

Arrestin function in G protein-coupled receptor endocytosis requires phosphoinositide binding

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Internalization of agonist-activated G protein-coupled receptors is mediated by non-visual arrestins, which also bind to clathrin and are therefore thought to act as adaptors in the endocytosis process. Phosphoinositides have been implicated in the regulation of intracellular receptor trafficking, and are known to bind to other coat components including AP-2, AP180 and COPI coatomer. Given these observations, we explored the possibility that phosphoinositides play a role in arrestin's function as an adaptor. High-affinity binding sites for phosphoinositides in β -arrestin (arrestin2) and arrestin3 (β -arrestin2) were identified, and dissimilar effects of phosphoinositide and inositol phosphate on arrestin interactions with clathrin and receptor were characterized. Alteration of three basic residues in arrestin3 abolished phosphoinositide binding with complete retention of clathrin and receptor binding. Unlike native protein, upon agonist activation, this mutant arrestin3 expressed in COS1 cells neither supported β_2 -adrenergic receptor internalization nor did it concentrate in coated pits, although it was recruited to the plasma membrane. These findings indicate that phosphoinositide binding plays a critical regulatory role in delivery of the receptor-arrestin complex to coated pits, perhaps by providing, with activated receptor, a multi-point attachment of arrestin to the plasma membrane.

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Introduction

The desensitization of signaling by G protein-coupled receptors (GPCRs) is a multi-level process initiated by phosphorylation of the agonist-activated receptors by the GPCR kinases. Receptor phosphorylation leads to recruitment of another protein, called arrestin. One of the consequences of arrestin binding to the receptor is an uncoupling of the receptor from the heterotrimeric G protein, believed to occur through direct competition between arrestin and G protein for the activated and phosphorylated receptor (Hargrave and McDowell, 1992; Sterne-Marr and Benovic, 1995). There are four known mammalian

arrestins, two of which (visual arrestin and cone arrestin) are restricted to the phototransduction pathway. Two other arrestins, β -arrestin (or arrestin2) and arrestin3 (or β -arrestin2), are ubiquitously expressed and are thought to regulate signaling of many different GPCRs (Krupnick and Benovic, 1998).

Another level of regulation of receptor signaling involves physical internalization of the receptor into an intracellular compartment in a process known as sequestration. GPCR kinases and arrestins are also thought to play a major role in the internalization of agonist-activated GPCRs (Ferguson *et al.*, 1996; Krupnick and Benovic, 1998). At least one of the pathways by which this internalization occurs is through receptor-mediated endocytosis carried out by clathrin-coated pits, specialized regions of the plasma membrane of all eukaryotic cells (Keen, 1990; Schmid, 1997). Clathrin is a trimeric protein arranged in a triskelion or three-legged shape and is the major structural protein of the characteristic polygonal lattice of the coated pit. Most of the protein comprises the outer shell of the lattice, with a globular portion at the end of each leg, denoted the terminal domain, protruding inward toward the membrane surface. Plasma membrane coated pits also contain AP-2 molecules which bind to clathrin and to the cytoplasmic tails of certain transmembrane receptors that are concentrated in coated pits, thereby functioning as adaptors in the endocytic pathway (Pearse, 1988; Keen, 1990; Sorkin and Carpenter, 1993; Schmid, 1997).

The non-visual arrestin2 and arrestin3 are thought also to function as adaptors by linking agonist-activated and phosphorylated β_2 -adrenergic receptors (β_2 ARs) with the clathrin-coated pit machinery (Goodman *et al.*, 1996). Biochemical studies have shown that the non-visual arrestins interact stoichiometrically and with high affinity with clathrin, interacting primarily with the clathrin terminal domain (Goodman *et al.*, 1997; Krupnick *et al.*, 1997a). In intact cells, β_2 ARs, arrestin2 and clathrin become co-localized upon activation by agonist (Goodman *et al.*, 1996). Additional evidence of an adaptor function for arrestins came from studies in which an arrestin mutant defective in clathrin binding was also found to be deficient in promoting internalization of β_2 ARs. Moreover, over-expression of a portion of arrestin containing the clathrin-binding domain acted as a dominant-negative regulator of β_2 AR internalization (Krupnick *et al.*, 1997b). Beyond these shared functional properties, both AP-2 (Murphy and Keen, 1992) and non-visual arrestins (Goodman *et al.*, 1997) bind to clathrin through the terminal domain region, as does another putative adaptor, AP-3 (Dell'Angelica *et al.*, 1998). Finally, phosphorylation/dephosphorylation of both AP-2 (Wilde and Brodsky, 1996) and arrestin2 (Lin *et al.*, 1997) have been reported to affect their binding to clathrin.

These similarities between the putative adaptor functions of AP-2 and non-visual arrestins prompted a consideration of other potential common regulatory mechanisms of their functions in endocytosis. Phosphoinositides, and particularly 3-phosphorylated phosphoinositides, have been implicated in multiple vesicular trafficking events (De Camilli *et al.*, 1996). These include the sorting of newly synthesized vacuolar (Schu *et al.*, 1993; Stack *et al.*, 1993; Stack and Emr, 1994) and lysosomal (Brown *et al.*, 1995) proteins in yeast and mammalian cells, respectively, in the endocytosis and post-endocytic trafficking of some growth factor receptors (Brown *et al.*, 1995), and in the incorporation of mannose 6-phosphate receptors into Golgi-derived transport vesicles (Gaffet *et al.*, 1997). In the case of AP-2, phosphoinositide binding modulates *in vitro* binding of AP-2 both to clathrin (Beck and Keen, 1991) and to receptor cytoplasmic tails (Rapoport *et al.*, 1997). Furthermore, we have shown recently that a functional phosphoinositide-binding site in AP-2 is required for its targeting to plasma membrane clathrin-coated pits in intact cells (I.Gaidarov and J.H.Keen, in preparation).

In this report, we investigated the potential role of phosphoinositides in the regulation of GPCR internalization by non-visual arrestins. The results show that non-visual arrestins, but not visual arrestin, contain a high-affinity phosphoinositide/phosphoinositol-binding site. Binding of the phosphoinositide phosphatidylinositol-(3,4,5)-trisphosphate [PI-(3,4,5)-P₃] and of inositol phosphates to this site have dissimilar functional effects on the interaction of arrestin3 with receptor and clathrin. By deletion analysis and site-directed mutagenesis, we identify specific amino acid residues critical for phosphoinositide binding to arrestin3. Using biochemical assays and immunofluorescence analyses in intact cells, we demonstrate that a functional phosphoinositide-binding site is necessary for arrestin3-mediated internalization of β_2 ARs. These observations reveal a critical regulatory role for phosphoinositides in arrestin/adaptor function in cells.

Results

Non-visual arrestins bind phosphoinositides

A previous report by Palczewski *et al.* (1991) indicated that visual arrestin prepared from bovine retinal tissue binds D-myo-inositol hexakisphosphate (IP₆) with moderate affinity ($K_D \sim 10 \mu\text{M}$). Using recombinant preparations of bovine visual arrestin, we obtained similar results, with a K_D for IP₆ of $\sim 18 \mu\text{M}$ (Figure 1A and Table I). We also tested recombinant forms of two non-visual arrestins, arrestin2 and arrestin3, in similar binding assays. Interestingly, both proteins also bound IP₆ but with ~ 200 -fold greater affinities ($K_D \sim 0.1 \mu\text{M}$) than that of visual arrestin (Figure 1A and Table I).

Many proteins that exhibit binding of IP₆ have been shown to have high-affinity interactions with phosphoinositides as well, and in many cases the latter are thought to be the physiologically relevant ligand (Beck and Keen, 1991; Garcia *et al.*, 1995; Lomasney *et al.*, 1996; Schiavo *et al.*, 1996; Frech *et al.*, 1997; Hao *et al.*, 1997; Rameh *et al.*, 1997; Chaudhary *et al.*, 1998). Accordingly, we evaluated binding of the non-visual arrestins to a number of short chain, soluble polyphosphoinositides and their derivatives. Both non-visual arrestins bound phospho-

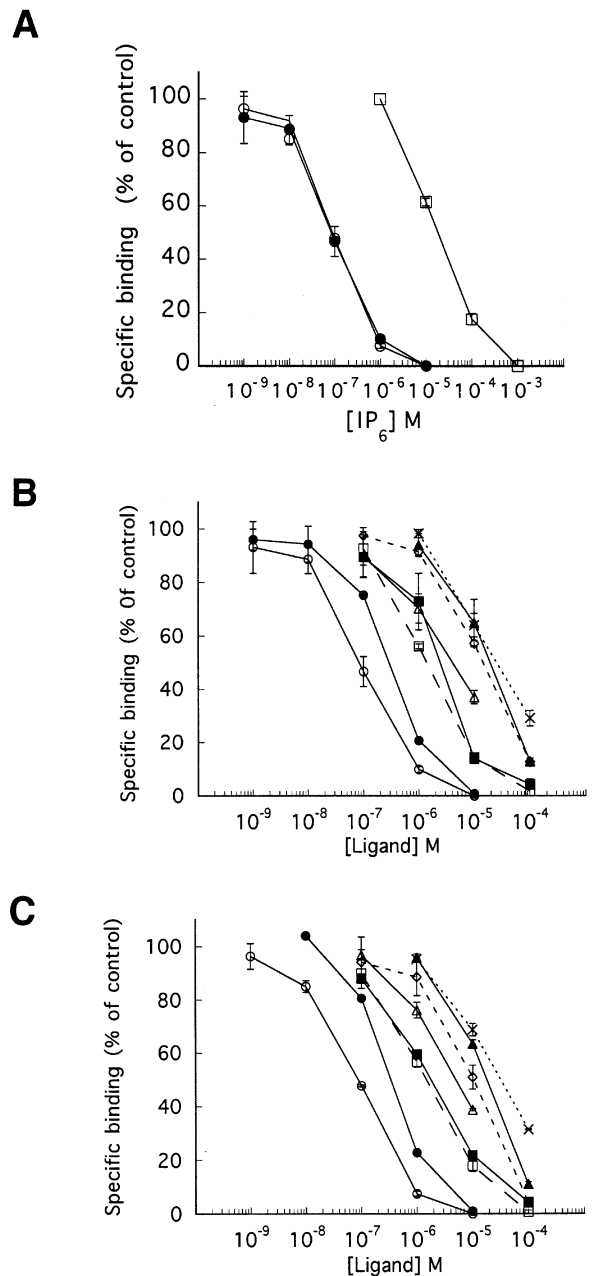


Fig. 1. Phosphoinositide and inositol phosphate binding to arrestins. (A) Specific binding of [³H]IP₆ to recombinant preparations of visual arrestin (□), arrestin2 or β -arrestin (●) and arrestin3 (○). (B and C) Binding of IP₆ (○); di-C₈-PI-3,4,5-P₃ (●); di-C₈-PI-4,5-P₂ (□, dashed lines); di-C₈-PI-3,4-P₂ (■, solid line); 1,3,4,5-IP₄ (△); 1,4,5-IP₃ (▲); di-C₈-PI-3-P (◇, short-dashed line); glycerylphosphoinositol-4,5-P₂ (-X-, dotted line) to recombinant arrestin2 (B) and arrestin3 (C), measured as inhibition of [³H]IP₆ binding. Error bars reflect mean and standard deviations from 4–6 replicates.

inositides as well as inositol phosphates, and the binding characteristics of arrestin2 and arrestin3 were both qualitatively and quantitatively similar (Figure 1B and Table I). In particular, both non-visual arrestins bound di-octyl phosphatidylinositol-3,4,5-P₃ [di-C₈-PI-(3,4,5)-P₃] with high affinity ($K_D \sim 0.3 \mu\text{M}$), roughly comparable with the binding of IP₆. Binding of di-C₈-PI-4,5-P₂ and di-C₈-PI-3,4-P₂ was somewhat weaker, while affinity for di-C₈-PI-3-P was even lower, indicating a preference for polyphosphoinositides. The comparable affinity for di-C₈-

Table I. Dissociation constants for phosphoinositide and inositol phosphate binding to arrestins

	Arrestin3 (μM)	Arrestin2 (μM)	Arrestin (μM)
IP ₆	0.085	0.085	18
PI-(3,4,5)-P ₃ ^a	0.33	0.28	ND
PI-(4,5)-P ₂ ^a	1.4	1.4	ND
PI-(3,4)-P ₂ ^a	1.8	2.3	ND
PI-3-P ^a	10	15	ND
(1,3,4,5)-IP ₄	4.8	4.0	ND
(1,4,5)-IP ₃	19	20	ND
gPI-(4,5)-P ₂	32	26	ND

^aSoluble dioctyl derivatives of these phosphoinositides were used (see Materials and methods).

PI-4,5-P₂ and di-C₈-PI-3,4-P₂ suggests no preferential recognition of the 3-phosphoinositide. However, the considerably higher affinity of the non-visual arrestins for di-C₈-PIP₃ and di-C₈-PIP₂ as compared with their polyphosphoinositol headgroups, D-myo-inositol 1,3,4,5-tetraphosphate (IP₄) and D-myo-inositol 1,4,5-triphosphate (IP₃), respectively, suggested that the proteins interact significantly with the diacylglycerol backbone of the inositides. This effect appears to be due largely or entirely to the diacyl chains, as both non-visual arrestins have comparable affinities for (1,4,5)-IP₃ and glycerylphosphatidylinositol (4,5)-P₂ (Table I).

Localization of the phosphoinositide-binding site on arrestin3

In preliminary experiments, we found that the binding of the non-visual arrestins to both clathrin and rhodopsin was affected by phosphoinositides (see below), suggesting a potential functional role for the arrestin–phosphoinositide interaction. Accordingly, we sought to localize the arrestin3 phosphoinositide-binding domain by assessing the activity of truncated forms of arrestin3 expressed as fusion proteins coupled at their N-terminus to GST (Figure 2A). A fusion protein containing the N-terminal half of arrestin3 (residues 1–210) exhibited specific binding of [³H]IP₆. However, its affinity was much lower than that of the parent protein, though comparable with that of visual arrestin (see below). In contrast, a fusion protein containing roughly the C-terminal half of arrestin3 (residues 182–409) displayed tight binding (K_D ~30–40 nM) comparable with that of the recombinant full-length protein, suggesting that this portion of the arrestin3 protein contains the high-affinity phosphoinositide-binding site.

To localize further the region responsible for phosphoinositide binding, the arrestin3 sequence was truncated progressively from the C-terminus (Figure 2A). A fusion protein containing arrestin3 residues 182–285 exhibited strong binding interactions, while that containing a more truncated segment, residues 182–253, displayed weaker but still detectable interaction. A smaller construct terminating at residue 226 was inactive in our assay. Similarly, successive truncations from the N-terminal region beyond residue 182 demonstrated that residues up to 223 need not be present to retain high-affinity binding. Collectively, these results implicate arrestin3 residues 223–285 as containing a discrete, high-affinity phosphoinositide-binding domain. Unfortunately, a GST–arrestin3 223–285

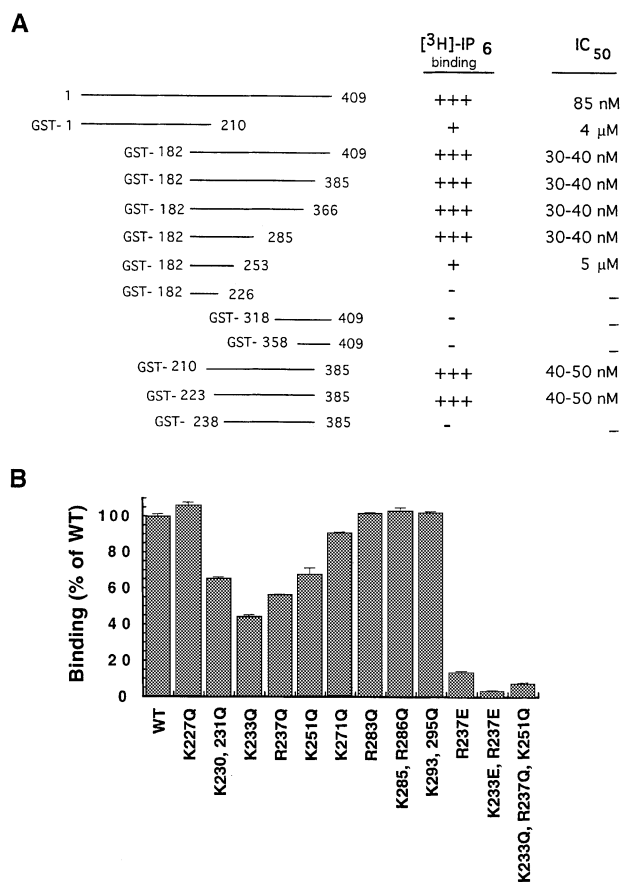


Fig. 2. Localization and site-directed mutagenesis of the phosphoinositide-binding site on arrestin3. (A) Full-length recombinant arrestin3 and truncated portions of the arrestin3 sequence coupled at their N-terminus to GST were analyzed for expression of [³H]IP₆ binding and approximate binding affinity. A high-affinity site is present within residues 223–285, while a lower affinity interaction is seen in the 1–210 fusion product. (B) Site-directed mutagenesis of the inositide-binding domain of arrestin3. A GST–arrestin3 182–385 fusion protein (WT) or mutants containing the indicated residue alterations were expressed and tested for retention of [³H]IP₆ binding. Error bars reflect mean and standard deviations from four replicates.

product was not soluble in our hands, but a fusion protein with residues 182–385 was expressed well and bound both PI-(3,4,5)-P₃ and PI-(4,5)-P₂ with high affinity (K_D ~1.3 and 2.6 μM , respectively; data not shown).

The 223–285 region of the arrestin3 sequence contains a number of basic amino acids, and these residues are likely candidates for interaction with the negatively charged phosphates of phosphoinositides. Accordingly, we prepared GST fusion proteins containing the arrestin3 182–385 sequence in which each of the 12 basic residues within the arrestin3 sequence from 223 to 295, individually or in pairs, were changed to uncharged glutamine residues and then tested for binding (Figure 2B). Little effect on binding was observed when individual basic residues in the flanking portion of this region (i.e. lysine residues at positions 227, 271, 285, 293 and 295, and arginines 283 and 286) were mutated to uncharged glutamines. Alteration of the adjacent lysine residues 230 and 231 to glutamines had a substantial effect. The greatest decrease in phosphoinositide binding on alteration of single residues was observed when lysyl residues at positions 233 and 251 and an arginine at position 237 were altered individually,

suggesting that these residues in the central portion of this region play a major role in the binding interaction. Interestingly, replacement of Arg237 with a glutamate residue, which normally is present at this position in visual arrestin, greatly inhibited binding; additional substitution of Lys233 with glutamate resulted in essentially undetectable binding. To avoid potential complications resulting from charge inversions, we also prepared a mutant arrestin3 in which Lys 233, Lys251 and Arg237 were replaced with uncharged glutamyl residues. This product, denoted arrestin3-KRK/Q, exhibited <10% of wild-type arrestin3 phosphoinositide-binding activity (Figure 2B), and was used for subsequent functional studies.

Mutant arrestin3 defective in phosphoinositide binding retains clathrin- and rhodopsin-binding activities

Native arrestin3 functions as an adaptor in the receptor-mediated endocytosis of GPCRs by virtue of its high-affinity interactions with both activated receptors and clathrin. The clathrin interaction domain has been mapped to the far C-terminal region (around residues 370–385; Krupnick *et al.*, 1997a), while the primary receptor-binding domain is in the N-terminal portion of the protein (Gurevich and Benovic, 1993). It seemed unlikely from the primary sequence that alteration of residues in the 233–251 region would directly affect either of these interactions. However, to evaluate the overall function of the arrestin3-KRK/Q mutant, we asked whether it retained the ability to bind to clathrin and receptor. Radiolabeled wild-type and arrestin3-KRK/Q were generated in an *in vitro* reticulocyte translation system and tested for binding to clathrin cages in a sedimentation assay (Goodman and Keen, 1995). Arrestin3-KRK/Q bound to clathrin indistinguishably from wild-type arrestin3 (Figure 3A).

Rhodopsin preparations provide a readily available experimental system for assessing receptor binding, and arrestin3 is known to bind to light-activated, phosphorylated rhodopsin almost as well as to β_2 AR (Gurevich *et al.*, 1995). When tested in this assay, *in vitro* translated wild-type and arrestin3-KRK/Q products bound comparably to activated, phosphorylated rhodopsin (Figure 3B). With the clathrin-binding results, these findings indicate that alteration of the three basic amino acids to glutamyl residues has only a local effect on the phosphoinositide-binding properties of the arrestin3 protein, and not a global effect on its overall structure.

PIP₃ and IP₆ have dissimilar effects on arrestin3

To explore the potential functional effects of inositide/inositol phosphate binding on arrestin3, we initially examined the effect of IP₆ on the binding of *in vitro* translated wild-type and mutant arrestin3 to clathrin. IP₆ inhibited wild-type arrestin3 binding, but only at concentrations much greater than the measured binding constant (Figure 4B). Interestingly, binding of the mutant arrestin3-KRK/Q to clathrin was affected by IP₆ but with essentially identical sensitivity to that of the wild-type protein, indicating that the high-affinity phosphoinositide binding of the arrestin3 protein is not responsible for these effects. These experiments utilized lightly cross-linked cages (see Materials and methods), but quantitatively similar results

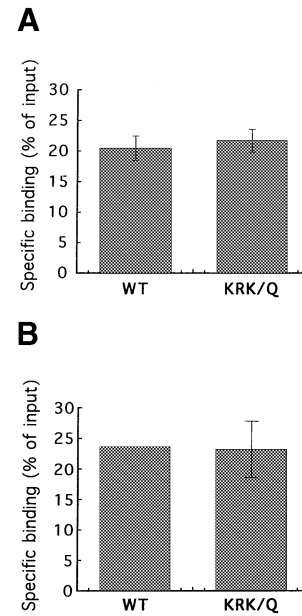


Fig. 3. Arrestin3-KRK/Q retains the ability to bind clathrin and rhodopsin. Radiolabeled wild-type and arrestin3-KRK/Q arrestin3 were expressed in a coupled *in vitro* transcription–translation system and tested for binding to clathrin cages (A) and to light-activated, phosphorylated rhodopsin (B). Error bars reflect mean and standard deviations from four replicates.

were obtained with native clathrin coat structures that contained AP-2 to stabilize them against dissociation at pH 7.3 (data not shown).

Palczewski and co-workers (1991) have reported that IP₆ inhibits visual arrestin binding to rhodopsin with a K_i of ~10 μ M, comparable with its binding constant for IP₆. We found that IP₆ also inhibited binding of both *in vitro* translated arrestin2 and arrestin3 to light-activated, phosphorylated rhodopsin (Figure 4A). However, much higher concentrations were required for this effect (K_i ~10 μ M) than for direct binding to the high-affinity site (K_D ~0.3 μ M). Again, the binding of arrestin3-KRK/Q to rhodopsin was also inhibited by 10 μ M IP₆ (Figure 4A). The similarity in the magnitude of IP₆ concentrations required for the effects with both arrestin and arrestin3, and the detection of an additional, lower affinity phosphoinositide site in the N-terminal half of the arrestin3 molecule (Figure 3) raise the possibility that a common phosphoinositide-binding site of comparatively lower affinity may exist in this region of the proteins. This site(s) has not been characterized further at this time.

As noted earlier, the physiological ligands for arrestin recruited to the plasma membrane by activated receptor would be expected to include phosphoinositides. As the most potent of the available phosphoinositides tested was di-C₈-PIP₃ (Table I), we examined its effects on recombinant arrestin3 interaction with clathrin and rhodopsin. Surprisingly, we found that di-C₈-PIP₃ at concentrations almost 30-fold greater than its binding constant had no effect on the binding of recombinant arrestin3 to clathrin cages (Figure 5A), although sensitivity to IP₆ was virtually identical to that seen using *in vitro* translated arrestin3 (Figure 4B).

Di-C₈-PIP₃ affected the interaction of arrestin3 with light-activated, phosphorylated rhodopsin very differently

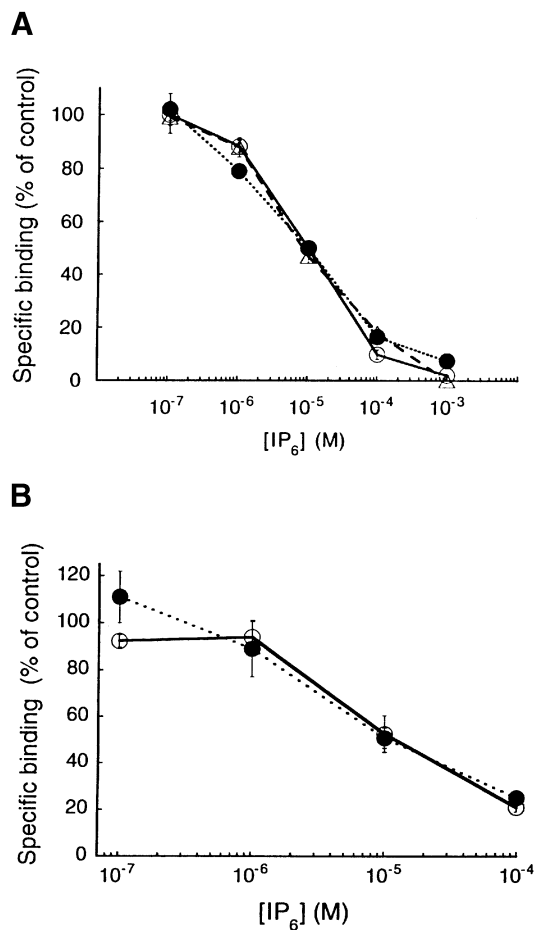


Fig. 4. The high-affinity phosphoinositide-binding site of arrestin3 is not responsible for IP₆ inhibition of arrestin3 binding to rhodopsin and clathrin. **(A)** Inhibition by IP₆ of binding to light-activated, phosphorylated rhodopsin of radiolabeled arrestin polypeptides expressed in a coupled *in vitro* transcription-translation system: arrestin3 (○, solid line), arrestin3-KRK/Q (●, dotted line), arrestin2 (△, dashed line). **(B)** Inhibition by IP₆ of binding to cross-linked clathrin cages by *in vitro* translated arrestin3 (○, solid line) or arrestin3-KRK/Q (●, dotted line). Quantitatively similar sensitivity to IP₆ of wild-type and arrestin3-KRK/Q translation product indicates that the high-affinity site is not responsible for this interaction (see text for details).

than IP₆. While high concentrations of IP₆ resulted in inhibition of rhodopsin binding (Figure 4A), di-C₈-PIP₃ actually stimulated wild-type arrestin3 binding by >2-fold (Figure 5B). Several observations suggest that this effect is mediated by occupancy of the high-affinity site. First, stimulation of receptor binding occurred at 10⁻⁷–10⁻⁶ M, in the range of the measured *K*_D for binding to arrestin3 (Table I). Di-C₈-PIP₂ elicited a similar effect at 5- to 10-fold higher concentrations, consistent with its lower affinity for arrestin 3 (Table I). The experimental system may underestimate the effects, as the exogenous phosphoinositides may be diluted in the vesicles. Finally, di-C₈-PIP₃ did not affect rhodopsin binding of arrestin3-KRK/Q, which lacks the high-affinity phosphoinositide-binding site (Figure 5B). Collectively, these results suggest that, in contrast to the low-affinity site which seems to have an inhibitory effect on arrestin3 interaction, occupancy of the high-affinity site by phosphoinositide has a permissive or even stimulatory effect on arrestin3 interactions with receptor and clathrin.

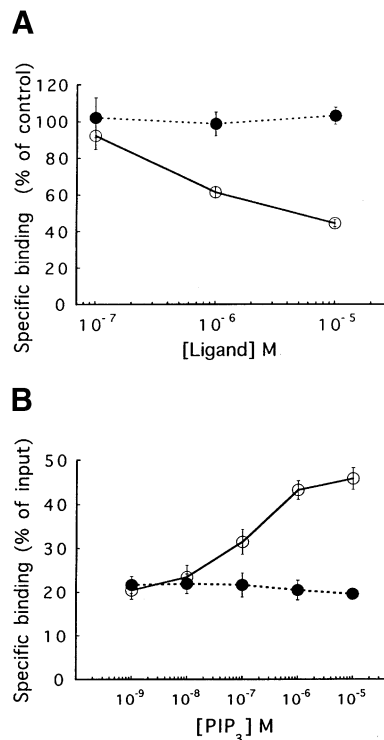


Fig. 5. PIP₃ and IP₆ have differing effects on arrestin3 binding to clathrin and rhodopsin. **(A)** Binding to cross-linked clathrin cages. Unlike IP₆ (○, solid line), di-C₈-PIP₃ (●, dotted line) does not inhibit recombinant arrestin3 binding. **(B)** Di-C₈-PIP₃ stimulates arrestin3 binding (○, solid line) to light-activated, phosphorylated rhodopsin. The mutant arrestin3-KRK/Q lacking high-affinity phosphoinositide binding does not show this effect (●, dotted line).

Mutant arrestin3-KRK/Q does not support efficient receptor internalization in intact cells

A major difference between the visual and non-visual G protein-coupled signal transduction systems is that there is substantial receptor internalization and trafficking of many non-visual GPCRs. We hypothesized that the existence of a high-affinity binding site for phosphoinositides in the non-visual arrestins, missing in arrestin, reflected a possible role for phosphoinositides in receptor trafficking. To explore this possibility, we transiently expressed β₂AR with either wild-type or arrestin3-KRK/Q in COS1 cells (Figure 6, inset) and evaluated their effects on receptor uptake. In the absence of exogenous arrestin3 expression, there is a low, basal level of internalization of β₂AR uptake in COS1 cells (Figure 6), presumably mediated by endogenous arrestin(s) or arrestin-like molecules. Expression of wild-type arrestin3 greatly enhances this uptake, with a >7-fold average increase, in the experiments shown (Figure 6). In contrast, expression of mutant arrestin3-KRK/Q was <20% as effective (Figure 6), suggesting that a fully functional phosphoinositide site is required for the efficient action of arrestin3 in the β₂AR internalization pathway. When overexpressed along with wild-type arrestin3 and β₂-receptor, arrestin3-KRK/Q also acted as a dominant-negative inhibitor, reducing receptor uptake by ~50% (data not shown).

Mutant arrestin3-KRK/Q is not recruited to clathrin-coated pits

The failure of arrestin3-KRK/Q to support β₂AR internalization indicated that inability to bind phosphoinositide with

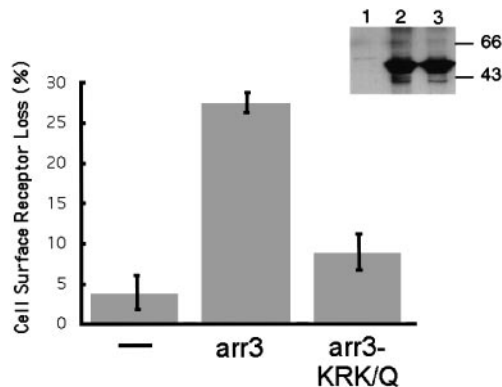


Fig. 6. Arrestin3-KRK/Q is defective in mediating internalization of β_2 -adrenergic receptor in COS1 cells. Loss of cell surface β_2 ARs in cells transfected with plasmid (12 μ g) for β_2 AR alone (—) or with receptor and accompanying plasmid (4 μ g) for either wild-type (arr3) or mutant arrestin3 (arr3-KRK/Q). Inset: arrestin3 levels were probed in COS1 cells that were untransfected (lane 1) or transfected with 4 μ g of plasmid coding for arrestin3 (lane 2) or arrestin3-KRK/Q (lane 3).

high affinity critically affected the endocytosis pathway. A readily recognizable step in this pathway is the recruitment of both β_2 ARs and arrestin to clathrin-coated pits following agonist stimulation in intact cells (Goodman *et al.*, 1996). The mutant protein was determined to be grossly defective in this step. We compared the distribution of wild-type and arrestin3-KRK/Q proteins with that of AP-2, a marker for plasma membrane clathrin-coated pits (Robinson, 1989). Under basal conditions, both transiently expressed and endogenous non-visual arrestins were present in a predominantly diffuse, cytoplasmic distribution (Figure 7A; Goodman *et al.*, 1996). On agonist treatment, wild-type arrestin3 rapidly appeared in clathrin-coated pits and co-localized with AP-2 on the bottom surface of cells (Figure 7A; Goodman *et al.*, 1996). However, in cells expressing arrestin3-KRK/Q, the mutant protein was not recruited to coated pits and remained almost entirely diffusely distributed; only occasionally could a small amount of arrestin3 signal be detected in coated pits (Figure 7A, right panels). Endocytosis in these cells, as indicated by uptake of fluorescently tagged transferrin, was indistinguishable from that in their non-expressing counterparts (data not shown).

While examination of the bottom surface is optimal for localization of signal in coated pits, recruitment of cytoplasmic arrestin to the plasma membrane is difficult to recognize on this plane. To examine this possibility more effectively, images were obtained through the middle portion of cells, considerably above the substrate. Upon agonist treatment, wild-type arrestin3 was recruited from a diffuse distribution throughout the interior of the cell to punctate dots on the cell periphery, corresponding closely to that of plasma membrane AP-2-containing coated pits (Figure 7B). Viewed in this way, on agonist treatment, mutant arrestin3-KRK/Q did undergo substantial relocalization to the plasma membrane and the peripheral region of the cell, though it is not recruited effectively to coated pits (Figure 7B). These results suggest that the failure of the mutant arrestin3 protein to support effective receptor internalization following agonist stimulation results from its inability to be recruited effectively to the coated pit.

Discussion

The results presented here demonstrate that non-visual arrestins have a high-affinity phosphoinositide-binding site and that occupancy of this site is critical to arrestin function in endocytosis of the β_2 AR. Truncation analysis identified a minimum region required for high-affinity phosphoinositide binding as existing between arrestin3 residues 223 and 285. Interestingly, fusion proteins containing these amino acids bound phosphoinositides with affinity comparable to that of the intact, parent protein, indicating that this region is probably a discrete folding domain within the arrestin3 protein. Further site-specific mutagenesis identified several basic residues that were critical for high-affinity phosphoinositide binding, including Arg237, Lys233 and Lys251 (Figure 2B). The virtually indistinguishable phosphoinositide-binding properties of arrestin2 and arrestin3 reflect the high degree of conservation of the two proteins in this region of their sequences (>80% identity) and complete retention of these basic residues. Conversely, the absence of high-affinity phosphoinositide binding in visual arrestin is consistent with considerably lower sequence similarity to arrestin3 in this region (41% identity). In particular, two of the three important basic residues identified in the arrestin3 sequence (R237 and K251) are altered in visual arrestin. Indeed, mutagenesis of just the arrestin3 Arg237 to the glutamate residue present in visual arrestin resulted in almost complete loss of high-affinity phosphoinositide binding (Figure 2B).

Granzin *et al.* (1998) recently have reported a crystal structure for bovine arrestin at 3.3 Å resolution. As bovine (visual) arrestin is 56% identical (and 75% similar) to arrestin3 overall, this structure provides a useful framework for interpretation of our binding results. Our truncation analysis suggests that the phosphoinositide site is in one of the two major domains of antiparallel β -pleated sheets, and primarily in the region corresponding to the sequence comprising β -strands S and T. This places the phosphoinositide site on the same side of the protein and near to the region hypothesized to interact with rhodopsin, an orientation toward the membrane surface and consistent with contact with bilayer phosphoinositides. Furthermore, the C-terminal end of the protein, which in the non-visual arrestins has been implicated in clathrin binding (Krupnick *et al.*, 1997a), is on the opposite side of the arrestin molecule. These properties probably explain why mutation of selective residues in the 230–250 region of the arrestin3 sequence abolishes high-affinity phosphoinositide binding, while not detectably affecting interaction with either clathrin or activated receptor. Orientation of these sites is also consistent with arrestin acting as an adaptor to bridge a transmembrane receptor to the surrounding clathrin lattice. Further structural work with the non-visual arrestins can be expected to refine our understanding of these interactions and their regulation.

Protein binding of both phosphoinositides and inositol phosphates, but with dissimilar functional consequences, has been observed previously for the pleckstrin homology (PH) domain of protein kinase B (also known as RAC or akt) (Frech *et al.*, 1997). In the case of arrestin3, IP₆ inhibited binding to clathrin and receptor. However, this effect was mediated by a locus other than the high-affinity

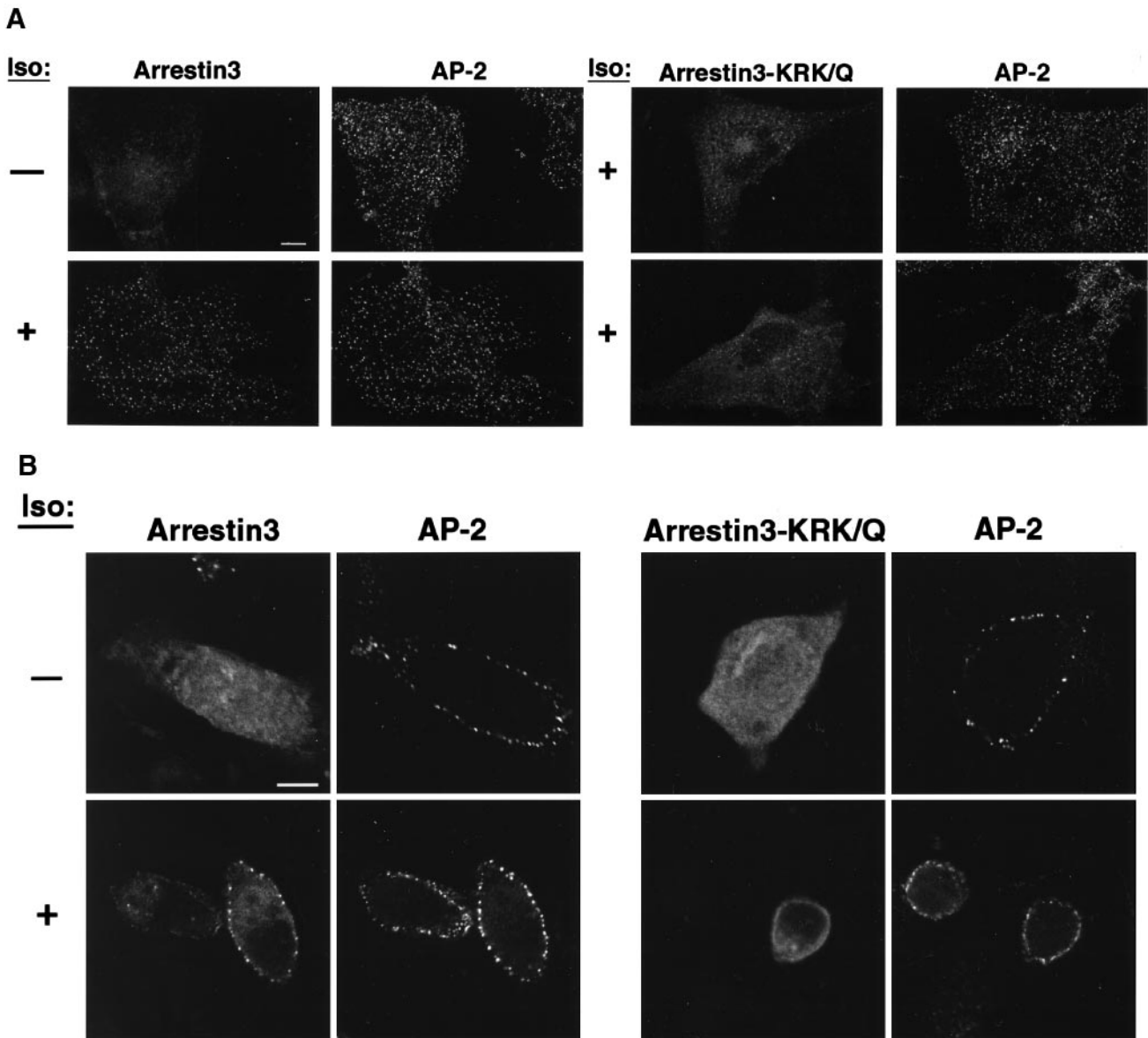


Fig. 7. Arrestin3-KRK/Q is defective in recruitment to clathrin-coated pits following agonist activation. (A) Left panels: arrestin3, initially in a predominantly diffuse distribution in the absence of agonist (- isoproterenol), redistributes efficiently to clathrin-coated pits on the lower surface of COS1 cells (marked by the presence of AP-2) upon stimulation (+ isoproterenol). Right panels: in cells expressing arrestin3-KRK/Q and stimulated with agonist, the inositol binding-defective arrestin3 localization on the bottom surface of the cell remains predominantly diffuse upon agonist stimulation, with very slight co-localization to clathrin-coated pits. (B) Left panels: confocal images through the interior of cells reveal redistribution of arrestin3 following agonist treatment to coated pits ringing the cell surface. Right panels: in contrast, on agonist treatment, arrestin3-KRK/Q is largely redistributed from a diffuse cytoplasmic localization toward the plasma membrane, but it does not co-localize with cell surface coated pits. Bar = 10 μ m.

phosphoinositide-binding site and required relatively high IP_6 concentrations. Total cell levels of IP_6 have been estimated to be in the micromolar concentration range (Sasakawa *et al.*, 1995; Shears, 1996), but chelation by cellular cations would be expected to reduce free concentrations substantially. Therefore, it is not certain, at this time whether these interactions are physiologically relevant. However, one possibility is that interaction with IP_6 and other soluble inositol phosphates is one of several mechanisms, possibly with phosphorylation (Lin *et al.*, 1997), that may contribute to impeding cytosolic arrestin interaction with clathrin or receptors in the absence of activation (Figure 8).

Interestingly, phosphoinositides stimulated arrestin3 interaction with receptor. This effect is mediated by the high-affinity phosphoinositide-binding site on arrestin3, and occurs at low concentrations. Comparison of the effects of phosphoinositides and their corresponding polyphosphate headgroups (Table I) indicated that the dioctyl side chains in the soluble phosphoinositides contributed significantly to the binding interaction. As naturally occurring phosphoinositides have much longer stearyl and arachidonyl side chains, interactions with physiological phosphoinositides may be considerably stronger. Recruitment of arrestin3 to the membrane through binding to phosphorylated, activated receptor would also

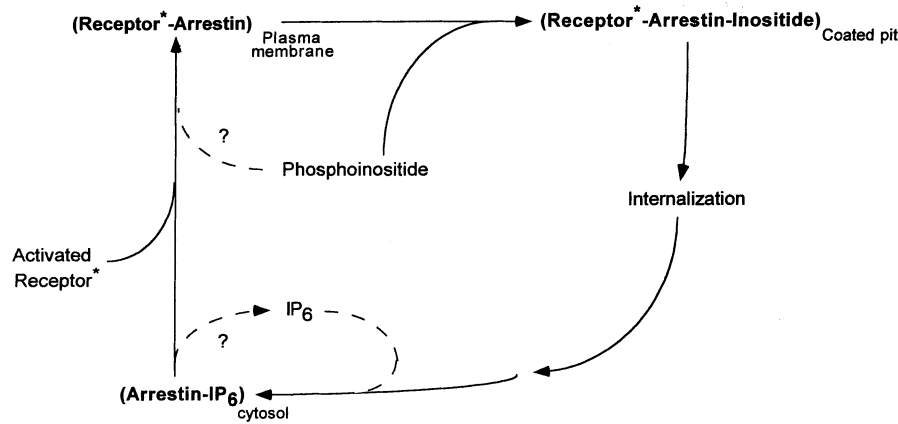


Fig. 8. A model for phosphoinositide function in receptor-mediated endocytosis of G protein-coupled receptors by arrestins (see text for details).

provide a higher local effective concentration of membrane phosphoinositides than in our experiments with soluble PI-(3,4,5)-P₃.

Though both arrestin3 and arrestin2 showed highest affinity for PI-(3,4,5)-P₃, pre-treatment of intact cells for 10 min with up to 500 nM wortmannin, an inhibitor of PI-3-kinase activity (Yano *et al.*, 1993), had no discernible effect on arrestin3 recruitment to coated pits following β_2 AR activation in COS-1 cells (data not shown). These findings are consistent with published reports of insensitivity of the initial uptake of several other receptors to wortmannin (Shepherd *et al.*, 1995; Martys *et al.*, 1996; Spiro *et al.*, 1996). In view of these observations and the much larger whole-cell concentrations of PI-(4,5)-P₂ compared with PI-(3,4,5)-P₃, the former is a likely candidate for binding to the non-visual arrestins, especially if interaction with activated receptor is sufficient to provide some driving force for membrane recruitment (see below). However, other pathways for formation of 3-phosphoinositides that involve wortmannin-insensitive enzymes have been reported (Domin *et al.*, 1997), as have novel forms of multiple phosphorylated 3-phosphoinositides such as PI-(3,5)-P₂ (Dove *et al.*, 1997), which might be ligands for the non-visual arrestins.

Based on our *in vitro* observations (Figure 5B), occupancy of the phosphoinositide site on arrestin3 would be expected to stabilize or enhance its interaction with activated GPCR. This supposition derives support from the experiments in intact cells which demonstrated that the arrestin3-KRK/Q lacking a functional phosphoinositide site, though recruited to the plasma membrane (Figure 7B), fails to support effective internalization of activated β_2 ARs. Furthermore, the requirement for inositide binding must occur early in the endocytosis pathway (Figure 8) as the mutant arrestin (and wild-type receptor; data not shown) are not recruited to coated pits. The molecular events occurring between activation of GPCRs and their concentration in coated pits are unknown, and it is conceivable that the phosphoinositide–arrestin interaction is required to promote one or more of these steps (Figure 8). One possibility is that interaction of arrestin3 with both activated receptor and a membrane phosphoinositide provide multi-point attachment to the membrane. This multivalency may be required to yield an interaction stable enough to deliver the activated, uncoupled receptor to a plasma membrane coated pit for internalization. However,

phosphoinositide also probably fulfills a key regulatory role in allowing the arrestin–receptor complex to move to a coated pit, perhaps from a restrained site elsewhere on the plasma membrane.

Multi-point attachment is consistent with binding of both receptor and phosphoinositide to separate domains, connected by a flexible hinge, on the same face of the arrestin molecule as suggested by the available crystal structure (Granzin *et al.*, 1998) (see above). A similar membrane-tethering function through multiple interactions has been suggested for phosphoinositide-binding PH domains (reviewed in Lemmon *et al.*, 1997) and for post-translational protein acylation modifications that occur on many signaling and trafficking proteins (Resh, 1996). Recently, Schlessinger and co-workers (Falasca *et al.*, 1998) have demonstrated that functional phosphoinositide binding by the phospholipase C γ PH domain is required for enzyme activation and PIP₂ hydrolysis following growth factor stimulation. Even in the absence of a functional PH domain, tyrosine phosphorylation of phospholipase C γ was detectable, suggesting its transient interaction with activated receptor. A similar, limited interaction can be inferred for arrestin3-KRK/Q and activated β_2 AR (Figures 3 and 7B).

Interestingly, all of these proposed membrane interactions involve components that can be readily and reversibly attenuated, either by cleavage of post-translational acylation products (Mumby, 1997; Wedegaertner, 1998) or by metabolism of bilayer lipid components. The presence of synaptojanin, an inositol-3-phosphate-5-phosphatase (Woscholski *et al.*, 1995; McPherson *et al.*, 1996), in clathrin-coated pits (Haffner *et al.*, 1997) provides evidence for the potential existence of such a pathway in cells. An obvious inference is that the multi-point arrestin (and more generally, adaptor)–membrane interaction needs to be closely regulated throughout its endocytic itinerary. This is a general theme in receptor trafficking and signaling, and clarification of the molecular steps involved in regulation will be important goals for future work.

Materials and methods

Materials

Clathrin was purified from bovine brain coated vesicles as described previously (Keen, 1987). Di-C₈-PIP₃ and di-C₈-PI-3,4-P₂ were synthesized as described by Kishta *et al.* (1995), and di-C₈-PI-4,5-P₂ and

di-C₈-PI-3-P were purchased from Echelon Research Laboratories (Salt Lake City, UT). IP₆, IP₄ and IP₃ were obtained from Calbiochem, [³H]-leucine and [³H]IP₆ were from DuPont NEN and [³⁵S]Translabel was from ICN. pGEX4T-2 and pGEX-2T vectors were from Pharmacia. Glutathione-agarose and Sepharose 2B were from Sigma. Restriction and modification enzymes were purchased from Boehringer Mannheim and TNT rabbit reticulocyte transcription-translation system was from Promega. All other chemicals were reagent grade or better and were purchased from Sigma or Fisher.

Inositol polyphosphate-binding assay

The binding of [³H]IP₆ to the recombinant proteins and its competition by unlabeled phosphoinositides were determined by a polyethylene glycol precipitation procedure as described previously (Gaidarov *et al.*, 1996).

Construction of GST fusion proteins encoding deletion and site-directed mutants of arrestin3

Specific fragments within the C-terminal half of arrestin3S were amplified using primers containing engineered *Sma*I restriction sites with stop codons. Fragments were digested with *Sma*I and subcloned into the vector pGEX4T-2 digested with *Sma*I. The GST fusion protein containing residues 1–210 of arrestin3S was generated by subcloning the *Nco*I–*Nco*I fragment of arrestin3 cDNA treated with Klenow fragment and dNTP into the pGEX-2T vector that had been digested with *Sma*I.

Site-directed mutants were made in the GST fusion construct containing residues 182–385 of arrestin3S. Mutagenesis was performed using PCR. All constructs were verified by sequencing (Sidney Kimmel Nucleic Acid Facility, Thomas Jefferson University).

Expression and purification of recombinant proteins in *Escherichia coli*

Recombinant arrestins were expressed and purified as described previously (Goodman *et al.*, 1996). GST fusion proteins were expressed and purified following the manufacturer's recommendations with some modifications. Briefly, 5 ml of an overnight culture of *Escherichia coli* BL-21 cells transformed with the recombinant plasmids were diluted into 500 ml of Luria-Bertani medium supplemented with 100 µg/ml ampicillin. Cells were grown for 3 h at 37°C (to OD₆₀₀ ~0.7) and expression induced with 100 µM isopropyl-β-D-galactopyranoside (IPTG). After 2 h, the bacteria were pelleted (6000 g, 20 min) and resuspended in 8 ml of ice cold phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and 2 mg/ml lysozyme. After incubation for 30 min on ice, the suspension was supplemented with 0.1% Triton X-100. Lysis was performed by two rapid freeze (in liquid N₂)–thaw cycles. The lysate was supplemented with 500 U of DNase I and incubated for 20 min on ice. After centrifugation at 226 000 g_{av} for 20 min, the supernatants were incubated with 0.5 ml of glutathione-agarose beads overnight at 4°C. The beads were washed three times with PBS + 0.1% Triton X-100, three times with PBS, and bound proteins were eluted with 1 ml of 50 mM Tris-HCl pH 8.0 containing 10 mM glutathione. Protein concentrations were determined by Bradford assay or by densitometric analysis of Coomassie-stained SDS-PAGE gels.

In vitro transcription-translation of wild-type and mutant arrestin3

A construct for *in vitro* transcription-translation of mutant arrestin3 was made by substituting an *Acc*I–*Xho*I fragment of arrestin3S cDNA in pGEM-2 with the corresponding fragment bearing the mutation.

Wild-type and mutant arrestin3 were expressed using a TNT rabbit reticulocyte lysate transcription-translation system performed according to the manufacturer's recommendations in the presence of [³⁵S]Translabel or [³H]-leucine. After incubation for 2 h at 30°C, the translation reactions were centrifuged at 100 000 r.p.m. in a TLA.100 rotor (Beckman) for 20 min at 4°C.

Cage-binding assays

To assess the ability of wild-type and mutant arrestin3 to bind to clathrin cages, assays were performed essentially as described previously (Goodman and Keen, 1995). Briefly, *in vitro* synthesized radiolabeled wild-type and mutant arrestins (~0.5 nM) were incubated for 30 min at room temperature with or without clathrin cages (200 nM trimers) in a final volume of 50 µl of 100 mM sodium MES, pH 6.8, containing 10 µg/ml each of leupeptin, chymostatin, antipain and pepstatin A. Samples were loaded on a 75 µl sucrose cushion (0.2 M sucrose in 0.1 M Na-MES, pH 6.8) and centrifuged for 5 min at 75 000 r.p.m. in

a TLA100 rotor (Beckman). Pellets were analyzed by SDS-PAGE and autoradiography.

To evaluate the effects of phosphoinositides on arrestin3 binding to clathrin, experiments were performed at physiological pH (7.3). Native cages readily dissociate above pH 6.8 (Nandi *et al.*, 1982), necessitating the use of cages stabilized by light chemical cross-linking: we have shown previously that non-visual arrestins interact well with these structures (Goodman *et al.*, 1996). Cross-linking of clathrin cages (~0.5 mg/ml) was performed exactly as described previously (Goodman *et al.*, 1996) with 0.5 mM 3,3'-dithiobis(sulfosuccinimidyl)propionate (Pierce Chem. Co.). The binding of *in vitro* translated or purified arrestins to cross-linked cages was performed in a final volume of 50 µl in the presence of the indicated concentration of IP₆ or di-C₈-PIP₃ in 20 mM Na-HEPES, pH 7.3, 120 mM potassium acetate. After incubation for 20 min at room temperature, the reaction mixture was loaded on a sucrose gradient and processed as described above. Subsequent analysis was performed by Coomassie Blue staining or autoradiography. Quantitation was done on PhosphorImager or Personal Densitometer (Molecular Dynamics).

Rhodopsin-binding assay

Bovine rod outer segment membranes containing >95% rhodopsin were prepared as described previously (Gurevich and Benovic, 1992). Phosphorylation of rhodopsin with rhodopsin kinase was done according to Krupnick *et al.* (1997a). The stoichiometry of phosphorylation was calculated to be ~1 mol phosphate/mol rhodopsin.

The rhodopsin-binding assay was performed essentially as described (Krupnick *et al.*, 1997a). Briefly, *in vitro* synthesized radiolabeled arrestins (~0.5 nM) were incubated with rod outer segment membranes (~100–200 nM phosphorylated rhodopsin) for 5 min at 37°C in a final volume of 50 µl in 50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 100 mM potassium acetate, 1.5 mM dithiothreitol and in the presence of the specified concentration of IP₆ or di-C₈-PIP₃. Receptor-arrestin complexes were separated from unbound arrestins by gel filtration on a Sepharose 2B column. Specific binding was calculated by subtracting non-specific binding obtained in the absence of rod outer segment membranes. No significant binding was observed to non-activated, unphosphorylated membranes, as observed previously (Gurevich *et al.*, 1995).

Transfection and internalization experiments in COS1 cells

The construct for bovine arrestin3S expression in the vector pBC12BI has been described previously (Goodman *et al.*, 1996). The construct for expression of mutant arrestin3-KRK/Q was made by substituting the *Acc*I–*Xho*I fragment of arrestin3 cDNA in the plasmid pBC12BI-arr3S with the corresponding fragment containing the mutations. The expression construct for FLAG-tagged human β₂AR in the vector pBC12BI was generously provided by Dr B.Kobilka (Stanford University, CA). COS1 cells were grown to 80–90% confluence in T75 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The cells were transfected with Lipofectamine (Gibco-BRL) following the manufacturer's recommendations. Briefly, DNAs were incubated with 65 µl of Lipofectamine in 5 ml of DMEM for 30 min at room temperature. Five millilitres of DMEM was then added and the mixture was applied to the DMEM-washed cells. After 5 h of incubation at 37°C, the mixture was replaced with complete medium. Cells were trypsinized 48 h after transfection, washed twice with ice-cold PBS and resuspended in 1.5 ml of ice-cold PBS containing 0.1 mM ascorbic acid. Aliquots of cells (0.5 ml) were incubated with or without 10 µM (-)isoproterenol at 37°C for 20 min, washed twice with 50 ml of ice-cold PBS and resuspended in 1.5 ml of ice-cold PBS. Cell surface receptors were measured directly by incubation of 0.5 ml aliquots with 10 nM [³H]CGP-12177 (Amersham) for 3 h at 14°C. Non-specific binding was measured in the presence of 10 µM (-)alprenolol. Bound and unbound ligands were separated on a Brandel cell harvester.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described previously (Goodman *et al.*, 1996). Briefly, cells were trypsinized 7–8 h after transfection and plated on 12-mm glass coverslips in a 24-well tissue culture plate (~75 000 cells/well). At 24 h after transfection, cells were incubated with or without 10 µM (-)isoproterenol for 10 min at 37°C, washed with PBS, fixed for 10 min with 3.7% formaldehyde in PBS and processed for immunofluorescence using monoclonal AP.6 anti-AP-2α (generously provided by Dr F.Brodsky, University of California at San Francisco) and affinity-purified rabbit anti-arrestin3

(182-6). The latter was prepared using as antigen a GST fusion protein containing the C-terminal 60 amino acids of bovine arrestin-3. The resulting antiserum was incubated with Sepharose 4B resin containing covalently coupled GST and GST- β -arrestin-(357-418) to remove GST and arrestin2 cross-reacting antibodies. Arrestin-3-specific antibodies were then purified on a GST-arrestin-3-(350-409) Sepharose 4B column, eluted with 0.1 M glycine, pH 2.5, and neutralized.

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