at a 1:1 molar ratio (Fig. 4, A and B). We also conducted a competition experiment to determine whether high concentrations of V2Rnp can compete with V2Rpp for binding to β-arrestin1. In the presence of a 1:1 molar ratio of V2Rnp, addition of a 100-fold molar excess of V2Rnp did not convert the V2Rpp pattern to the control pattern, clearly demonstrating that the V2Rnp is incapable of competing with V2Rpp binding (Fig. 4C, lane 6).

MALDI-TOF MS Analysis of Limited Tryptic Proteolysis Fragments of β-Arrestin1—Limited proteolysis of β-arrestin1 clearly demonstrates a conformational change in the presence of V2Rpp. However, details of these conformational changes could not be obtained because of the low resolution of SDS-PAGE analysis of tryptic fragments. To map precisely the regions of β-arrestin1 involved in these conformational changes, we employed MALDI-TOF MS to determine sites of proteolysis by measuring the accurate masses of tryptic fragments. Taking advantage of the high mass accuracy of MALDI-TOF MS, most of the species observed by SDS-PAGE were precisely assigned by comparing the experimental mass value of a protein fragment to its theoretical value (Table 1). For those fragments that could not be definitively assigned by MALDI-TOF MS, we used the more accurate LC/ESI-MS with a higher mass accuracy (within less than 1.0 Da for a 5-kDa peptide) to help with assignments. Additionally, to assign some fragments, we also conducted Western blot analysis with antibodies that recognize different domains of β-arrestin1 (N- or C-domain). MALDI-TOF MS analysis from over the range of m/z 20,000–50,000 was conducted on tryptic fragments from β-arrestin1 alone or in the presence of either V2Rnp or V2Rpp. At an early time point (5 min), the spectra for β-arrestin1 alone or in the presence of V2Rnp are identical, and we have therefore shown spectra of β-arrestin1 with V2Rpp only. The major peaks are 48 and 47 kDa and minor peaks at 25 and 21 kDa (Fig. 5A, left panel). In the presence of V2Rpp, the major peaks are left-shifted with masses of 45 and 44 kDa, and a minor peak is also observed at 21 kDa (Fig. 5A, bottom of left panel). The most notable difference in spectra collected at an early time point is the accelerated proteolysis of β-arrestin1 in the presence of V2Rpp resulting in a left-shift of the major peaks.

MALDI-TOF MS of an early time point (5 min) of β-arrestin1 tryptic fragments in the presence of V2Rpp indicates that the major species is full-length β-arrestin1 (Gly\(^8\)–Arg\(^{418}\)), which has an experimental mass (m/z) of 47,840 ± 41 Da (Fig. 5A). Full-length β-arrestin1 is then slowly proteolyzed to residues Leu\(^1\)–Arg\(^{418}\) because of an N-terminal clip at position Arg\(^{418}\) (Fig. 5A). In the presence of V2Rpp, β-arrestin1 proteolysis is initially accelerated and then slow and continuous over
time giving rise to a 45-kDa fragment (45,014 ± 28 Da) corresponding to amino acids Gly^{8}–Arg^{417} as is evident in both the MALDI-TOF spectra and by SDS-PAGE (Fig. 5, A and B). The assignment for this fragment was confirmed by LC/ESI-MS, which gave a mass of 44,992 Da (Table 1). We further confirmed the assignment of this 45-kDa peak (Gly^{8}–Arg^{417}) by the presence of the 25-amino acid C-terminal peptide, Arg^{393}–Arg^{417}, which had a mass of 2854.3 Da by LC/ESI-MS (data not shown). In addition, we also identified a peptide corresponding to residues Asp^{38}–Asp^{44} (data not shown). Interestingly, fragment Leu^{1}–Arg^{285} is more readily proteolyzed in the presence of V_{2}Rpp, as assessed by SDS-PAGE (Fig. 5D), and two species are observed by MALDI-TOF MS with masses of 31,920 ± 41 and 32,342 ± 9 Da, respectively (Fig. 5A, bottom spectrum). There are five candidate fragments for the 31-kDa species that occur in the presence of V_{2}Rpp (Table 1), which cannot be assigned; however, the most likely assignment of this fragment is residues Gly^{5}–Arg^{285} because it represents further proteolysis of the already assigned 32-kDa species (Leu^{1}–Arg^{285}). Fig. 5C shows the location of Arg^{385} on the crystal structure of bovine β-arrestin1 (Protein Data Bank code 1G4R).

The most striking feature of our β-arrestin1 study is the rapid appearance of a 21-kDa species in the presence of V_{2}Rpp, which persists even up to 2 h after digestion (Fig. 3A, panel III) and the complete absence of a 25-kDa peak in the MALDI-TOF MS. MALDI-TOF analysis over the range m/z 20,000–30,000 revealed that there are in fact two species, with masses of 25 and 21 kDa, for β-arrestin1 alone or in the presence of V_{2}Rnp (Fig. 5A, right panel). The 25-kDa species in all experiments is not visible by SDS-PAGE; however, this low abundance species is visible by MALDI-TOF analysis in the late time point (Fig. 5A, right-hand spectra). Experimental masses for the 25-kDa species were determined to be 25,742 ± 17 and 25,736 ± 41 for β-arrestin1 alone or in the presence of V_{2}Rnp, respectively (Table 1). There are three candidate fragments (Asp^{26}–Lys^{256}, Val^{171}–Lys^{400}, and Gln^{397}–Arg^{417}) for the 25-kDa species, and it is visible by Western blot

### TABLE 1
Assignment of the limited tryptic proteolytic fragments of β-arrestin1

| Final assignment of tryptic fragments | Candidate fragments (theoretical) | MALDI-TOF m/z (experimental) | Apparent mass by SDS-PAGE | Confirmed by ESI-MS |
|--------------------------------------|-----------------------------------|-----------------------------|--------------------------|-------------------|
| Gly^{5}–Arg^{118} | Gly^{8}–Arg^{118} | 47,830 | 47,841 ± 35 | 47,853 ± 32 | 48 |
| Leu^{1}–Arg^{118} | Gly^{8}–Arg^{118} | 47,003 | 47,022 ± 19 | 46,995 ± 28 | 47 |
| Gly^{5}–Arg^{109} | Gly^{8}–Arg^{109} | 44,993 | 44,875 ± 30 | NA | 44 |
| Leu^{1}–Arg^{109} | Leu^{1}–Lys^{109} | 45,007 | 45,014 ± 28 | NA | 45 |
| Leu^{1}–Arg^{283} | Leu^{1}–Arg^{283} | 44,165 | NA | NA | + |
| Leu^{1}–Arg^{285} | Leu^{1}–Arg^{285} | 40,744 | 40,739 ± 24 | 40,725 ± 24 | 40 |
| Leu^{1}–Arg^{363} | Leu^{1}–Arg^{363} | 32,335 | 32,321 ± 21 | 32,342 ± 9 | 32 |
| Gly^{5}–Arg^{393} | Leu^{1}–Arg^{363} | 31,905 | 31,900 ± 37 | 31,920 ± 41 | 32 |
| Gly^{5}–Arg^{393} | Gly^{5}–Arg^{393} | 31,922 | NA | 25 |
| Lys^{1}–Arg^{385} | Gly^{5}–Arg^{393} | 31,922 | 25,742 ± 17 | 25,736 ± 41 | 21 |
| Lys^{1}–Arg^{385} | Gly^{5}–Arg^{393} | 31,934 | 21,256 ± 19 | 21,262 ± 23 | 21 |

**NA indicates not applicable.**

**Recognized by F4C1.**
The Active Conformation of β-Arrestin 1

Functional Consequences of Conformational Changes in β-Arrestin1—To assess the functionality of our β-arrestin1:V2Rpp system and the biological ramifications of conformational changes induced in β-arrestin1, we tested the in vitro binding of β-arrestin to clathrin (Fig. 6). Clathrin was bound to protein A beads through a monoclonal antibody that recognizes clathrin heavy chain, and β-arrestin1 was then incubated with the clathrin beads (clathrin-protein A beads) either in the absence or presence of V2R peptides. We assessed β-arrestin1 binding to clathrin by Western blot analysis. Fig. 6A shows a Western blot for clathrin to ensure equal loading of clathrin for all experimental conditions tested. Quantitation of β-arrestin1 binding to either empty protein A beads or clathrin-protein A beads from five independent experiments is shown in Fig. 6D. β-Arrestin1 binding to clathrin-protein A beads is normalized to 100% and shows enhanced binding over β-arrestin1 in the presence of V2Rnp (29.7 ± 4.1%) or absence of ligand (21.3 ± 4.5% for β-arrestin1 alone and 29.1 ± 1.5% for β-arrestin1 with clathrin).

The “Active” Conformations of β-Arrestin1 and -2 Are Different—A previous study from our laboratory reported conformational changes in β-arrestin2 upon its interaction with V2Rpp (29). However, this study did not include information on tryptic fragments below 30 kDa, which would exclude information on the individual N- and C-domains of β-arrestin2. Thus, we conducted limited tryptic proteolysis on β-arrestin2 as described under “Experimental Procedures” to directly compare conformational differences between the two β-arrestin isoforms (Fig. 7). Fig. 7A shows a limited tryptic digestion of...
The Active Conformation of \(\beta\)-Arrestin1

\(\beta\)-Arrestins, initially discovered for their role in terminating 7TMR signaling, have been shown more recently to interact with over a dozen nonreceptor partners and thereby serve as scaffolds for MAPK cascades initiating a second wave of signaling independently of G-proteins (37). The majority of all \(\beta\)-arrestin functions are receptor activation-dependent, and the obvious corollary for this is that \(\beta\)-arrestins undergo a conformational change when bound to agonist-occupied 7TMRs. The solved crystal structure of \(\beta\)-arrestin1 shows that both the N and C termini are in close proximity in the overall fold of the molecule and that intramolecular interactions of these termini stabilize the basal conformation of the protein (16). The C terminus of \(\beta\)-arrestin1 contains both clathrin- and AP2-binding sites, and the release of this terminus from the fulcrum of the molecule is required to expose these sites (3). Clathrin and AP2 recruitment to 7TMRs via \(\beta\)-arrestin1 occurs only when 7TMRs are both active and phosphorylated, which demonstrates that \(\beta\)-arrestin1 must be in its active conformation for this now

\[
\begin{array}{cccccc}
\text{Protein A beads} & + & + & + & + & + \\
\text{Ligand} & - & - & \text{V2Rnp} & \text{V2Rpp} & - \\
\text{Clathrin} & - & + & + & + & + \\
\text{\(\beta\)-arrestin1} & + & + & + & + & - \\
\end{array}
\]

\[
\text{Enhancement of clathrin binding to \(\beta\)-arrestin1 in the presence of V2Rpp, panel I, and the representation of a \(\beta\)-arrestin1 digestion in the presence of V2Rpp (panel II). The major species present in all \(\beta\)-arrestin1 digestions is a 42-kDa species, which was previously reported as Gly\(^{7-}\text{Arg}^{364}\), and this species is significantly protected in the presence of V2Rpp (Fig. 7A, compare panels I and II). Residues Gly\(^{7-}\text{Arg}^{364}\) of \(\beta\)-arrestin2 include both the N-domain and most of the C-domain; thus, in the presence of V2Rpp, the majority of \(\beta\)-arrestin2 is protected over time with only the last 52 C-terminal residues missing from this fragment. This is, however, not the case for \(\beta\)-arrestin1 as only the N-domain itself, Leu\(^{7-}\text{Arg}^{364}\), is protected in the presence of V2Rpp (Fig. 7B, panel III). Taken together, these data suggest that the V2Rpp-bound, or active, conformations of \(\beta\)-arrestin1 and -2 are different.

DISCUSSION

\(\beta\)-Arrestins, initially discovered for their role in terminating 7TMR signaling, have been shown more recently to interact with over a dozen nonreceptor partners and thereby serve as scaffolds for MAPK cascades initiating a second wave of signaling independently of G-proteins (37). The majority of all \(\beta\)-arrestin functions are receptor activation-dependent, and the obvious corollary for this is that \(\beta\)-arrestins undergo a conformational change when bound to agonist-occupied 7TMRs. The solved crystal structure of \(\beta\)-arrestin1 shows that both the N and C termini are in close proximity in the overall fold of the molecule and that intramolecular interactions of these termini stabilize the basal conformation of the protein (16). The C terminus of \(\beta\)-arrestin1 contains both clathrin- and AP2-binding sites, and the release of this terminus from the fulcrum of the molecule is required to expose these sites (3). Clathrin and AP2 recruitment to 7TMRs via \(\beta\)-arrestin1 occurs only when 7TMRs are both active and phosphorylated, which demonstrates that \(\beta\)-arrestin1 must be in its active conformation for this now
classical function. This study clearly demonstrates that, in the presence of V₂Rpp, both the N and C termini are more flexible and, furthermore, that the C terminus is released as evidenced by accessibility of the previously shielded Arg^{393} and enhanced clathrin binding.

The mechanism by which visual arrestins and β-arrestins interact with activated, phosphorylated 7TMRs has been studied through a series of mutagenesis and biochemical studies in addition to solved crystal structures (7–10, 12, 16, 18–19, 22–23, 25–29, 38–40). One of the most striking features of all arrestin structures is the presence of a distinctive polar core, a series of five interacting charges completely shielded from water and embedded at the center of the molecule. Disruption of this polar core is necessary for arrestin activation, and we have demonstrated disruption of the polar core of β-arrestin1 in the presence of V₂Rpp by increased accessibility of Arg^{285}. This residue is actually located in what is termed the “lariat loop” (Arg^{282}–Gly^{309}) region. The lariat loop, in part, maintains the basal conformation of β-arrestin because it contains Asp^{290}, the primary counterion for Arg^{169} in the polar core (Fig. 1C). The primary sequence of the lariat loop is not conserved among the four mammalian arrestins; however, the secondary structure appears to be conserved in all solved crystal structures of arrestin family members to date (9, 16, 18, 21, 25). Arg^{169} of β-arrestin1 is the primary phosphate sensor because a charge reversal mutation (R169E) results in a phosphorylation-independent mutant. Our data represent the first direct biochemical evidence that “activation” of β-arrestin1 is dependent on both the disruption of the polar core of β-arrestin1 and thereby release of its C terminus to carry out functions such as clathrin binding.

Previous studies with both visual arrestin and β-arrestin1 have shown that the N-domain of the molecule contains the main phosphate sensor for phosphorylated 7TMRs (reviewed in Ref. 15). These studies have localized the main sensor to a single residue, Arg^{169}. To date, only mutagenesis studies and structural activation models have been used to localize the phosphate sensor of β-arrestin. One of the most notable features of our β-arrestin1 limited tryptic proteolysis study is the rapid appearance and protection of a 21-kDa species in the presence of V₂Rpp. MALDI-TOF MS analysis of this tryptic fragment confirms that it is indeed the N-domain of β-arrestin1 (Leu^{1}–Arg^{188}), and this fragment persists even up to 2 h post-digestion, which indicates great stability of this domain when in complex with V₂Rpp. Although our study does not show direct biophysical evidence of V₂Rpp binding to the N-domain of β-arrestin1, our data are in accordance with previously published models based on crystal structures indicating that the more flexible N-domain of β-arrestin1 is responsible for recognizing the phosphate elements of 7TMRS, whereas the more rigid C-domain most likely serves as a structural scaffold for β-arrestin1 binding partners (16). The intact C-domain itself is never apparent as a separate entity once cleaved from the N-domain, which leads us to believe that a binding partner such as clathrin would be necessary to stabilize this domain and protect it from complete digestion. Our study represents the first direct evidence that the N-domain itself is in fact responsible for phosphate recognition.

One of the most valuable aspects of our in vitro model system is that it can be used to study conformational changes in β-arrestins with various phosphoreceptor peptides and, perhaps more interestingly, can also be used to study conformational differences between β-arrestin1 and -2. Numerous crystal structures have been solved for β-arrestin1, but the crystal structure of β-arrestin2, however, remains elusive, so any information on structural differences between the two isoforms will have to be garnered from direct biochemical assays. Conformational changes in β-arrestin2 with V₂Rpp have been previously described from our laboratory (29). Briefly, this study similarly demonstrated the release of the C terminus of β-arrestin2 from the fulcrum of the molecule by accessibility of Arg^{394} (homologous to Arg^{393} of β-arrestin1) and disruption of the polar core of β-arrestin2 by increased accessibility of Arg^{285} (homologous to Arg^{286} of β-arrestin1). This previous study, however, contained no information on the tryptic fragments of β-arrestin2 below 30 kDa, which would preclude any information of individual N- and C-domains of β-arrestin2.

Accordingly, we directly compared tryptic fragments below 30 kDa for both β-arrestin1 and -2 (Fig. 7). The major protected species for β-arrestin1 is the N-domain (Leu^{1}–Arg^{188}), and the major protected species for β-arrestin2 is both the N- and C-domains (a 42-kDa species) corresponding to residues Gly^{7}–Arg^{364}. Although similar N-domain fragments are observed for β-arrestin2 (25- and 21-kDa species), they are not more rapidly generated nor protected over time in the presence of V₂Rpp. These data taken together suggest that although the overall activation mechanism is the same for β-arrestin1 and -2, the final conformations in the presence of V₂Rpp are in fact different. We first need to exclude two obvious explanations for the observed differences in the active conformations, namely stoichiometric differences in peptide binding to the β-arrestins and primary sequence differences that would result in altered final conformations.

First, the stoichiometry of the binding of V₂Rpp to β-arrestin1 and -2 may be different. If, for example, β-arrestin2 were to bind two phosphopeptides, then it would make sense that both the N- and C-domains (Gly^{7}–Arg^{364}) are protected, whereas β-arrestin1 binding a single peptide would explain protection of only the N-domain (Leu^{1}–Arg^{188}). We have shown in this study, by limited proteolysis, that different molar ratios of V₂Rpp:β-arrestin1 do not alter the digestion pattern. A 1:1 molar ratio of V₂Rpp:β-arrestin1 is both necessary and sufficient to induce the observed conformational differences. Similarly, this same experiment in a previous study with β-arrestin2 demonstrated that increasing the molar ratio of V₂Rpp:β-arrestin2 did not alter the digestion pattern (29). These data taken together suggest that a 1:1 molar ratio of V₂Rpp to either β-arrestin1 or -2 is sufficient to induce the active conformation. Moreover, we also performed native PAGE of β-arrestin1 and -2 in the presence of increasing amounts of V₂Rpp and have determined via a mobility shift that a 1:1 molar ratio of V₂Rpp:β-arrestin suffices for the same shift that we observe at a 5:1 molar ratio (data not shown). These data indicate that the stoichiometry of binding for V₂Rpp to β-arrestin1 and -2 is in fact 1:1, and thus the conformational differences observed between
the two isoforms is not because of a difference in the stoichiometry of peptide binding to the two β-arrestins.

Second, differences in the active conformation of β-arrestin1 and -2 could simply be due to differences in their primary sequences. Fig. 2 shows the sequence alignment for recombinant rat β-arrestin1 and -2 used in this study. The two isoforms are 78% identical, and the two sequences are well conserved up to the residue numbered 340. We observed a 40-kDa fragment in all β-arrestin1 digests that corresponds to residues Leu1–Arg361. This fragment was protected in the early time points but was not the major protected species for β-arrestin1. In the case of β-arrestin2, cleavage was observed at Arg189 producing a 42-kDa fragment that was significantly protected over time in the presence of V2Rpp. Although these two arginines in β-arrestin1 (Arg189) and β-arrestin2 (Arg186) do not align, we still see a somewhat similar pattern of digestion in this portion of their C termini. The striking difference, however, is that β-arrestin2 digests show significant protection of the 42-kDa species, whereas β-arrestin1 digests do not show as dramatic a protection of the corresponding 40-kDa species (Leu1–Arg361). We do not believe that differences seen in the digestion patterns of β-arrestin1 and -2 are because of primary sequence differences, and therefore, we conclude that the observed differences are in fact because of conformational differences in the final fold of the two proteins.

Because the major product in β-arrestin1 digestions in the presence of V2Rpp is Leu1–Arg188, we also inspected the sequence alignment of β-arrestin1 and -2 in this region (Fig. 2). The sequence alignment of β-arrestin1 and -2 containing Arg188 and Arg189, respectively, is just upstream of a flexible 12-residue interdomain hinge (boxed in Fig. 2) that connects the N- and C-domains. The primary sequence of the hinge region is very well conserved, and Arg188 of β-arrestin1 aligns perfectly with Arg189 of β-arrestin2. We do see tryptic fragments in the digestion of β-arrestin2 with V2Rpp that correspond to the N-domain, but these fragments are generated more slowly from the 42-kDa parent fragment and are only slightly protected over time. Conversely, in the digestion of β-arrestin1 with V2Rpp, the N-domain is dramatically protected over time and persists up to 2 h post-digestion. The protection of the hinge region tryptic residues in the final fold of β-arrestin2 makes this region, specifically Arg189, less susceptible to tryptic proteinolysis. In summary, the major protected species for β-arrestin1 in the presence of V2Rpp is the N-domain (Leu1–Arg188), whereas for β-arrestin2 it is both the N- and C-domains (Gly7–Arg364). These data suggest that the conserved hinge region becomes more accessible in β-arrestin1 (as evidenced by proteinolysis at Arg188) than β-arrestin2 because proteinolysis at the corresponding Arg189 is less rapid in the presence of V2Rpp. This study shows, for the first time, that the flexibility of this hinge region is in fact different for the two β-arrestin isoforms. Moreover, other studies, including mutagenesis and solved crystal structures, have suggested that the two domains of β-arrestin move relative to one another upon binding 7TMRs, and this hinge region is responsible for the movement of the two domains (9, 15–16, 18, 21, 25–27, 40). Interestingly, successive shortening of the hinge region of the visual arrestin abolishes its ability to bind light-activated, phosphorylated rhodopsin (26). We have evidenced here that this movement in β-arrestin1 and -2 is in fact different when bound to V2Rpp.

Because structure dictates function, it follows that the two isoforms have different final conformations because evidence exists demonstrating that β-arrestins1 and -2 are functionally nonredundant, and such individual, nonoverlapping roles in 7TMR regulation are very compatible with distinct receptor-bound conformations. The first evidence to suggest that β-arrestin1 and -2 are in fact not functionally redundant lies in their differential binding to 7TMRs. 7TMRs are broadly broken down into two classes, class A and B (37). Class A receptors, such as the β2AR, have a higher affinity for β-arrestin2 than β-arrestin1; however, the interaction of these 7TMRs with β-arrestin is transient in that internalization of class A receptors leads to dissociation of the β-arrestin-receptor complex. Class B receptors, such as the AT1aR and V2 vasopressin receptor, display equal affinity for both β-arrestin isoforms and form more stable β-arrestin-receptor complexes. This preference for one isoform versus the other in receptor binding was the first evidence to suggest that β-arrestins do not serve redundant roles, but rather that the two isoforms have nonoverlapping, distinct functions.

In addition to differential receptor regulation, the two β-arrestin isoforms also display functional differences with nonreceptor partners. β-Arrestins are multidomain adaptor scaffold proteins that regulate 7TMR signaling, and multiple lines of recent evidence have shown that β-arrestins1 and -2 are also nonredundant in this function (reviewed in Ref. 41, 42). Over a dozen nonreceptor partners have been shown to interact with β-arrestins, and the majority of these interactions occur in an agonist-dependent manner indicating that β-arrestins must be in the active or receptor-bound conformation to elicit these functions. The first evidence that β-arrestins play a role in 7TMR signaling stemmed from the discovery that β-arrestin1 specifically, and not β-arrestin2, interacts with c-Src, a nonreceptor tyrosine kinase (43, 44).

Studies following the discovery of the role of β-arrestin1 in c-Src recruitment and ERK activation led to what is now a newly appreciated paradigm, the idea that β-arrestins may in fact play a role in scaffolding MAPK cascades. In terms of ERK signaling, it appears as though different 7TMRs prefer one β-arrestin isoform over the other. In the case of the protease-activated 2 receptor, it has been demonstrated that agonist stimulation leads to complex formation containing receptor, β-arrestin1, Raf-1, and active ERK (44). However, for angiotensin 1A receptor (AT1aR) stimulation of ERK activation via β-arrestins, β-arrestin2 carries the signal, whereas β-arrestin1 actually functions antagonistically (4, 33). It has also been recently reported that in some receptor systems such as the parathyroid hormone receptor (PTH1R), agonist stimulation of ERK through β-arrestin requires both isoforms (32). Thus, in the case of β-arrestin scaffolding of ERK via 7TMR stimulation, it appears as though different receptor systems, in general, display differential preference for either β-arrestin1 or -2, and in some cases both isoforms may be necessary. Although the ERK MAPK cascade has
emerged as the prototypical β-arrestin scaffold function studied, it has also been demonstrated that β-arrestins directly scaffold the activation of JNK3 and possibly indirectly p38 activation, although these systems have not been studied to the same extent as the role of β-arrestins in ERK signaling (34, 35, 45, 46). Nonetheless, the scaffolding of the JNK3 MAPK cascade has only been shown for β-arrestin2, yet again indicating isoform specificity and functional non-redundancy for the β-arrestins. Additionally, β-arrestin1, but not β-arrestin2, has been shown to signal coordinately with Goq/11 to activate RhoA, a small G-protein, through AT1aR activation (30).

The different active conformations documented here for β-arrestins1 and -2 are thus consistent with numerous functional differences demonstrated by a multitude of studies. Furthermore, the differences in the conformational changes observed in both β-arrestins1 and -2 may in fact be due to differences in the flexibility of their interdomain hinge regions. This hypothesis could be easily tested by simply swapping the hinge regions of the two isoforms and determining if their functions in various systems are reversed. The model system used in this study provides an excellent means for determining conformational differences not only between β-arrestin1 and -2 upon interaction with a given 7TMR, but also in comparing the conformational differences that may occur when β-arrestin1 and -2 are bound to phosphopeptides from various 7TMRs, which in turn may aid in delineating why some receptor systems (as in the case of ERK activation) prefer one of the β-arrestins or both. β-Arrestins1 and -2 are clearly not functionally redundant, and this study provides the first evidence to suggest that these physiological, functional differences may in fact be due differences in their active conformations.

Acknowledgments—We thank Donna Addison and Elizabeth Hall for excellent secretarial assistance and Darrell Capel for technical help. We are indebted to Dr. Homme Hellinga of the Biochemistry Department of the Duke University Medical Center for excellent secretarial assistance and Darrell Capel for technical help. We are indebted to Dr. Homme Hellinga of the Biochemistry Department of the Duke University Medical Center for excellent secretarial assistance and Darrell Capel for technical help. We are indebted to Dr. Homme Hellinga of the Biochemistry Department of the Duke University Medical Center for excellent secretarial assistance and Darrell Capel for technical help. We are indebted to Dr. Homme Hellinga of the Biochemistry Department of the Duke University Medical Center for excellent secretarial assistance and Darrell Capel for technical help.
Physiol. 69, 483–510
42. Gurevich, V. V., and Gurevich, E. V. (2003) Structure (Camb.) 11, 1037–1042
43. Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) Science 283, 655–661
44. DeFea, K. A., Vaughn, Z. D., O’Bryan, E. M., Nishijima, D., Dery, O., and Bunnett, N. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11086–11091
45. Bruchas, M. R., Macey, T. A., Lowe, J. D., and Chavkin, C. (2006) J. Biol. Chem. 281, 18081–18089
46. Miller, W. E., Houtz, D. A., Nelson, C. D., Kolattukudy, P. E., and Lefkowitz, R. J. (2003) J. Biol. Chem. 278, 21663–21671