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Arrestin Effects on Internalization of Vasopressin Receptors

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ABSTRACT

Arrestins have been shown to facilitate the recruitment of G protein-coupled receptors to the clathrin-coated vesicles that mediate their internalization. After ^{8}Arg-vasopressin-induced internalization, the human V2 vasopressin receptor failed to recycle to the cell surface, whereas the vasopressin type 1a receptor (V1a) subtype did. The possibility that the lack of recycling could identify a novel role for arrestins was investigated by examining the effect of coexpressing wild-type and dominant negative arrestins on the recycling of wild-type and mutant V2 and V1a receptors. Coexpression of the V1a or V2 receptors with the last 100 amino acids of arrestin reduced significantly their internalization, whereas coexpression of wild-type and mutant arrestins had diverse effects on internalization. Arrestin3 but not arrestin2 increased the internalization of the V1aR without altering its recycling pattern. Both nonvisual arrestins enhanced vasopressin type 2 receptor (V2R) internalization, inducing the appearance of a pool of recycling receptor in addition to the nonrecycling pool. The effect of arrestins on the internalization of the chimeric V1a/V2 receptor and its reciprocal chimera was specified by the identity of the carboxyl-terminal segment. The S363A mutation that confers recycling to the V2R did not alter its interaction with arrestins. Truncation of the carboxyl-terminal segment of the V2R impaired ligand-induced internalization that could be fully restored by wild-type arrestins. Internalization of the V2 and V1a receptors required dynamin GTPase activity.

The interaction of G protein-coupled receptors with their agonists promotes activation and signaling through G proteins (Birnbaumer and Birnbaumer, 1996), and in many G protein-coupled receptors phosphorylation of intracellular segments of the receptors. Loss of receptors from the cell surface is preceded by phosphorylation that facilitates binding of arrestins and internalization (Freedman and Lefkowitz, 1996). Studies carried out primarily with the β2-adrenergic and other receptors of the family, identified G protein-coupled receptor kinases (GRKs) (Premont et al., 1995), arrestins (Ferguson et al., 1996), clathrin (Goodman et al., 1996), and dynamin (van der Bliek and Meyerowitz, 1991) as some of the proteins participating in this process. The correlations observed between ligand-triggered receptor phosphorylation and sequestration contributed toward the development of a model that sought to define the sequential steps of ligand-promoted receptor traffic (Zhang et al., 1997). The model proposes that the agonist-bound receptor acquires a conformation that promotes its phosphorylation by G protein-coupled receptor kinases, followed by binding of arrestins to the phosphorylated receptor. Arrestins behave as adaptor proteins facilitating the recruitment of receptors to the plasma membrane domains where the clathrin-coated pits develop. The reduced binding of V53D mutant arrestin2 to rhodopsin present in disks from rod outer segments identified the amino-terminal domain of arrestin as the one interacting with receptors, whereas the carboxyl-terminal domain by itself bound to purified clathrin cages (Goodman et al., 1997; Krupnick et al., 1997a). This dual binding recruits the receptors to the clathrin lattice, in a process that requires the participation of the adaptor protein AP-2 (Laporte et al., 1999). Once the receptor containing coated pits have been formed, the GTPase activity of dynamin is required to "pinch" the vesicles from the membrane, allowing sequestration of the receptors into a compartment usually devoid of G proteins and effectors (Damke, 1996; Urrutia et al., 1997). The newly formed vesicles, termed endosomes, contain proton pumps that acidify their lumen, promoting ligand dissociation and facilitating the cleavage of cytoplasmic phosphate esters by cellular phosphatases. The identity and regulation of these phosphatases are the subject of speculation because no details of the process are known (Krueger et al., 1997). The dephosphorylated receptor returns to the cell surface ready to be activated and internalized once more (Morrison et al., 1996).

The human V2 vasopressin receptor expressed in transfected cells undergoes agonist-induced internalization but fails to recycle to the cell surface after removal of the ligand from the medium and the surface of the cells (Innamorati et al., 1998). Lack of recycling under similar circumstances has

ABBREVIATIONS: GRK, G protein-coupled receptor kinase; V2R, vasopressin type 2 receptor; V1a, vasopressin type 1a receptor; DMEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; HEK, human embryonic kidney; AVP, ^{8}Arg-vasopressin; PBS, phosphate-buffered saline; WT, wild-type; AR, adrenergic receptor; GST, glutathione.

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been reported for the M2 muscarininc acetylcholine receptor by Voegler et al. (1998) without information about the fate of the protein, and for the thrombin- and protease-activated receptors (Trejo and Coughlin, 1999) that are targeted to lysosomes for degradation. It has been demonstrated for the V2R that the permanence of the receptor inside the cell is determined by the identity of the amino acids at its carboxyl terminus that are phosphorylated by G protein-coupled receptor kinases (Innamorati et al., 1997). Trapping of the receptor within the cell requires phosphorylation at key residues, because mutagenesis that eliminates specific acceptor sites confers recycling properties to the V2R (Innamorati et al., 1998). It has been proposed that trapping of the V2R was determined by its sustained binding to arrestins because the mutations that confer recycling to the receptor reduced the time arrestin was associated with V2-containing endosomes (Oakley et al., 1999), but those studies did not examine the impact of additional arrestin on trapping of the V2R.

We investigated the role of arrestins in the internalization of the wild-type human V2R and its recycling mutants, as well as the ability of aspartic or glutamic acids to mimic the effect of phosphorylated serines and threonines (Gaponenko et al., 1999). The effect of dominant negative arrestins (Krupnick et al., 1997b) was tested also on wild-type V1a, and chimeric V2/V1a and V1a/V2 vasopressin receptors.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (DMEM), Hanks' buffered salt solution, Dulbecco's phosphate-buffered saline (D-PBS), penicillin/streptomycin, 0.05% trypsin/0.5 mM EDTA, and fetal bovine serum (FBS) were from Life Technologies, Grand island, NY; cell culture plasticware from Costar, Cambridge, MA; and arginine vasopressin and (−)-isoproterenol from Sigma, St. Louis, MO. [^{3}H]Arginine vasopressin, specific activity 60 to 80 Ci/mmol, was from American Radiolabeled Chemicals, Inc., St. Louis, MO; (−)-CGP-12177,[5,7^{3}H], specific activity 30 to 60 Ci/mmol, was from PerkinElmer Life Science Products, Boston, MA. The cDNAs encoding arrestin2, arrestin3, arrestin2 V53D, and arrestin3 V54D in pCMV plasmids were a generous gift from Dr. Marc Caron (Duke University, Durham, NC), whereas the cDNAs encoding wild-type and K44D-dynamin were a generous gift from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). The 319-418 arrestin2 construct was a generous gift of Dr. John H. Walsh (UCLA, Los Angeles, CA).

Plasmid Preparation. Preparation of the mutant V2R cDNAs G345ter and S363A has been reported (Innamorati et al., 1997, 1998). The mutant cDNAs encoding for nine aspartic or nine glutamic acids substituting for the serines and threonines present in the last 15 amino acids of the V2R were introduced by *Apa*I (codon 358)/*Xba*I ligation of synthetic oligonucleotides encoding for the desired amino acid composition. cDNAs encoding V2/V1a and V1a/V2 chimeric receptors were obtained by splicing the full-length carboxyl-terminal segment of one receptor cDNA onto the other at the codons corresponding to the palmitoylated cysteines. All cDNAs encoding for receptors or K44D-dynamin were cloned into pcDNA3 (Invitrogen, Boston, MA), for expression in HEK 293 cells. The arrestin cDNAs were used in their pCMV vectors.

Cell Culture. HEK 293-T cells were grown in DMEM-high glucose, supplemented with 10% heat-inactivated FBS, penicillin (50 units/ml), and streptomycin (50 μg/ml).

Transient Expression in Cells. Subconfluent HEK 293-T cells were plated at a density of 3.5×10^6 cells/100-mm dish and transfected by a modification of the method of Luthman and Magnusson (1983). After removing the growth medium each plate received 6.4 ml of DMEM/10% FBS containing 3 μg of plasmid DNA (1.5 μg of plasmid encoding the receptors plus 1.5 μg of plasmids encoding either β-galactosidase or the different arrestin cDNAs) mixed with 0.25 mg/ml DEAE-Dextran plus 100 μM chloroquine. After 2 h at 37°C the cells were exposed to 10% dimethyl sulfoxide in D-PBS for 1 min, rinsed twice with D-PBS, and returned to growth medium at 37°C. Control cells were transfected with the cDNAs encoding the wild-type receptor and β-galactosidase cloned into pcDNA3 to obtain unaltered level of expression of the receptor. For the β2AR experiments, COS 7 cells were transfected with the same protocol with 5 μg of pcDNA3-human β2AR plus 5.5 μg of plasmids encoding either β-galactosidase or the different arrestins at the proportions described in the legend to the figure.

Hormone Treatments. Transfected cells were plated 24 h after transfection in poly-D-lysine-coated 24-well plates (4.0×10^5 cells/well), the next day cells were treated with 100 nM AVP or 10 μM (−)-isoproterenol in medium for 20 min at 37°C to promote receptor sequestration. After AVP treatments the hormone remaining on the cell surface was removed by two washes with PBS, two washes with 150 mM NaCl/5 mM acetic acid, and three washes with PBS, all at 4°C. The acid washes were omitted after the (−)-isoproterenol treatment. For the recycling experiments fresh DMEM/10% FBS was added, the cells returned to the 37°C incubator and the receptor present on the plasma membrane was measured with [^{3}H]AVP at the indicated times. Addition of the protein synthesis inhibitor cycloheximide at 5 mg/ml did not alter the outcome of the recycling or the nonrecycling experiments, indicating that de novo protein synthesis did not alter the abundance of cell surface receptors.

Hormone Binding to Intact Cells. Vasopressin receptor numbers were determined as described previously (Innamorati et al., 1998). Cells were exposed to 20 nM [^{3}H]AVP for 2 h at 4°C, washed twice with ice-cold D-PBS, and the bound radioactivity extracted adding 0.5 ml of 0.1 N NaOH/well. After 30 min at 37°C, the fluid from each well was transferred to a scintillation vial containing 3.5 ml of ULTIMA-FLO M (Packard, Meriden, CT) scintillation fluid for radioassay. Nonspecific binding was determined under the same conditions in the presence of 10 μM unlabeled AVP. β2-Adrenergic receptors were measured following the procedure of Morrison et al. (1996) by exposing the receptor to 6 nM (−)-CGP-12177,[5,7^{3}H] for 90 min at 4°C followed by washes in D-PBS and lysis as described above. Nonspecific binding of the radioligand was determined by parallel incubations in the presence of 3 μM propranolol. The data are expressed as means ± S.E.M. Receptor abundance is expressed as number of receptors per cell.

Preparation of Cell Lysates Containing Arrestins. HEK 293 cells were transiently transfected with 1.5 μg of pCMV5 containing the arrestin cDNAs as described above; 48 h later cells were rinsed once with cold PBS and collected with a rubber policeman in 1 ml of cold PBS. Cells were centrifuged at 2000 rpm for 3 min, washed one time with 1 ml of cold PBS, and suspended in 200 μl of 50 mM Tris-HCl pH 7.5, 0.5 mM $MgCl_2$, 150 mM potassium acetate, 1% Nonidet-40, 1.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl flouride, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Cells were lysed by passing the suspension five times through a 20-gauge needle and five times through a 25-gauge needle. The mixtures were allowed to sit for 30 min on ice. The lysates were centrifuged at 3000 rpm for 5 min in a refrigerated microcentrifuge, and the supernatants were separated and stored at 4°C. Protein concentration was measured by the Bradford assay on an aliquot of the cell lysate diluted 1:5 and the expression of arrestins was assessed by immunoblotting with an anti-arrestin monoclonal antibody (Research Diagnostics, Flanders, NJ) that detects the wild-type and mutant forms of both arrestins.

Results

Effect of Arrestins in Wild-Type V2R and V1aR Internalization. As control of the cotransfection experiments, the levels of expression of arrestins were assessed by immu-

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noblotting lysates prepared from transfected cells. Forty-eight hours after transfection, samples were analyzed using a commercial monoclonal antibody that detects all arrestins. Figure 1 exemplifies in lane 1 the amount of endogenous arrestin present in 50 μg of lysate from naive HEK 293 cells, whereas lanes 2 and 3 illustrate the amount of arrestin present in 0.5 μg of lysate prepared from cells transfected with plasmids expressing arrestin2 or arrestin3. As observed here, the content of arrestin in the cells was increased more than 100-fold by transfection. Lanes 4 to 7 of Fig. 1 exemplify the quantity of wild-type and mutant arrestins present in 0.5 μg of transfected cell lysate: arrestin2 (lane 4), V53D arrestin2 (lane 5), arrestin3 (lane 6), and V54D arrestin3 (lane 7), respectively, all expressed at similar level.

Plasmids expressing wild-type and mutant arrestins were cotransfected with the wild-type V2R into HEK 293 cells and their effect on ligand-promoted receptor internalization was assessed 48 h after transfection. As shown in Fig. 2, arrestin2 and arrestin3 almost double the fraction of the V2R that internalized, and the same effect was observed upon cotransfection with V53D mutant arrestin2. The V54D mutant arrestin3 did not change V2R internalization, but expression of the carboxyl-terminal segment of arrestin decreased significantly the fraction of internalized receptor. As shown in Fig. 3, internalization of the recycling S363A-V2R was enhanced by V53D arrestin2 and reduced by 319-418 arrestin2, as seen with WT V2R. These data confirmed the involvement of arrestin in the internalization of the V2 receptor and suggested a problem with our V53D arrestin2 plasmid.

The V53D arrestin2 plasmid was cotransfected with the β2-adrenergic receptor cDNA into COS 7 cells to test its effect on the internalization of this receptor. As illustrated in Fig. 4, the V53D arrestin2 construct successfully reduced the internalization of the β2-AR promoted by wild-type arrestin as reported by Krupnick et al. (1997b).

As illustrated in Fig. 5, internalization of the V1aR was enhanced only by arrestin3, with arrestin2 being ineffective. As observed with the V2R, the last segment of arrestin was effective in reducing V1aR internalization although neither valine mutant was effective.

Examining the effect of overexpressed arrestins on V2R recycling revealed the appearance of a pool of V2R that recycled to the cell surface after removal of ligand from the medium, whereas the fraction of nonrecycling receptor remained unchanged. Thus, as illustrated in Fig. 6, the additional V2 receptor internalized when arrestins were overexpressed followed a pathway within the cell different from the one followed by the nonrecycling V2R, indicating that arrestin could direct the V2R in HEK 293 cells to a recycling or to a nonrecycling path. Similar data were obtained when the V2R was coexpressed with arrestin3 (data not shown). Arrestin3 increased the fraction of V1aR that was internalized without altering receptor recycling (data not shown).

Internalization of a Truncated V2R. The G345ter-V2R lacks the last 26 amino acids that constitute the carboxyl terminus after the palmitoylated cysteines. This protein had no acceptor sites for GRK phosphorylation, displayed reduced internalization compared with the wild-type, and recycled to the cell surface after removal of the ligand (Innamorati et al., 1997, 1998). This mutant was used to examine whether arrestins could alter the internalization of a receptor lacking the carboxyl terminus. As illustrated in Fig. 7,

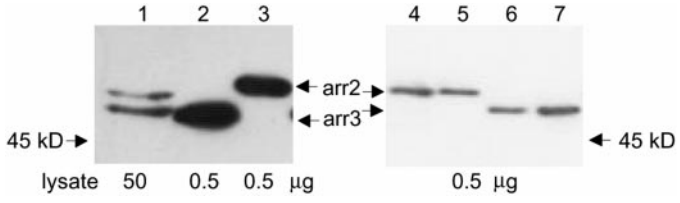

Fig. 1. Expression of arrestins in HEK 293 cells. Levels of arrestins present in lysates from naive and transfected cells were assessed by immunoblotting with an anti-arrestin antibody. Lane 1 contains 50 μg of lysate prepared from naive cells, whereas the other lanes contain 0.5 μg each of lysate prepared from cells transfected with plasmids encoding wild-type arrestin2 (lanes 3 and 4), V53D arrestin2 (lane 5), wild-type arrestin3 (lanes 2 and 6), and V54D arrestin3 (lane 7). Immunoblots were visualized with the enhanced chemiluminescence method, the image of lanes 1 to 3 was developed for longer time to improve visualization of the less abundant endogenous arrestins in HEK cells; the migration of ovalbumin (45-kDa molecular mass marker) is shown.

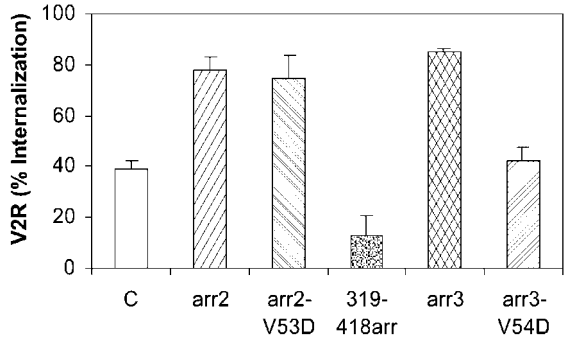

Fig. 2. Arrestins and V2R internalization. HEK 293 cells were cotransfected as described under *Experimental Procedures* with 1.5 μg of human V2R cDNA in pcDNA3 plasmid mix either with 1.5 μg of β-gal/cDNA3 plasmid or 1.5 μg of pCMV5 containing the cDNAs encoding for either the wild-type, the mutant arrestins, or the 319-418 arrestin2. Receptor internalization was measured 48 h after transfection exposing the cells to 100 nM AVP for 20 min at 37°C. After washes with cold isotonic acid solutions, the number of receptors remaining on the cell surface was determined as described under *Experimental Procedures*. The data are reported as the mean ± S.E.M. of three independent experiments. V2 receptors were 1.50 to 1.75 × 10^6 sites/cell in the presence of β-galactosidase, wild-type, or mutant arrestins.

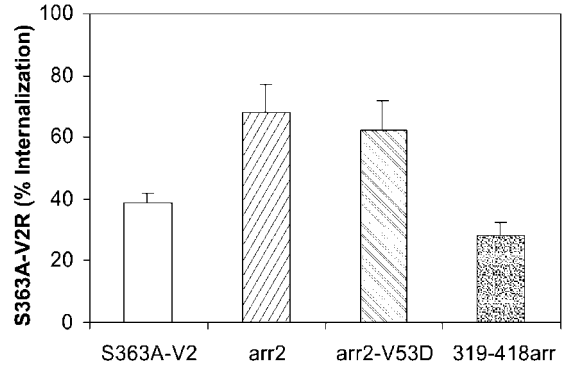

Fig. 3. Arrestins and S363A-V2R internalization. The experiment was performed as described in the legend for Fig. 2, cotransfecting cDNA encoding the recycling mutant V2R with the different arrestin cDNAs. HEK 293 cells were cotransfected as described under *Experimental Procedures* with 1.5 μg of cDNA encoding the human S363A-V2R in pcDNA3 plasmid mix either with 1.5 μg of β-gal/cDNA3 plasmid or 1.5 μg of pCMV5 containing the cDNAs encoding for either the wild-type, the mutant arrestins, or the 319-418 arrestin2. The data are reported as the mean ± S.E.M. of three independent experiments. S363A-V2 receptors were 1.45 to 1.62 × 10^6 sites/cell in the presence of β-galactosidase, wild-type, or mutant arrestin2.

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both arrestins increased the internalization of the truncated receptor to levels similar to those observed with the wild-type V2R plus arrestins, with arrestin3 being the most effective. This strong augmentation of internalization observed in the absence of the carboxyl terminus implied that a region of the receptor separate from the tail was mediating the interaction. Ferguson et al. (1996) reported that a truncated β2-adrenergic receptor lacking phosphorylation sites exhibits a 50% reduction in ligand-induced internalization. Similar to our data with the V2R, coexpression of this mutant β2-AR with arrestins restored internalization to the same values obtained with the full-length receptor (Ferguson et al., 1996). Internalization of the truncated V2R was sensitive to the mutant forms of arrestin, with V54D arrestin3 being slightly more effective than V53D arrestin2, suggesting that elimination of the last segment of the receptor exposed cytoplasmic segments of the nonphosphorylated V2R able to discriminate between WT and mutant forms of the protein.

Arrestins and Chimeric Receptors Internalization. Substituting the last 30 amino acids of the V2R by the last segment of the V1aR produced a chimeric receptor that recycled readily to the cell surface after ligand-induced internalization (Innamorati et al., 1998). As shown in Fig. 8, coexpression of wild-type arrestins promoted only a slight increase in the internalization of the V2/V1a chimeric receptor, whereas the valine mutants were ineffective. Similar to our findings with wild-type V2R, internalization of the V1a/V2 chimera was enhanced by coexpression of arrestin2, V53D arrestin2, and arrestin3 but not by V54D arrestin3. As expected internalization of the chimeric receptors was reduced by 319-418 arrestin.

A few receptors of this superfamily are internalized via dynamin-independent pathways (Zhang et al., 1996) as shown by the lack of effect of GTPase-deficient dynamin on

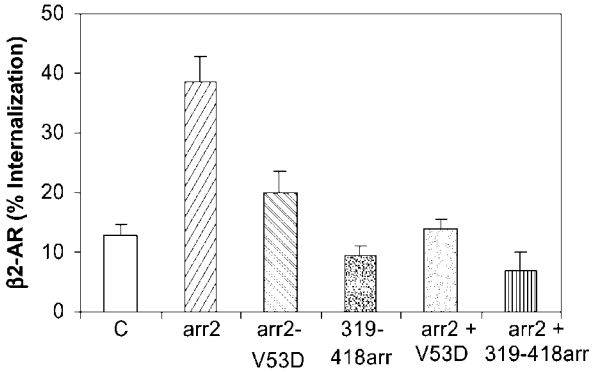

Fig. 4. Arrestins effect on β2-AR internalization. COS 7 cells were cotransfected as described under *Experimental Procedures* with 5 μg of human β2-AR cDNA in pcDNA3 plasmid with plasmids encoding β-galactosidase 5 μg (C), or arrestin2 0.5 μg, or V53D arrestin2 5 μg, or 319-418 arrestin2 5 μg, or arrestin2 0.5 μg plus V53D arrestin2 5 μg, or arrestin2 0.5 μg plus 319-418 arrestin2 5 μg. Receptor internalization was measured 48 h after transfection exposing the cells to 10 μM (−)-isoproterenol for 20 min at 37°C. After washes with cold isotonic solutions, the number of receptors remaining on the cell surface was determined as described under *Experimental Procedures*. The data are reported as the mean ± S.E.M. of three independent experiments. β2-AR receptors were 8.0 to 8.8 × 10^5 sites/cell in the presence of β-galactosidase, wild-type, or mutant arrestin2, or the indicated mixtures of wild-type and arrestin2 mutants.

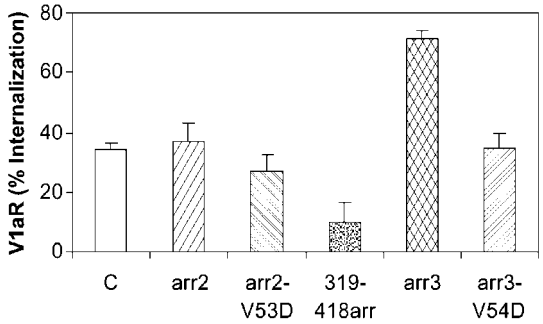

Fig. 5. Arrestins and V1aR internalization. The experiment was performed as described in the legend for Fig. 2, cotransfecting pcDNA3 containing a cDNA that encodes the rat V1a receptor with plasmids encoding the different arrestins. The data are reported as the mean ± S.E.M. of three independent experiments. V1a receptors were 5.8 to 7.3 × 10^5 sites/cell in the presence of β-galactosidase, wild-type, or mutant arrestins.

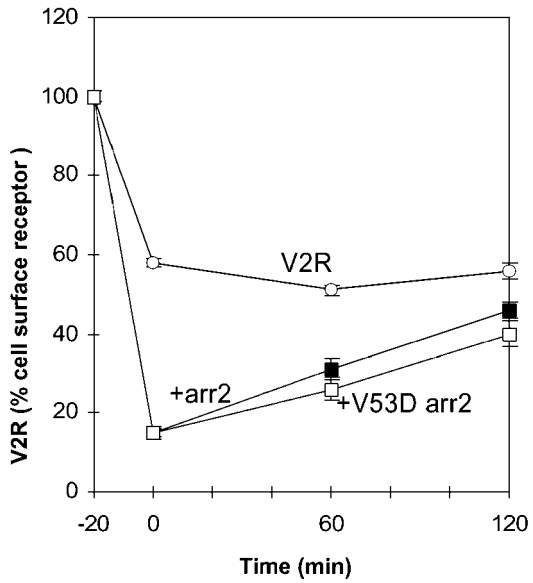

Fig. 6. Arrestin effect on V2R recycling. HEK 293 cells were transfected and treated with 100 nM AVP as described in the legend for Fig. 2. After ligand-induced internalization the cells were subjected to cold acid washes, and returned to the incubator with growth medium. At the times indicated in the figure, the number of receptors present on the cell surface was measured to assess the extent of recycling of the proteins. The effect of arrestin2 and the arrestin2 V53D mutant are illustrated. The data are reported as the mean ± S.E.M. of three independent experiments. V2 receptors were 1.4 × 10^6 sites/cell in the presence of β-galactosidase, wild-type, or mutant arrestin2.

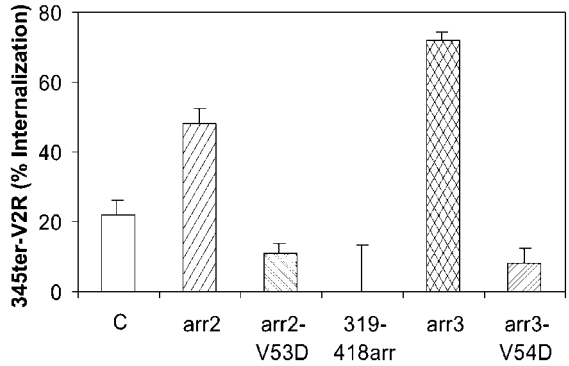

Fig. 7. Arrestin effect on internalization of G345ter-V2R. The experiment was performed as described in the legend for Fig. 2. The enhancing effect of arrestins2 and 3 and the competitive effect of the mutant arrestins on internalization are illustrated. The data are reported as the mean ± S.E.M. of three independent experiments. 345ter-V2 receptors were 6.5 to 7.9 × 10^5 sites/cell in the presence of β-galactosidase, wild-type, or mutant arrestins.

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their internalization. As illustrated in Fig. 9, ligand-promoted internalization of the V2R, the V1aR, and the G345ter-V2R was significantly diminished by the presence of the inactive dynamin mutant K44A. A mild reduction in receptor internalization was also observed for the G345ter-V2R when wild-type dynamin was expressed. These data are similar to those reported by Paals-Rylaarsdam et al. (1997) and Werbonat et al. (2000) for the M2 muscarinic acetylcholine and the angiotensin II 1A receptors.

Discussion

The ability of 319-418 arrestin2 to reduce the internalization of the V1a and V2 receptors demonstrated the participation of arrestins in recruiting both receptors to the site of endosome formation. As previously reported by Krupnick et al (1997b), substitution by aspartic acid of 53 or 54 valine in arrestin2 or arrestin3 generates a weak dominant negative arrestin (Ferguson et al., 1996). The V53D mutation in arrestin2 did not reduce binding to the WT or mildly mutagenized V2R, causing an increase in internalization rather than a reduction. Similar to our finding, Yu and Hinkle (1999) reported enhancement of ligand-induced internalization of the thyrotropin-releasing hormone receptor when coexpressed with wild-type or V53D arrestin2. For the V2R, the carboxyl-terminal segment was responsible for this interaction because the V1a/V2R chimera also failed to distinguish between the two proteins. The same V53D arrestin2 demonstrated dominant negative activity when cotransfected with wild-type arrestin and the β2-adrenergic receptor in COS 7 cells, as described by other laboratories (Krupnick et al., 1997b; Zhang et al., 1997).

Coexpression of arrestins increased the fraction of receptor that was internalized but did not confer recycling properties to the fraction of the V2R internalized by endogenous arrestin, resulting in the appearance of pool of recycling receptors. It is possible that the capacity of the HEK 293 cells to retain the internalized V2R cannot accommodate the additional receptor internalized upon arrestin overexpression. Such an interpretation would imply that arrestin is not responsible for trapping of the receptor and other proteins might be responsible for this phenomenon. Cell imaging data recently published by Oakley et al. (1999) lead these authors to correlate the lack of recycling of the V2R with the ability of the receptor to recruit arrestin to endosomal vesicles. Confocal microscopy immunofluorescence demonstrating colocalization of recycling S363A-V2R and endogenous arrestin in the perinuclear recycling compartment of HEK 293 indicated that sustained binding to arrestin is compatible with receptor recycling (Innamorati et al., 2001).

Wild-type arrestins and V53D arrestin2 did not alter recycling of the S363A-V2R mutant (data not shown). Thus, the mutation of the phosphorylation site that bestowed recycling properties to the V2R did not allow the protein to distinguish between the wild-type and V53D mutant arrestin2. Substitution of serines for aspartic or glutamic acids has been shown to mimic in some instances the presence of phosphorylated amino acids (Gaponenko et al., 1999). The possibility that negatively charged amino acids at the carboxyl terminus could trap the internalized V2R within the cell was tested by substituting all possible phosphate acceptor sites between codons 357 and 371 of the V2R. Two mutants were produced, one containing aspartic acid (V2R/allD), the other containing glutamic acid (V2R/allE), at all relevant positions in the carboxyl terminus. The mutant receptors had levels of expression similar to the wild-type receptor, indicating that the negative charges had not promoted constitutive internalization, and coupled to G_s with the same efficiency as the wild-type (data not shown). The negatively charged amino acids reduced slightly ligand-induced internalization, but neither receptor was trapped inside the cell, signifying that these negative charges could not mimic the presence of phosphate groups on the receptor (Innamorati et al., 1998).

Internalization of the 345ter-V2R that lacks most of the carboxyl terminus and thus the GRK phosphorylation sites was enhanced by wild-type arrestins, identifying a second site in the receptor protein able to interact with the adaptor proteins. These findings suggested the existence of two sites of interaction of the V2R with arrestins: one at the carboxyl

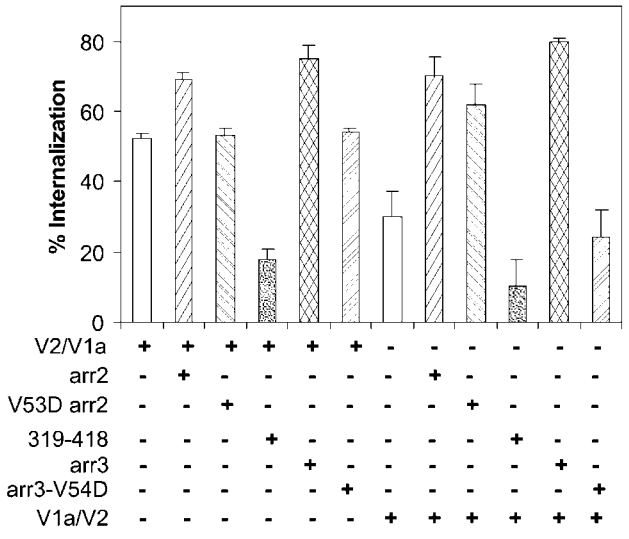

Fig. 8. Effect of arrestins on internalization of V2/V1aR and V1a/V2R chimeras. The experiments were performed as described in the legend for Fig. 2. The data are reported as the mean ± S.E.M. of three independent experiments. The V2/V1a and the V1a/V2 chimeric receptors were 3.5 to 4.6 and 7.5 to 8.3 sites/cell, respectively, in the presence of β-galactosidase, wild-type, or mutant arrestins.

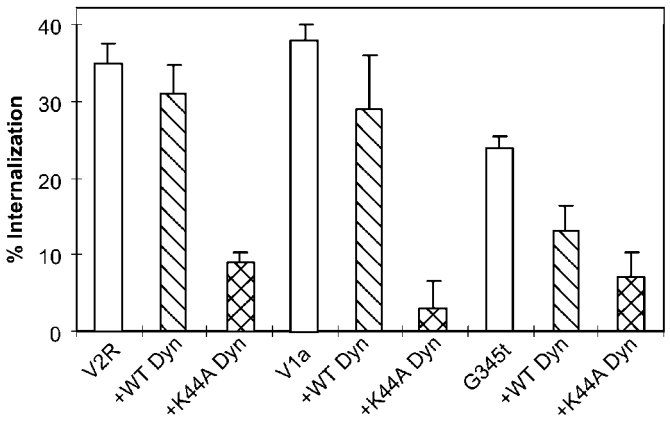

Fig. 9. Effect of wild-type and K44A dynamin on internalization of V2, V1a, and G345ter-V2 receptors. The experiments were performed as described in the legend for Fig. 2. The data are reported as the mean ± S.E.M. of three independent experiments. In a representative experiment receptor abundance was 1.2×10^6 for the V2, 7.2×10^5 for the V1a, and 6.0×10^5 for the G345ter-V2, expressed as sites per cell.

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terminus, the other present in the 345ter-V2R formed by the intracellular loops of the protein. Similar findings have been reported by Ferguson et al. (1996) for the β2-adrenergic receptor truncated at the palmitoylated cysteine and thus lacking the entire carboxyl terminus that contains the GRK phosphorylation sites. The truncated β2-AR internalized only 50% as well as the wild-type receptor, but coexpression of arrestin increased the fraction internalized to values comparable with the wild-type β2-AR (Ferguson et al., 1996).

We attempted to identify the site of interaction between arrestin and the intracellular loops of the V2R by expressing these segments as GST-fusion proteins and testing their ability to bind arrestin present in lysates of transfected HEK 293 cells. Although there was reproducible retention of arrestin by loops 2 and 3, more than by loop 4 and GST, the amount retained represented only 1% of the arrestin present during the binding assay, raising doubts as to the significance of these observations (data not shown). These results could be due to the existence of a cytosolic protein that mediates the association between the G345ter-V2R and arrestins, or alternatively, to the distorted conformation of the intracellular loops when attached to GST, unable to mimic the structure presented by these segments when adjacent to the inner layer of the plasma membrane.

The data obtained with the G345ter-V2R corroborated that the presence of phosphorylated amino acids was not required for arrestin to enhance internalization of the receptor protein, as previously demonstrated by Ferguson et al. (1996), for the truncated the β2-adrenergic receptor. Thus, it was puzzling that the internalization of the V2/V1a chimeric receptor was poorly enhanced by arrestins, although it contained the intracellular segments present in the G345ter-V2R. It is conceivable that accessibility of cytoplasmic proteins to the intracellular loops was reduced by the presence of the long V1a carboxyl terminus.

The V1a/V2 chimeric receptor confirmed that the carboxyl terminus could retain the V1aR inside the cell, similar to what has been reported for the V2/β2-adrenergic receptor chimera spliced at the same location after (−)-isoproterenol-promoted internalization (Oakley et al., 1999), indicating that the trapping properties of the V2R tail are dominant and can promote the retention of other receptors inside the cell. The V1a/V2 chimeric receptor did not recycle to the cell surface as fast as the wild-type V1a (data not shown), but it was not trapped within the cell as detected for the β2/V2 receptor chimera, suggesting that the protein context can modify the effectiveness of the V2R signal. A possible explanation for this difference is that the V2R segment in the chimeric protein was not phosphorylated to the same extent as it would have been in the context of the wild-type V2R. The V54D arrestin3 mutant reduced the internalization of the chimeric V1a/V2 receptor, although it did not alter the internalization of either wild-type receptor.

The data from transfected cells invite speculations as to whether the V2 receptor recycles in the human kidney. Several factors may result in minimal impact of lack of recycling in vivo. First, the extent of phosphorylation is concentration-dependent and the kidney is not likely to be exposed to saturating concentrations of AVP (Innamorati et al., 1997). Consequently, only a few receptor molecules will be completely phosphorylated in vivo; therefore, the fraction of receptor refractory to recycling may be small. Second, kidney cells may contain phosphatases plus other factors that are more efficient than the transfected cells at hydrolyzing phosphate from the GRK-phosphorylated sites.

In summary, the data presented demonstrated that arrestin did not alter trapping of the V2R within the cell as exemplified by the appearance of the recycling pool of receptors. The results clearly demonstrate the presence of two regions of the V2R able to interact with arrestin independently from each other: the carboxyl terminus and a domain defined by the intracellular segments of the protein.

References

Birnbaumer M and Birnbaumer L (1996) Signal transduction by G protein coupled receptors, in *Molecular Biology of Membrane Transport Disorders* (Schultz SG, Andreoli TE, Brown AM, Fambrough DM, Hoffman JF, and Welsh MJ eds) pp 321–366, Plenum Press, New York.

Damke H (1996) Dynamin and receptor-mediated endocytosis. *FEBS Lett* **389:**48–51.

Ferguson SS, Downey WE III, Colapietro AM, Barak LS, Menard L and Caron MG (1996) Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science (Wash DC)* **271:**363–366.

Freedman NJ and Lefkowitz RJ (1996) Desensitization of G protein-coupled receptors. *Rec Prog Horm Res* **51:**319–351.

Gaponenko V, Abusamhadneh E, Abbott MB, Finley N, Gasmi-Seabrook G, Solaro RJ, Rance M and Rosevear PR (1999) Effects of troponin I phosphorylation on conformational exchange in the regulatory domain of cardiac troponin C. *J Biol Chem* **274:**16681–16684.

Goodman OB Jr, Krupnick JG, Gurevich VV, Benovic JL and Keen JH (1997) Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J Biol Chem* **272:**15017–15022.

Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH and Benovic JL (1996) β-Arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor. *Nature (Lond)* **383:**447–450.

Innamorati G, Le Gouill C, Balamotis M and Birnbaumer M (2001) The long and the short cycle: alternative routes for G-protein coupled receptors trafficking. *J Biol Chem* **276:**13096–13103.

Innamorati G, Sadeghi H, Eberle AN and Birnbaumer M (1997) Phosphorylation of the V2 vasopressin receptor. *J Biol Chem* **272:**2486–2492.

Innamorati G, Sadeghi H, Tran NT and Birnbaumer M (1998) A serine cluster prevents recycling of V2 vasopressin receptor. *Proc Natl Acad Sci USA* **95:**2222–2226.

Krueger KM, Daaka Y, Pitcher JA and Lefkowitz RJ (1997) The role of sequestration in G protein-coupled receptor resensitization. *J Biol Chem* **272:**5–8.

Krupnick JG, Goodman OB Jr, Gurevich VV, Benovic JL and Keen JH (1997a) Arrestin/clathrin interaction. Localization of the clathrin binding domain of non-visual arrestins to the carboxy terminus. *J Biol Chem* **272:**15011–15016.

Krupnick JG, Santini F, Gagnon AW, Keen JH and Benovic JL (1997b) Modulation of the arrestin-clathrin interaction in cells. Characterization of β-arrestin dominant-negative mutants. *J Biol Chem* **272:**32507–32512.

Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG and Barak LS (1999) The β2-adrenergic receptor/β-arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci USA* **96:**3712–3717.

Luthman H and Magnusson G (1983) High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res* **11:**1295–1308.

Morrison KJ, Moore RH, Carsrud ND, Trial J, Millman EE, Tuvim M, Clark RB, Barber R, Dickey BF and Knoll BJ (1996) Repetitive endocytosis and recycling of the β2-adrenergic receptor during agonist-induced steady state redistribution. *Mol Pharmacol* **50:**692–699.

Oakley RH, Laporte SA, Holt JA, Barak LS and Caron MG (1999) Association of β-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem* **274:**32248–32257.

Paals-Rylaarsdam R, Gurevich VV, Lee KB, Ptasienski JA, Benovic JL and Hosey MM (1997) Internalization of the m2 muscarinic acetylcholine receptor. Arrestin-independent and -dependent pathways. *J Biol Chem* **272:**23682–23689.

Premont RT, Inglese J and Lefkowitz RJ (1995) Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J* **9:**175–182.

Trejo J and Coughlin SR (1999) The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling. *J Biol Chem* **274:**2216–2224.

Urrutia R, Henley JR, Cook T and McNiven MA (1997) The dynamins: redundant or distinct functions for an expanding family of related GTPases? *Proc Natl Acad Sci USA* **94:**377–384.

van der Bliek AM and Meyerowitz EM (1991) Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature (Lond)* **351:**411–414.

Voegler O, Bogatkewitsch GS, Wriske C, Krummenerl P, Jakobs KH and van Koppen CJ (1998) Receptor subtype-specific regulation of muscarinic acetylcholine receptor requestration by dynamin. Distinct sequestration of m2 receptors. *J Biol Chem* **273:**12155–12160.

Werbonat Y, Kleutges N, Jakobs KH and van Koppen CJ (2000) (1974) Essential role of dynamin in internalization of M2 muscarinic acetylcholine and angiotensin AT_{1A} receptors. *J Biol Chem* **275:**21969–21974.

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Yu R and Hinkle PM (1999) Signal transduction and hormone-dependent internalization of the thyrotropin-releasing hormone receptor in cells lacking G_q and G_{11}. *J Biol Chem* **274:**15745–15750.
Zhang J, Barak LS, Winkler KE, Caron MG and Ferguson SS (1997) A central role for β-arrestins and clathrin-coated vesicle-mediated endocytosis in β2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types. *J Biol Chem* **272:**27005–27014.
Zhang J, Ferguson SS, Barak LS, Menard L and Caron MG (1996) Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem* **271:**18302–18305.

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