Regulation of Muscarinic Acetylcholine Receptor Sequestration and Function by β-Arrestin*

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Oliver Vögler, Bettina Nolte, Matthias Voss, Martina Schmidt, Karl H. Jakobs, and Chris J. van Koppen‡
From the Institut für Pharmakologie, Universitätsklinikum Essen, D-45122 Essen, Germany

After activation, agonist-occupied G protein-coupled receptors are phosphorylated by G protein-coupled receptor kinases and bind cytosolic β-arrestins, which uncouple the receptors from their cognate G proteins. Recent studies on the β2-adrenergic receptor have demonstrated that β-arrestin also targets the receptors to clathrin-coated pits for subsequent internalization and activation of mitogen-activated protein kinases. We and others have previously shown that muscarinic acetylcholine receptors (mACHRs) of the m1, m3, and m4 subtype require functional dynamin to sequester into HEK-293 tsA201 cells, whereas m2 mACHRs sequester in a dynamin-independent manner. To investigate the role of β-arrestin in mACHR sequestration, we determined the effect of overexpressing β-arrestin-1 and the dominant-negative inhibitor of β-arrestin-mediated receptor sequestration, β-arrestin-1 V53D, on mACHR sequestration and function. Sequestration of m1, m3, and m4 mACHRs was suppressed by 60–75% in cells overexpressing β-arrestin-1 V53D, whereas m2 mACHR sequestration was affected by less than 10%. In addition, overexpression of β-arrestin-1 V53D as well as dynamin K44A significantly suppressed m1 mACHR-mediated activation of mitogen-activated protein kinases. Finally, we investigated whether mACHRs sequester into clathrin-coated vesicles by overexpressing Hub, a dominant-negative clathrin mutant. Although sequestration of m1, m3, and m4 mACHRs was inhibited by 50–70%, m2 mACHR sequestration was suppressed by less than 10%. We conclude that m1, m3, and m4 mACHRs expressed in HEK-293 tsA201 cells sequester into clathrin-coated vesicles in a β-arrestin- and dynamin-dependent manner, whereas sequestration of m2 mACHRs in these cells is largely independent of these proteins.

Exposure of many G protein-coupled receptors (GPCRs) to their agonists results within seconds to minutes in attenuation of receptor responsiveness. An important step in this process of receptor desensitization is the rapid phosphorylation of agonist-bound receptors by G protein-coupled receptor kinases (1). These kinases phosphorylate serine and threonine residues located in the third cytoplasmic loop (i.e. m1 and m2 muscarinic acetylcholine receptors (mACHRs)) (2, 3) or in the cytoplasmic carboxyl-terminal tail of the receptors (for example, β2-adrenergic receptors) (4). After phosphorylation, cytosolic β-arrestins bind with increased affinity to the receptors and sterically inhibit further coupling of the receptors with G proteins (1). To date, two β-arrestin isoforms, β-arrestin-1 (arrestin 2) and β-arrestin-2 (arrestin 3) have been identified, each undergoing alternative splicing (1). Both isoforms are ubiquitously expressed, with β-arrestin-1 being the major β-arrestin expressed in many tissues (1). Recent evidence indicates that β-arrestins do not only bind to GPCRs but also associate with nanomolar affinity with clathrin heavy chains and target β-arrestin-bound GPCRs to the clathrin-coated pits, leading to receptor internalization (5, 6). This process has been particularly well studied for the β2-adrenergic receptors. Overexpression of β-arrestin-1 or β-arrestin-2 in the presence of sufficient G protein-coupled receptor kinases augments internalization of β2-adrenergic receptors, whereas overexpression of the dominant-negative β-arrestin-1 V53D mutant, which binds with high affinity to clathrin cages but is significantly impaired in its ability to interact with β2-adrenergic receptors, suppresses β2-adrenergic receptor internalization (5, 7–9).

The budding of clathrin-coated vesicles from the plasma membrane into the cytosol is catalyzed by the monomeric G protein dynamin. This protein oligomerizes at the neck of the invaginated clathrin-coated pits and pinches off the pits from the plasma membrane (10). Overexpression of the dominant-negative dynamin mutant K44A, which is not able to bind guanine nucleotides, effectively blocks β2-adrenergic receptor internalization, indicating that β2-adrenergic receptors sequester into clathrin-coated vesicles in an arrestin- and dynamin-dependent manner (8). The primary function of internalization of the β2-adrenergic receptors is to allow resensitization of desensitized receptors in endosomes before their return to the plasma membrane (11, 12). Interestingly, receptor internalization via clathrin-coated vesicles has recently been reported to be essential for β2-adrenergic receptor-induced activation of the mitogen-activated protein (MAP) kinase pathway (13).

The mACHRs have been subject of a large number of studies on the regulation of GPCRs by G protein-coupled receptor kinases and β-arrestins as well (1). In contrast to β2-adrenergic receptors, which couple predominantly to G proteins of the Gs family, mACHRs efficiently activate G proteins of the Gi and Gq family. The family of mACHRs consists of five mammalian subtypes, with m1, m3, and m5 mACHRs predominantly activating phospholipase C via Gi proteins and m2 and m4 mACHRs efficiently inhibiting adenyl cyclase by activation of Gq proteins. We and others have recently shown that the monomeric GTPase dynamin is essential for internalization of m1, m3, and m4 mACHRs in HEK-293 cells, whereas internalization of the m2 mACHRs is dynamin-independent (14, 15). These results indicate that m1, m3, and m4 mACHRs sequester by a dynamin-dependent trafficking pathway, probably similar as

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† To whom correspondence should be addressed. Tel.: 49-201-723 3462; Fax: 49-201-723 5968; E-mail: van.koppen@uni-essen.de.
‡ The abbreviations used are: GPCR, G protein-coupled receptor; mACHR, muscarinic acetylcholine receptor; MAP, mitogen-activated protein; NMS, N-methylscopolamine.

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used by β2-adrenergic receptors. Previous studies have shown that β-arrestins can interact with peptide sequences derived from the third intracellular loop of the m2 and m3 mAChRs in vitro (16, 17). By analogy on the regulation of internalization of β2-adrenergic receptors, we hypothesized that β-arrestins are essential for the internalization of mAChR subtypes as well. In this study, we overexpressed the dominant-negative β-arrestin-1 mutant V53D in HEK-293 tsA201 cells and examined the effect on m1, m2, m3, and m4 mAChR sequestration. In addition, we investigated, using β-arrestin-1 V53D and dynamin K44A as inhibitors of clathrin-mediated endocytosis, whether receptor sequestration is required for m1 mAChR-mediated MAP kinase activation.

EXPERIMENTAL PROCEDURES

Materials—N-(2,3)-[3H]Methylscopolamine ([3H]NMS, specific activity 84 Ci/mmol) was purchased from NEN Life Science Products. DNA encoding mouse m1 mAChR (18), porcine m2 mAChR (19), human m3 mAChR (20), and mouse m4 mAChR (21) were subcloned into pCD-PS expression vector. The cDNAs encoding bovine β-arrestin-1 wild type in pBLC (22) and rat β-arrestin-1 V53D in pcDNA-1 Amp (5) were generously provided by Drs. M. J. Lohse and R. J. Lefkowitz, respectively. (Bordetella pertussis) fluid before being generated by polymerase chain reaction amplification using the sense and antisense primers as described by Krupnick et al. (23). The PCR product was purified, digested with HindIII and BamHI, purified, and then ligated into HindIII-BamHI-cut pcDNA3 (Invitrogen). The authenticity of the mutant was confirmed by DNA sequencing of both strands (Sequence Laboratories Goettingen, Germany). The cDNA encoding the T7 epitope-tagged Hub fragment in pCDMS (24) was provided by Dr. P. M. Brodsky. Mouse anti-α-munan monoclonal antibody F4C1 was a gift of Dr. L. A. Donoso (Wills Eye Hospital, Philadelphia, PA). Rabbit anti-ERK1 antibody (C-16), mouse anti-T7 Tag antibody, and rabbit anti-phosphospecific p44/p42 MAP kinase antibody were purchased from Santa Cruz Biotechnology, Novagen, and New England Biolabs, respectively. The goat peroxidase-conjugated anti-mouse and goat peroxidase-conjugated anti-rabbit antibodies were obtained from DiNovo (Hamburg) and Sigma, respectively.

Cell Culture and Transfection—HEK-293 tsA201 cells (25) were grown in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal calf serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO2. Cells were maintained at 37 °C and 5% CO2. Actinomycin D (1 μg/ml) was added to the culture medium 8 h before the harvesting of the cells. Cells on 150-mm plates were transfected with either 12.5 μg (m1, m2) or 25 μg (m3, m4) of pCD-PS containing mAChR DNA, together with 15 μg of pBLC/β-arrestin-1, pcDNA-1 Amp/β-arrestin-1 V53D, pcDNA3/β-arrestin-1 (319–418), or control vector (pRK5) using the calcium phosphate method. In some experiments, cells were transfected with 150 μg instead of 25 μg of pCD-PS/m1 mAChR to increase m2 mAChR expression from ~90% to ~90% of total protein.

Immunoblot Analysis of β-Arrestin and Hub Expression—Cells on 150-mm plates were washed twice with phosphate-buffered saline (150 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 6.5 mM Na2HPO4, pH 7.4) and lysed by the addition of 1.0 ml of lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4). Lysate was transferred to a microcentrifuge tube and boiled for 5 min. After 5 passages through a 25-gauge needle, samples were centrifuged for 5 min to remove insoluble material and diluted with lysis buffer to an equal amount of protein as measured by the BCA method (Pierce). One hundred μl of electrophoresis sample buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% 2-mercaptoethanol) were added to 100 μl of the diluted samples and boiled for another 5 min. After SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels, protein was blotted onto nitrocellulose. Nitrocellulose was then blocked with 10 μl Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and 5% bovine serum albumin (Fraction V, Sigma). After washing three times for 5 min in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, the blot was incubated with mouse anti-α-arrestin monoclonal antibody (diluted 1: 2000) or mouse anti-T7 Tag monoclonal antibody (0.1 μg/ml) in blocking buffer for 1 h. After three washes with 5 min, the blot was incubated with peroxidase-conjugated goat anti-mouse antibody (0.16 μg/ml) at room temperature. After 1 h, the blot was washed again, and immunoreactivity was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

MAP Kinase Assay—Forty-eight h after transfection, HEK-293 tsA201 cells on 100-mm plates were serum-starved overnight in Dulbecco’s modified Eagle’s medium/F12 medium before stimulation with 10 μM carbachol or 1 μM phorbol 12-myristate 13-acetate. After stimulation for 5 min at 37 °C, cells were lysed in 0.5 ml of lysis buffer and processed as described above. After SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels, phosphorylated MAP kinases on nitrocellulose filters were detected using a rabbit anti-phosphospecific MAP kinase antibody (diluted 1: 1000) and goat peroxidase-conjugated anti-rabbit antibody (diluted 1:5000). Expression of the total amount of MAP kinases was detected by incubation of nitrocellulose blots with rabbit anti-ERK1 antibody (0.1 μg/ml), which recognizes p44 and p42 MAP kinases, and goat peroxidase-conjugated anti-rabbit antibody (diluted 1:5000). Immunoreactivity was visualized by enhanced chemiluminescence.

mAChR Sequestration Assay—As described before (14), 24 h after transfection, cells from 150-mm plates were replated on poly-L-lysine-coated 24-well plates and allowed to reattach and grow for another 24 h. The cells were then incubated with and without carbachol for 0–60 min in 25 mM HEPS-buffered Dulbecco’s modified Eagle’s medium/F-12 medium. For each manipulation, 6 wells of cells were taken. After washing with ice-cold phosphate-buffered saline, cells were incubated with 2 μl [3H]NMS in 500 μl of ice-cold phosphate-buffered saline with and without 30 μM atropine to measure total and nonspecific binding, respectively. After 4 h of incubation at 4 °C, cells were washed with ice-cold phosphate-buffered saline, solubilized in 1% Triton X-100, sonicated, and transferred into scintillation vials, which received 3.5 ml of scintillation fluid before radioactivity counting. Sequestration is expressed as (1 – quotient of cell surface receptors of carbachol-treated and untreated cells) × 100%. Untransfected HEK-293 tsA201 do not express detectable levels of mAChR.

RESULTS

Effect of β-Arrestin-1 on m1 mAChR Sequestration—Western blot analysis demonstrated equal overexpression of β-arrestin-1 and β-arrestin-1 V53D in HEK-293 tsA201 cells transiently transfected with the expression vector encoding either β-arrestin (Fig. 1A). In the absence of overexpressed β-arrestins, agonist stimulation led to significant internalization of m1 mAChRs (Fig. 1B). A 10-min incubation with 1 mM carbachol reduced cell surface receptor number by 21 ± 4%, with 35 ± 2 and 38 ± 3% of receptors internalized after 30 min and 60 min of incubation, respectively. Overexpression of β-arrestin-1 modestly increased the extent of m1 mAChR sequestration. After 10 min of incubation with 1 mM carbachol, m1 mAChRs were sequestered by 27 ± 4%, and after 60 min of incubation, by 45 ± 3%. An increase in m1 mAChR sequestration of similar magnitude was observed at a lower carbachol concentration of 10 μM carbachol (Fig. 1C). Overexpression of β-arrestin-1, however, did not appear to decrease the EC50 value of carbachol for inducing m1 mAChR internalization. In contrast to wild-type β-arrestin-1, β-arrestin-1 V53D significantly suppressed m1 mAChR sequestration. Receptor sequestration in β-arrestin-1 V53D-overexpressing cells was only 19 ± 3% after 60 min of incubation with 1 mM carbachol. Furthermore, inhibition of m1 mAChR sequestration was evident at lower concentrations of carbachol as well. Incubation of control cells with 10 μM carbachol for 60 min led to a 23 ± 3% loss of cell surface receptor number, whereas receptor sequestration in β-arrestin-1 V53D-overexpressing cells was only 6 ± 2%.

Role of Receptor Internalization in m1 mAChR-induced MAP Kinase Stimulation—Daaka et al. (15) recently demonstrated that overexpression of β-arrestin-1 V53D or dynamin K44A in HEK-293 cells inhibits activation of MAP kinases by β2-adrenergic receptors and lysophosphatidic acid receptors, suggesting that receptor internalization into clathrin-coated vesicles is required for receptor-mediated activation of MAP kinase. As m1 mAChRs appear to internalize by the same β-arrestin- and dynamin-dependent internalization pathway as utilized by β2-adrenergic receptors, we examined the effect of overexpressing β-arrestin-1 V53D and dynamin K44A on m1 mAChR-mediated activation of MAP kinase. As shown in Fig. 2, pCD-PS/m1 mAChR-transfected cells showed a significant increase in phos-
were transfected with empty pRK5 only, did not show phosphorylation of MAP kinase in response to 1 μM carbachol (mean ± S.E. of six sets of independent experiments are shown. Expression of m1 mAChRs in untreated cells transfected with pRK5, pcDNA-1 Amp/β-arrestin-1 V53D, or empty pRK5 were incubated in the absence and presence of 1 mM carbachol for the indicated periods of time (B) or with the indicated concentrations of carbachol for 60 min (C) at 37 °C. Sequestration was assessed by [3H]NMS binding to intact cells at 4 °C. Data are the mean ± S.E. of six sets of experiments each. Specific [3H]NMS binding to intact cells transfected with pRK5, pBC/β-arrestin-1, and pcDNA-1 Amp/β-arrestin-1 V53D was 290 ± 35, 229 ± 44, and 226 ± 28 fmol/mg of protein, respectively.

**Fig. 1.** Overexpression of β-arrestin-1 wild-type and V53D mutant in HEK-293 tsA201 cells. Effects on m1 mAChR sequestration. A, detection of β-arrestins in total lysates of HEK-293 tsA201 cells grown on 150-mm plates and transiently transfected with pRK5 (pRK5), pBC/β-arrestin-1 (WT), or pcDNA-1 Amp/β-arrestin-1 V53D (V53D) by immunoblotting. Equal amounts of cell lysates (15 μg of protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis, and β-arrestins were detected using the anti-arrestin monoclonal antibody F4C1. B, and C, HEK-293 tsA201 cells transiently transfected with pCD-PS/m1 mAChR together with pBC/β-arrestin-1 (WT), pcDNA-1 Amp/β-arrestin-1 V53D (V53D), or empty pRK5 (pRK5) were serum-starved overnight before a 5-min incubation with 10 μM carbachol (CARB) or 1 μM phorbol 12-myristate 13-acetate (PMA) at 37 °C in 25 mM HEPES-buffered Dulbecco’s modified Eagle’s medium/F12 medium. Cells were lysed, and cellular protein (75 μg of protein) was subjected to SDSPolyacrylamide gel electrophoresis and blotted onto nitrocellulose. Phosphorylation of MAP kinases was determined by immunoblotting with a phosphospecific MAP kinase antibody. Representative Western blots of 3 (K44A) or 6 (V53D) independent experiments are shown. Expression of m1 mAChRs in untreated cells transfected with pRK5, pcDNA-1 Amp/β-arrestin-1 V53D, and pRK5/dynamin K44A were 275 ± 44, 62 fmol/mg of protein, respectively. Treatment of HEK-293 tsA201 cells, which were transfected with empty pRK5 only, did not show phosphorylation of MAP kinase in response to 1 mM carbachol (n = 3 independent experiments). NS, nonstimulated.

**Fig. 2.** Effects of β-arrestin-1 V53D and dynamin K44A on m1 mAChR-mediated activation of MAP kinases. HEK-293 tsA201 cells transiently transfected with pCD-PS/m1 mAChR together with either empty pRK5 (left panel), pcDNA-1 Amp/β-arrestin-1 V53D (middle panel), or pRK5/dynamin K44A (right panel) were serum-starved overnight before a 5-min incubation with 10 μM carbachol (CARB) or 1 μM phorbol 12-myristate 13-acetate (PMA) at 37 °C in 25 mM HEPES-buffered Dulbecco’s modified Eagle’s medium/F12 medium. Cells were lysed, and cellular protein (75 μg of protein/lane) was subjected to SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Phosphorylation of MAP kinases was determined by immunoblotting with a phosphospecific MAP kinase antibody. Representative Western blots of 3 (K44A) or 6 (V53D) sets of independent experiments are shown. Expression of m1 mAChRs in untreated cells transfected with pRK5, pcDNA-1 Amp/β-arrestin-1 V53D, and pRK5/dynamin K44A were 275 ± 50, 244 ± 52, and 559 ± 62 fmol/mg of protein, respectively. Treatment of HEK-293 tsA201 cells, which were transfected with empty pRK5 only, did not show phosphorylation of MAP kinase in response to 1 mM carbachol (n = 3 independent experiments). NS, nonstimulated.

Regulation of mAChR Sequestration by β-Arrestin

Effects on m1 mAChR sequestration—As shown in Fig. 3, overexpression of β-arrestin-1 V53D only marginally inhibited m2 mAChR sequestration, whereas m2 mAChR sequestration was only modestly stimulated by overexpression of wild-type β-arrestin-1. Comparison of the extent of receptor sequestration after 60 min of incubation with 10 μM and 0.1 μM carbachol showed that overexpression of either β-arrestin-1 inhibited sequestration of m1 mAChR was without any significant effect on m2 mAChR sequestration at lower carbachol concentrations either (results not shown). However, under conditions of higher m2 mAChR expression (i.e. 737 ± 79 versus 112 ± 22 fmol/mg of protein), overexpression of wild-type β-arrestin-1 significantly augmented m2 mAChR internalization. After 60 min of incubation with 10 μM and 1 mM carbachol, m2 mAChR internalization in control cells was 24 ± 9 and 22 ± 8%, whereas in β-arrestin-1-transfected cells, m2 mAChR internalized by 43 ± 9 and 51 ± 3%, respectively (mean ± S.E., n = 6–9 independent experiments).

In contrast to the m2 mAChRs, sequestration of m3 and m4 mAChRs, which like m1 mAChRs sequester in a dynamin-dependent manner (14, 15), was β-arrestin-dependent. As depicted in Fig. 4, overexpression of β-arrestin-1 V53D inhibited m3 and m4 mAChR sequestration by 68 and 64%, respectively, after 60 min of incubation with 1 mM carbachol. Overexpression of wild-type β-arrestin-1 had no (significant) stimulatory effect on the maximal extent of sequestration of either receptor subtype. In addition to investigating the effect of overexpressing β-arrestin-1 V53D, we also examined the influence of another dominant-negative inhibitor of β-arrestin-mediated receptor sequestration, β-arrestin-1 (319–418). Overexpression of β-arrestin-1 (319–418) reduced sequestration of m1, m3, and m4 mAChRs from 43 ± 4, 40 ± 2, and 47 ± 3% to 6 ± 4, 13 ± 2, and 12 ± 2%, respectively, following 60 min of incubation with 1 mM carbachol (mean ± S.E., n = 6–8 independent experiments).

Role of Clathrin in mAChR Sequestration—Two recent stud-
ies have indicated that dynamin not only catalyzes the budding of clathrin-coated vesicles but of caveolae as well (26, 27). To directly test whether m1, m3, and m4 mAChRs sequester into clathrin-coated vesicles, we took advantage of the recent availability of a dominant-negative form of clathrin, termed Hub (24). Hub comprises the carboxyl-terminal third of the clathrin heavy chain (residues 1073–1675) and specifically blocks clathrin-mediated endocytosis by depletion of clathrin light chains, causing clathrin-coated pits to be frozen at the plasma membrane. Transfection of HEK-293 tsA201 cells with pCD-PS containing T7 epitope-tagged Hub led to a large expression of Hub (Fig. 5A). Expression of Hub caused a 50−70% inhibition of m1, m3, and m4 mAChR sequestration. In contrast, sequestration of m2 mAChRs was not affected (Fig. 5B).

**DISCUSSION**

In the present study, we determined the role of β-arrestin in mAChR sequestration and function using β-arrestin-1 wild type and the dominant-negative inhibitor of β-arrestin-mediated receptor sequestration, β-arrestin-1 V53D. This β-arrestin-1 mutant was chosen for its increased ability to interact with clathrin and its impaired capacity to bind to agonist-bound phosphorylated GPCRs (9, 23).

Overexpression of β-arrestin-1 V53D suppressed sequestration of m1, m3, and m4 mAChRs in HEK-293 tsA201 cells by 60−75%, indicating that these mAChR subtypes sequester predominantly in a β-arrestin-dependent manner. On the other hand, overexpression of β-arrestin-1 wild type only slightly stimulated m1, m3, and m4 mAChR internalization. The small magnitude of stimulation may be related to the possibility that other downstream partners involved in receptor internalization are rate-limiting, so additional β-arrestin is hardly able to promote receptor internalization further. In line with our observation that m1, m3, and m4 mAChRs sequester in a β-arrestin-dependent manner, overexpression of Hub, a dominant-negative clathrin mutant (24), significantly blocked sequestration of m1, m3, and m4 mAChRs. These data lend strong support to the hypothesis that m1, m3, and m4 mAChRs utilize the same sequestration pathway as β2-adrenergic receptors in HEK-293 cells. Our findings are consistent with immunocytochemical and biochemical studies on the internalization of m1, m3, and m4 mAChRs in a number of cells including HEK-293 cells. In these studies, internalized m1 mAChRs were found to colocalize with clathrin (28), or perturbation of clathrin distribution inhibited m3 and m4 mAChR internalization (29, 30). In contrast, m2 mAChR sequestration was hardly affected by overexpression of wild-type β-arrestin-1, β-arrestin-1 V53D, or Hub under conditions of low m2 mAChR expression levels (i.e. 0.1–0.2 pmol/mg of protein). At higher levels of receptor expression (i.e. ~0.75 pmol/mg of protein), overexpression of wild-type β-arrestin-1 strongly stimulated m2 mAChR sequestration in HEK-293 tsA201 cells, in accordance with a previous study by Pals-Rylaarsdam et al. (16). These results suggest that at low m2 mAChR levels, the endogeneous internalization components are in excess over the number of m2 mAChRs, and the receptors sequester via the β-arrestin- and dynamin-independent pathway. At higher levels of receptor expression, the capacity of this internalization pathway becomes saturated and its components become rate-limiting, so overexpressed β-arrestin now supports sequestration of m2 mAChRs by the other, less efficient β-arrestin- and dynamin-independent pathway in HEK-293 tsA201 cells (16).

During the course of this study, Lee et al. (15) reported that overexpression of another β-arrestin-1 mutant, termed arrestin 2- (319–418), did not lead to inhibition of m1, m3, and m4 mAChR internalization in HEK-293 tsA201 cells, whereas internalization of β2-adrenergic receptors was significantly suppressed. This β-arrestin mutant, which encodes the last 100 amino acids of β-arrestin-1 as the major clathrin binding determinants, binds weakly to phosphorylated agonist-activated GPCRs and blocks internalization via clathrin-coated vesicles.

![Fig. 3. Effects of β-arrestin-1 wild type and V53D mutant on sequestration of m2 mAChRs in HEK-293 tsA201 cells.](image1)

**Fig. 3.** Effects of β-arrestin-1 wild type and V53D mutant on sequestration of m2 mAChRs in HEK-293 tsA201 cells. HEK-293 tsA201 cells transiently transfected with pCD-PS/m2 mAChR together with pBC/β-arrestin-1 (WT), pcDNA-1 Amp/β-arrestin-1 V53D (V53D), or empty pRK5 (pRK5) were incubated in the absence and presence of 1 mM carbachol for 60 min at 37 °C. Sequestration was assessed by [3H]NMS binding to intact cells. Data are the mean ± S.E. from six sets of experiments. Specific [3H]NMS binding to untreated cells transfected with pRK5, pBC/β-arrestin-1, and pcDNA-1 Amp/β-arrestin-1 V53D was 252 ± 60, 112 ± 22, and 183 ± 68 fmol/mg protein, respectively.

![Fig. 4. Effects of β-arrestin-1 wild-type and V53D mutant on sequestration of m3 and m4 mAChRs in HEK-293 tsA201 cells.](image2)

**Fig. 4.** Effects of β-arrestin-1 wild-type and V53D mutant on sequestration of m3 and m4 mAChRs in HEK-293 tsA201 cells. HEK-293 tsA201 cells transiently transfected with pCD-PS/m3 or pCD-PS/m4 mAChR together with pBC/β-arrestin-1 (WT), pcDNA-1 Amp/β-arrestin-1 V53D (V53D), or empty pRK5 (pRK5) were incubated with 1 mM carbachol for 60 min at 37 °C. Sequestration was assessed by [3H]NMS binding to intact cells. Data are the mean ± S.E. from five sets of experiments each. Expression of m3 mAChRs in cells transfected with pRK5, pBC/β-arrestin-1, and pcDNA-1 Amp/β-arrestin-1 V53D was 234 ± 75, 308 ± 69, and 186 ± 57 fmol/mg of protein, respectively. Expression of m4 mAChRs in cells transfected with pRK5, pBC/β-arrestin-1, and pcDNA-1 Amp/β-arrestin-1 V53D was 346 ± 93, 242 ± 63, and 154 ± 60 fmol/mg of protein, respectively.
Regulation of mACHR Sequestration by β-Arrestin

Effect of Hub on sequestration of mACHR subtypes in HEK-293 tsA201 cells. A, detection of T7 Hub in total lysates of HEK-293 tsA201 cells transiently transfected with pCDM8-T7 Hub (Hub) or empty pRK5 (pRK5) by immuno blotting with a T7 epitope-tag-specific monoclonal antibody (75 µg of protein/lane). B, HEK-293 tsA201 cells transiently transfected with pCD-P5 containing mACHR DNA together with pCMDS-T7 Hub or empty pRK5 were incubated for 60 min with 1 mM carbachol. Specific [3H]NMS binding to intact untreated pRK5-transfected cells expressing m1, m2, m3, and m4 mACHRs was 268 ± 78, 169 ± 34, 262 ± 34, and 341 ± 31 fmol/mg of protein, respectively. Specific [3H]NMS binding to intact untreated T7 Hub-transfected cells expressing m1, m2, m3, and m4 mACHRs was 342 ± 70, 130 ± 54, 253 ± 76, and 516 ± 83 fmol/mg of protein, respectively. Data are the mean ± S.E. of 3–6 independent experiments.

In summary, the present study demonstrates an important role for β-arrestin (or a β-arrestin-like protein) in the internalization of m1, m3, and m4 mACHRs in HEK-293 tsA201 cells. The lack of effect of β-arrestin V53D overexpression on the internalization of m2 mACHRs suggests that desensitization of m2 mACHRs can be β-arrestin-independent as well. Wu et al. (17) recently showed that a peptide sequence derived from the third cytoplasmic loop of m2 mACHRs and containing the G protein-coupled receptor kinase phosphorylation sites and a putative β-arrestin binding site (16) does not bind β-arrestins derived from an enriched brain cytosol fraction, whereas a peptide sequence from the third cytoplasmic loop of m3 mACHRs is able to do so (17). As the m2 receptor peptide sequence was able to bind to purified β-arrestins, perhaps there are other cytosolic proteins that preferentially bind to the m2 mACHR and effectively compete with β-arrestin. Like the β-arrestins, association of these unidentified proteins to the m2 mACHR might uncouple the receptor from its cognate G proteins and target the m2 mACHR to the clathrin-independent internalization pathway. It is, however, noteworthy, that m2 mACHRs (and the other mACHR subtypes likely as well) can use alternative sequestration pathways, dependent on the cell species involved (14, 33–35). This underscores the plasticity of the molecular mechanisms of receptor trafficking within the family of GPCRs.

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