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Palmitoylation of the V2 Vasopressin Receptor Carboxyl Tail Enhances β-Arrestin Recruitment Leading to Efficient Receptor Endocytosis and ERK1/2 Activation*

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A large number of G protein-coupled receptors are palmitoylated on cysteine residues located in their carboxyl tail, but the general role of this post-translational modification remains poorly understood. Here we show that preventing palmitoylation of the V2 vasopressin receptor, by site-directed mutagenesis of cysteines 341 and 342, significantly delayed and decreased both agonist-promoted receptor endocytosis and mitogen-activated protein kinase activation. Pharmacological blockade of receptor endocytosis is without effect on the vasopressin-stimulated mitogen-activated protein kinase activity, excluding the possibility that the reduced kinase activation mediated by the palmitoylation-less mutant could result from altered receptor endocytosis. In contrast, two dominant negative mutants of β-arrestin which inhibit receptor endocytosis also attenuated vasopressin-stimulated mitogen-activated protein kinase activity, suggesting that the scaffolding protein, β-arrestin, represents the common link among receptor palmitoylation, endocytosis, and kinase activation. Co-immunoprecipitation and bioluminescence resonance energy transfer experiments confirmed that inhibiting receptor palmitoylation considerably reduced the vasopressin-stimulated recruitment of β-arrestin to the receptor. Interestingly, the changes in β-arrestin recruitment kinetics were similar to those observed for vasopressin-stimulated receptor endocytosis and mitogen-activated protein kinase activation. Taken together the results indicate that palmitoylation enhances the recruitment of β-arrestin to the activated V2 vasopressin receptor thus facilitating processes requiring the scaffolding action of β-arrestin.

Among the molecular mechanisms regulating G protein-coupled receptor (GPCR)[1] function, post-translational modifica-

tions such as phosphorylation and palmitoylation have been the subject of numerous studies. The role of phosphorylation in receptor desensitization is now well established (1). Upon activation, GPCRs become phosphorylated by both second messenger-activated and GPCR kinases on serine and threonine residues located in the third intracellular loop and/or carboxyl-terminal tail of the receptors. These phosphorylation events prevent further G protein interaction through the involvement of β-arrestin proteins preferentially binding to receptors phosphorylated by GPCR kinases. In addition to promoting receptor/G protein uncoupling, the β-arrestins target such desensitized receptors to clathrin-coated pits for endocytosis by functioning as adaptor proteins that link the receptors to components of the endocytic machinery. Furthermore, β-arrestin acts as a scaffolding protein directly linking the receptors to the mitogen-activated protein kinases (MAPK) signaling pathways, a process that has been shown to play an important role in GPCR-mediated MAPK activation (2).

On the other hand, GPCR palmitoylation has also been shown to affect receptor function, but its precise role is not well defined. This post-translational modification, resulting from the covalent linkage of palmitic acid through a thioester bond, usually occurs at the level of conserved cysteine residues in the carboxyl tail of receptors. GPCR palmitoylation has been found to affect differentially a broad spectrum of biological processes, including G protein coupling efficiency and selectivity, receptor phosphorylation and desensitization, receptor endocytosis, cell surface expression and trafficking, and receptor down-regulation (3). Despite the role that β-arrestin plays in several of these processes, the influence of receptor palmitoylation on the recruitment of the scaffolding protein has never been investigated.

For the V2 vasopressin receptor (V2R), palmitoylation has been shown to occur on cysteine residues 341 and 342 in the carboxyl tail of the receptor (4). In two studies, mutation of these palmitoylation sites had no effect on the arginine vasopressin (AVP) binding affinity or agonist-stimulated adenylyl cyclase activity (4, 5). Similarly, no difference in agonist-promoted desensitization was observed between wild-type and the palmitoylation-less mutant V2R (4). In contrast, Schülein *et al.* (5) also reported that lack of palmitoylation was associated with a reduced rate of agonist-promoted endocytosis. However, this finding was not confirmed by Sadeghi *et al.* (4), where wild-type V2R and two palmitoylation-less V2R mutants

[1] The abbreviations used are: GPCR, G protein-coupled receptor; AVP, arginine vasopressin; β2AR, β2-adrenergic receptor; BRET, bioluminescence resonance energy transfer; 2-BrP, 2-bromopalmitic acid; BSA, bovine serum albumin; DSP, dithiobis(succinimidyl propionate);

ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; HEK293, human embryonic kidney 293 cells; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; P-ERK1/2, phosphorylated ERK1/2; V2R, V2 vasopressin receptor; wt, wild-type.

V2R Palmitoylation and β-Arrestin Recruitment

(C341G/C342G, C341S/C342S) were reported to have identical AVP-promoted endocytosis.

Although stimulation of adenylyl cyclase through G_s is the best characterized signaling cascade engaged by the V2R, extracellular signal-regulated kinases 1 and 2 (ERK1/2) have also been shown to be activated upon activation of the V2R by its natural ligand, AVP (6). Among diverse mechanisms, β-arrestin recruitment and receptor endocytosis have often been proposed to be implicated in the activation of these MAPKs by GPCR. Despite the growing recognition that activation of MAPK by GPCR plays important roles in downstream signaling events (7) and the putative role of palmitoylation in agonist-promoted endocytosis, very little is known of the potential role of receptor palmitoylation in MAPK activation.

In the present study, we investigated the role of V2R palmitoylation on AVP-stimulated MAPK activation, receptor endocytosis, and β-arrestin recruitment. We report that the lack of palmitoylation at cysteines 341 and 342 significantly decreases the rate and extent of β-arrestin recruitment to the V2R, thus leading to slower and reduced receptor endocytosis and MAPK activation. Our results also indicate that although the scaffolding function of β-arrestin plays important roles in both AVP-stimulated endocytosis and MAPK activation, the latter can occur independently of receptor internalization. We thus suggest that V2R palmitoylation serves to induce and/or stabilize a particular receptor conformation, optimizing the interaction of β-arrestin with the receptor and facilitating β-arrestin-dependent downstream events such as ERK1/2 activation and endocytosis.

EXPERIMENTAL PROCEDURES
Materials

Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin, streptomycin, glutamine, Fungizone, G418, and phosphate-buffered saline (PBS) were all from Wisent, Inc. Cell culture plates and dishes were from Corning. Bovine serum albumin (BSA), AVP, 3-isobutylmethylxanthine, *o*-phenylenediamine dihydrochloride tablets (OPD peroxidase substrate), and tunicamycin were from Sigma. The Bio-Rad D_C Protein Assay kit and the Bradford reagent were from Bio-Rad Laboratories. [^{3}H]Adenine, [^{3}H]palmitic acid, $^{32}P_i$, and ECL were from PerkinElmer Life Sciences. Antibodies recognizing ERK1/2 and their phosphorylated forms (P-ERK1/2), anti-myc mouse monoclonal 9E10, and rabbit polyclonal A14 IgGs were from Santa Cruz Biotechnology Inc. Anti-human (h)V2R AS435 antibody was a generous gift from W. Muller-Esterl (University of Frankfurt Medical School, Frankfurt).

Expression Vectors

wtV2R and C341A/C342A-V2R—Wild-type hV2R was subcloned 3′ to the myc epitope sequence (MEQKLISEEDLNA) into the pBC12BI mammalian expression vector as described previously (8). The myc epitope-tagged hV2R (mycV2R) was then subcloned into a pcDNA3 vector in which the cytomegalovirus promoter had been replaced by the Rous sarcoma virus (RSV) promoter. The V2R mutant lacking the palmitoylation sites cysteine 341 and cysteine 342 (C341A/C342A-V2R) was constructed by PCR site-directed mutagenesis of the cysteines into alanines using the wild-type pcDNA3-RSV-mycV2R.

V2R-GFP and C341A/C342A-V2R-GFP—The coding sequence of the green fluorescent protein variant GFP10 (9) was inserted in-frame 3′ of the wild-type V2R coding sequence into pcDNA3.1-V2R so that a 14-amino acid linker (GSGTAGPGSPPVAT) separated the carboxyl tail of the V2R and the initiator methionine of GFP10. The palmitoylation sites cysteine 341 and cysteine 342 were then replaced by alanine residues by PCR site-directed mutagenesis to generate the pcDNA3.1-C341A/C342A-V2R-GFP.

β-Arrestin Constructs—For the β-arrestin-*R*luc, the rat β-arrestin2 (arrestin3) coding sequence was inserted in-frame 5′ of the *Renilla* luciferase sequence in the p*R*luc vector (PerkinElmer Life Sciences) so that a 7-amino acid linker (GAGALAT) separated the carboxyl terminus of β-arrestin and the initiator methionine of *R*luc. For the β-arrestin-GFP, the rat β-arrestin2 coding sequence was subcloned in-frame 5′ of the GFP10 coding sequence within the pcDNA3.1-V2R-GFP (see above) after removing the V2R coding sequence. This led to a construct in

which the carboxyl terminus of the β-arrestin was separated from the initiator methionine of GFP10 by a 6-amino acid linker (GSGTGS). β-Arrestin (319–418) and β-arrestin V53D (10) were generously provided by J. Benovic (Thomas Jefferson University, Philadelphia).

All constructs were confirmed by sequencing.

Cell Culture and Transfections

Human embryonic kidney 293 cells (HEK293) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine, 0.1 unit/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml Fungizone. Stable transfections of myc-tagged wt- or C341A/C342A-V2R were performed using the calcium phosphate precipitation method, and neomycin-resistant cells were selected in the presence of 450 μg/ml G418. Resistant clones were screened for V2R expression by radioligand binding assay. In cases where receptors and accessory proteins needed to be overexpressed, transient transfections were made, and cells were harvested 48 h after transfection. In bioluminescence resonance energy transfer (BRET) assays, the calcium phosphate precipitation method (11) was used, whereas FuGENE 6 transfection reagent (Roche Applied Science) was utilized, according to the manufacturer's protocol, in all other cases.

[³H]AVP Radioligand Binding Assay

Radioligand binding assays were carried out in both whole cell and purified membrane preparations using [^{3}H]AVP (PerkinElmer Life Sciences) as radioligand. In the case of membrane binding, cells were washed twice with cold PBS, lysed in 15 mM Tris-HCl, 2 mM MgCl$_2$, 0.3 mM EDTA, pH 7.4, using a Polytron (three times for 5 s). The supernatant resulting from a 5-min 200 × g centrifugation was recentrifuged at 40,000 × g for 20 min. The pelleted membranes were washed once in 50 mM Tris-Cl, 5 mM MgCl$_2$, pH 7.4, and a binding assay was carried out in the same buffer. 15 μg of membrane proteins were incubated for 30 min at room temperature in the presence of increasing concentrations of [^{3}H]AVP (0.1–40 nM) and 1 mg/ml BSA, in a total volume of 300 μl. Nonspecific binding was determined in the presence of 10 μM cold AVP. For whole cell binding, cells were detached from 100-mm dishes using 5 mM EDTA in PBS. Cells (40 μg of cell proteins) were then resuspended in ice-cold PBS and the radioligand binding initiated by adding a saturating concentration of [^{3}H]AVP (20 nM) in the presence or absence 10 μM cold AVP for 2 h at 4 °C. In both cases, the reaction was stopped by rapid filtration over glass fiber (GF/C) filters (Whatman) and bound radioligand detected by scintillation counting.

[³H]Palmitic Acid Labeling and V2R Purification

Cells were grown in 100-mm dishes, preincubated for 1 h in serum-free medium, and labeled with 0.4 mCi/ml [^{3}H]palmitic acid for 2 h at 37 °C. Where indicated, cells were also pretreated for 16 h in the presence of 100 μM 2-bromopalmitic acid (2-BrP) (Fluka Chemie) or 4 h with 30 μM tunicamycin at 37 °C. After metabolic labeling, V2Rs were purified by immunoprecipitation using the AS435 antibody raised against the carboxyl-terminal portion of the human V2R (peptide ARG29, ARGRTPPSLGPQDESCTTASSSLAKDTSS). The selectivity of the antibody was confirmed by the fact that a band was identified only in cells transfected with the V2R and that the myc-tagged V2R was recognized by both AS435 and the anti-myc 9E10 antibodies. Labeled cells were washed twice with cold PBS and lysed by sonication (twice for 15 s) in 25 mM Tris-HCl, 2 mM EDTA, pH 7.4, in the presence of protease inhibitors (10 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin) and 5 mM *N*-ethylmaleimide at 4 °C. The lysate was centrifuged for 5 min at 500 × g and then 20 min at 40,000 × g at 4 °C. The recovered membranes were then washed once in the same buffer and solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, with protease inhibitors and *N*-ethylmaleimide) for 1 h at 4 °C under gentle agitation. The solution was then centrifuged 1 h at 145,000 × g at 4 °C to get rid of insoluble material, and the supernatant was preincubated with a suspension of *Staphylococcus aureus* (Pansorbin; Calbiochem) for 30 min at 4 °C before incubation with 5 μl of AS435 antibody in the presence of 1% BSA for 16 h at 4 °C. Antigen-antibody complexes were then isolated by incubation with 40 μl of Pansorbin for 2 h at 4 °C followed by centrifugation at 4,000 × g for 2 min. Precipitates were washed five times in RIPA buffer and proteins eluted in 50 μl of SDS-PAGE loading buffer (125 mM Tris-HCl, pH 6.5, 4% SDS, 1 M urea, 5% glycerol, 0.1% bromphenol blue). Proteins were then resolved on SDS-PAGE in nonreducing conditions and transferred to nitrocellulose membranes. Tritium-labeled proteins were detected using EA-Wax (EABiotech Ltd., Interscience) and exposing the membranes to Kodak

X-Omat film for 1 week. Detection of the receptors by immunoblots was performed using the anti-myc monoclonal 9E10 IgG as a primary antibody followed by an anti-mouse horseradish peroxidase-conjugated IgG (Amersham Biosciences) for chemiluminescence detection.

Intracellular cAMP Accumulation Measurement

Cells were grown in 6-well plates and incubated in the presence of 2 μCi/ml [³H]adenine in complete Dulbecco's modified Eagle's medium for 16 h. They were then washed twice with PBS containing 1 mM isobutylmethylxanthine before being incubated in the presence of increasing concentration of AVP for 15 min at 37 °C. The reaction was stopped by adding 1 ml of ice-cold 5% trichloroacetic acid and 1 mM unlabeled cAMP to decrease enzymatic degradation of [³H]cAMP. The cells were scraped off the plates and centrifuged at $800 \times g$ for 20 min at 4 °C to clear the lysate. The [³H]cAMP was then separated by sequential chromatography over Dowex and Alumina columns as described previously (56). The cAMP accumulation was calculated as ([³H]cAMP cpm/([³H]cAMP cpm + [³H]ATP cpm)) × 1,000 and expressed as a percentage of the maximal AVP-stimulated cAMP production for the wtV2R.

ERK1/2 Phosphorylation and Immunoblots

Cells were grown in 6-well plates and rendered quiescent by serum starvation for 24 h prior to stimulation with 10% FBS or different concentrations of AVP for the indicated time. Cells were then placed on ice, washed twice with ice-cold PBS, and solubilized directly in 100 μl of Laemmli sample buffer containing 50 mM dithiothreitol. The samples were sonicated for 10 s, heated for 5 min at 95 °C, and microcentrifuged 5 min before fractionation of the proteins on SDS-PAGE. ERK1/2 phosphorylation was detected by protein immunoblotting using P-ERK1/2-specific antibodies and horseradish peroxidase-conjugated IgG as secondary antibody for chemiluminescence detection. After quantification of phosphorylation by autoradiography, nitrocellulose membranes were stripped of immunoglobulins and reprobed using anti-ERK1/2. ERK1/2 phosphorylation was normalized according to the loading of proteins by expressing the data as a ratio of P-ERK1/2 over total ERK1/2.

Receptor Internalization Assay

Receptor internalization was measured as described previously (12). Cells were grown in 24-well plates and washed twice with 0.2 M Hepes in Dulbecco's modified Eagle's medium prior to incubation with AVP for the indicated time. Stimulations were stopped on ice, and cells were washed twice with cold PBS and blocked for 30 min with 1% BSA in PBS before being incubated for 1 h with the anti-myc 9E10 antibody. Cells were then washed three times with 1% BSA in PBS and fixed for 15 min at room temperature with 3% paraformaldehyde in PBS. They were then washed twice with PBS, reblocked for 15 min, and incubated with an anti-mouse horseradish peroxidase antibody. Antibody binding was then visualized after three additional washes by adding 0.5 ml of OPD peroxidase substrate diluted in PBS. Reactions were stopped by adding 0.1 ml of 3 N HCl, and the absorbance of the samples was read at 492 nm in a spectrophotometer.

BRET

The BRET between the V2R-GFP and β-arrestin2-*R*luc was measured as described previously (13). Briefly, 48 h post-transfection, cells were detached and washed twice with PBS at room temperature. Cells (40 μg of proteins) were then distributed in a 96-well microplate (white Optiplate from Packard Bioscience) and incubated in the presence or absence of 1 μM AVP for the indicated time. DeepBlueC™ coelenterazine (Packard Bioscience) was added at a final concentration of 5 μM, and readings were collected using a modified top count apparatus (BRETCount) that allows the sequential integration of the signals detected in the 370–450 and 500–530 nm windows using filters with the appropriate band pass (Chroma). The BRET signal was determined by calculating the ratio of the light emitted by the receptor-GFP (500–530 nm) to the light emitted by the β-arrestin2-*R*luc (370–450 nm). The values were corrected by subtracting the background signal detected when the β-arrestin2-*R*luc construct was expressed alone. Where indicated, cells were pretreated for 16 h in the presence of 100 μM 2-BrP or 4 h with 30 μM tunicamycin at 37 °C before being detached.

β-Arrestin2-GFP Coimmunoprecipitation

Coimmunoprecipitation of covalently cross-linked β-arrestin to V2Rs was performed as described previously (14). Cells were transiently transfected with β-arrestin2-GFP and myc-tagged wt- or C341A/C342A-V2R. 48 h post-transfection, cells were incubated with or without 1 μM AVP in PBS for 15 min at 37 °C, and stimulations were terminated by the addition of the membrane-permeable and reversible cross-linking agent DSP at a final concentration of 2 mM. Cells were then incubated for 30 min at room temperature under gentle agitation; washed twice with 50 mM Tris-HCl, pH 7.4 in PBS to neutralize unreacted DSP; lysed in 0.5 ml of 50 mM Hepes, 50 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 2 mM EDTA, 100 μM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and *N*-ethylmaleimide; and clarified by centrifugation. 25-μl aliquots of whole cell lysate was removed and mixed with an equal volume of 2× reducing loading buffer (with dithiothreitol at a final concentration of 50 mM). To isolate V2R-bound β-arrestin2-GFP, BSA was added to each lysate to a final concentration of 1%, and immunoprecipitation was performed for 16 h at 4 °C using the anti-myc monoclonal 9E10 antibody precoated on protein G-Sepharose beads (Amersham Biosciences). Immune complexes were washed three times with glycerol lysis buffer and eluted in 1× reducing loading buffer 15 min at 45 °C. Proteins were resolved on SDS-PAGE and transferred to nitrocellulose for immunoblotting. Immunoblotting of β-arrestin2-GFP was performed using rabbit polyclonal anti-GFP IgG (Clontech), and immunoblotting of mycV2Rs was performed using rabbit polyclonal anti-myc A14 IgG. Immune complexes were then visualized by chemiluminescence detection using anti-rabbit horseradish peroxidase-conjugated IgG.

Receptor Phosphorylation Assay

Cells were grown in 100-mm dishes, preincubated for 1 h in 0.2 M Hepes in phosphate-free medium, and labeled with 0.5 mCi/ml ³²Pᵢ for 2 h at 37 °C with or without 1 μM AVP for the last 15 min. Labeled cells were washed twice with cold PBS and incubated in RIPA buffer for 1 h under gentle agitation. The solution was then centrifuged for 1 h at $145,000 \times g$ at 4 °C to get rid of insoluble material, and the V2Rs were purified by overnight immunoprecipitation at 4 °C using the anti-myc monoclonal 9E10 antibody precoated on protein G-Sepharose beads. Immune complexes were washed three times with RIPA buffer and eluted in 1× loading buffer 15 min at 45 °C. Proteins were then resolved on SDS-PAGE and transferred to nitrocellulose membranes where ³²P-labeled proteins were detected using the Molecular Imager® FX PhosphorImager (Bio-Rad). Detection of the receptors by immunoblots was performed using rabbit polyclonal anti-myc A14 IgG. Immune complexes were then visualized by chemiluminescence detection using anti-rabbit horseradish peroxidase-conjugated IgG.

Protein Determination

Protein concentrations were determined using either the Bio-Rad D_C Protein Assay or the Bradford quantification assay using BSA as standard.

Data Analysis

Dose-response curves and saturation experiments were analyzed by nonlinear regression using Prism (GraphPad Software), and the EC₅₀ values were derived from the curves. Affinity constant (K_d) and maximal binding (B_{max}) values of the radioligand were derived from the curve fitting. Immunoreactivities were determined by densitometric analysis of the films using NIH Image software. The extent of receptor phosphorylation was determined by digital analysis of the images generated by the PhosphorImager using the Quantity One software (Bio-Rad). The MAPK activation, internalization, and β-arrestin recruitment rates were evaluated by analyzing the linear portion of the curves with the following rate equation

$$q(t) = q(t \to \infty) + q(t = 0)e^{(-R)t} \qquad \text{(Eq. 1)}$$

where t is the time of incubation (in min), R is the rate, and q represents the level of MAPK activation, internalized receptors, or β-arrestin recruitment. The data in the initial part of the curves were plotted as in Equation 2.

$$f(t) = \ln(q(t)/q(0)) \qquad \text{(Eq. 2)}$$

The half-times were estimated as t where $q(t) = 50\%$ of the maximal effect in each data set. Statistical significances of the differences were carried out using unpaired Student's t test. $p < 0.05$ was considered statistically significant.

RESULTS

Expression and Palmitoylation State of Wild-type and Mutant V2Rs—To study the role of palmitoylation of the V2Rs, HEK293 cells were stably transfected with myc-tagged wtV2Rs
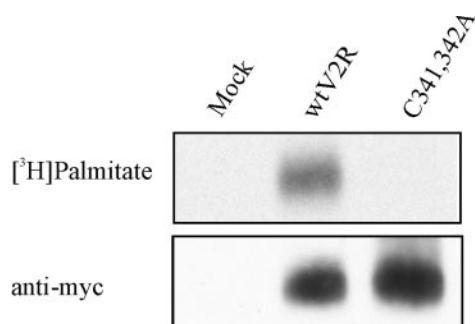
FIG. 1. **[³H]palmitate incorporation into wt- and C341A/C342A-V2R.** HEK293 cells stably expressing equivalent numbers of myc-wtV2R or myc-C341A/C342A-V2R (*C341,342A*) were metabolically labeled with [³H]palmitate and the receptor purified by immunoprecipitation using the anti-V2R antibody (polyclonal antibody AS435) and resolved on SDS-PAGE. [³H]Palmitate incorporation was revealed by autoradiography after transfer to nitrocellulose. The expression level of each receptor was assessed by Western blot analysis of the same samples using the 9E10 anti-myc antibody. Immunoprecipitation using untransfected HEK293 cells was carried out as a control (*Mock*). The data shown are representative of three experiments.

or mutant receptors in which the potential palmitoylation sites (cysteines 341 and 342) were replaced by alanines (C341A/C342A-V2R). Clonal cell lines expressing similar and physiological levels (wtV2R, 350 ± 20 fmol/mg; C341A/C342A, 290 ± 20 fmol/mg) of V2Rs as determined by [³H]AVP binding were selected and used in the various assays. Saturation binding experiments performed on isolated membranes of these clones confirmed, as reported previously (5), that the replacement of cysteines 341 and 342 by alanines does not affect the affinity of the receptor for AVP (K_d = wtV2R, 0.8 ± 0.1 nM *versus* C341A/C342A, 0.7 ± 0.1 nM).

The palmitoylation state of the V2Rs was then verified by metabolic labeling of the cells with [³H]palmitic acid followed by immunoprecipitation of the receptors as described under "Experimental Procedures." Fig. 1 shows that mutation of cysteines 341 and 342 in the V2R greatly reduced the level of tritium incorporation in the receptor, confirming that these cysteines represent the major palmitoylation sites (4).

Role of V2R Palmitoylation in Intracellular Signaling—To assess the importance of palmitoylation in receptor signaling, we investigated the effect of mutating cysteines 341 and 342 on the ability of the V2R to activate adenylyl cyclase and MAPK pathways. As reported previously (4, 5), replacing cysteines 341 and 342 with alanine residues did not affect AVP-stimulated adenylyl cyclase activity. Indeed, neither the efficacy nor the potency of AVP for this signaling pathway was affected by the mutation in HEK293 cells stably expressing comparable amounts of receptors (Fig. 2). In contrast, significant differences in the pattern of the ERK1/2 MAPK stimulation were observed between wt- and C341A/C342A-V2R (Fig. 3). Although an AVP-mediated increase in ERK1/2 activity was observed with both receptors, as assessed by immunoblotting using phospho-specific ERK1/2 antibodies, a delayed and reduced activation was found for the palmitoylation-less mutant (Fig. 3*A*). However, AVP was found to promote a more sustained ERK1/2 activation through the C341A/C342A-V2R. Quantitative assessment of these differences using total ERK1/2 immunoreactivity to normalize the phosphorylation levels is shown in Fig. 3*B*. The maximal level of wtV2R-mediated ERK1/2 phosphorylation was reached as early as 2 min after the addition of AVP and returned to basal level after 10 min, whereas the C341A/C342A-V2R-mediated phosphorylation of the kinases peaked after ~4 min and only returned to basal level after 20 min. Kinetic analysis of the initial ERK1/2 activation curves revealed a significantly ($p < 0.05$) reduced
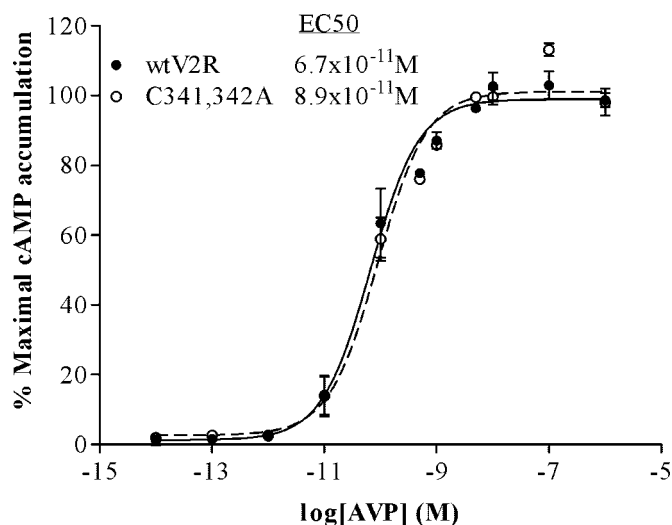

FIG. 2. **AVP-stimulated cAMP accumulation in HEK293 cells stably expressing wt- or C341A/C342A-V2R.** [³H]cAMP accumulation was assessed in whole cells prelabeled with [³H]adenine and treated with increasing concentrations of AVP for 10 min at 37 °C. cAMP production was calculated as ([³H]cAMP dpm/[³H]ATP dpm + [³H]cAMP dpm) $\times$ 1,000 and expressed as percentage of the maximal accumulation observed for the wild-type receptor. EC$_{50}$, concentration of AVP producing half-maximal cAMP accumulation. The *curves* shown represent the mean $\pm$ S.D. of two independent experiments.

rate of C341A/C342A-mediated kinase activation compared with wtV2R (Fig. 3*C*; $t_{1/2}$: wtV2R, 0.7 ± 0.1 min *versus* C341A/C342A, 1.1 ± 0.1 min). It thus appears that the cellular response leading to ERK1/2 activation develops more slowly through the C341A/C342A-V2R than with the wild-type receptor.

To characterize further the differences between wt- and C341A/C342A-V2R-mediated ERK1/2 activation, AVP dose-response curves were carried out for stimulation time corresponding to the peak activation of each receptor (*i.e.* 2 min for wtV2R and 4 min for C341A/C342A; Fig. 4*A*). Consistent with the lack of effect of the mutation on the affinity of the receptor for AVP, no difference in the EC$_{50}$ was observed (Fig. 4*B*). However, the maximal response mediated by C341A/C342A-V2R was lower than that of the wild-type receptor by ~35%. Taken together, these results suggest that the V2R-linked MAPK pathway, but not the adenylyl cyclase one, is affected by the palmitoylation state of the receptor.

Internalization of V2Rs—We then examined the potential role of palmitoylation on AVP-promoted V2R endocytosis by assessing the influence of cysteine 341 and 342 mutations on receptor internalization. For this purpose, the effect of AVP on cell surface receptor expression was measured by enzyme-linked immunosorbent assay using an anti-myc antibody detecting the amino-terminally myc-tagged cell surface receptors. Internalization was determined as the AVP-promoted decrease in cell surface imunoreactivity. As shown in Fig. 5*A*, the extent of endocytosis was reduced significantly for the palmitoylation-less mutant, reaching only $69 \pm 4\%$ compared with $87 \pm 3\%$ for the wild-type receptor. More importantly, C341A/C342A-V2R was found to undergo AVP-promoted internalization at a significantly ($p < 0.01$) reduced rate compared with the wild-type receptor (Fig 5*B*; $t_{1/2}$: wtV2R, 5.4 ± 0.5 min *versus* C341A/C342A-V2R, 9.1 ± 0.5 min). It follows that, as was found for the AVP-stimulated ERK1/2 activity, both the kinetic and the extent of V2R endocytosis are modulated by the receptor palmitoylation state.

β-Arrestin-mediated ERK1/2 Activation—Because both endocytosis and MAPK activation were found to be decreased and
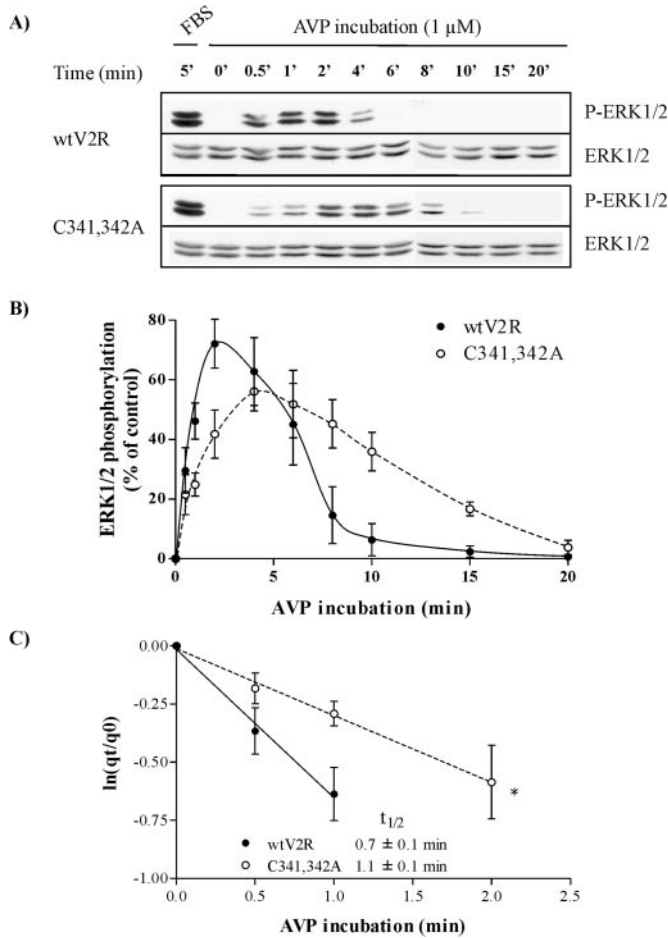
A)

B)

C)

FIG. 3. **Time course of AVP-induced ERK1/2 activity.** *A*, HEK293 cells stably expressing myc-wtV2R or myc-C341A/C342A-V2R (*C341,342A*) were stimulated with 1 μM AVP or 10% FBS for the indicated times. MAPK activity was then detected using phospho-specific anti-ERK1/2 antibodies (*P-ERK1/2*). Levels of the MAPK were controlled using antibodies directed against the total kinase population (*ERK1/2*). *B*, graphic representation of the data expressed as P-ERK1/2 over ERK1/2 in percentage of the levels observed with 10% FBS used as the reference control. The *curves* shown represent the mean ± S.E. of at least four independent experiments. *C*, rate of ERK1/2 phosphorylation determined using the initial portion of the curves presented in *B*. *, indicates $p < 0.05$ between the half-times.

delayed by mutating cysteines 341 and 342, and given the proposed linked between endocytosis and MAPK activation (1, 15), we then investigated the potential implication of V2R endocytosis in the AVP-stimulated ERK1/2 activation. For this, the effect of a pharmacological blocker (concanavalin A, a lectin that interferes with endocytosis by binding to the carbohydrate moieties (16)) and of two dominant negative mutants of β-arrestin with impaired receptor binding capacities (a truncated form, β-arrestin (319–418) (10), and a point mutant, β-arrestin V53D (10, 17)) were assessed. As shown in Fig. 6*A*, the concanavalin A treatment almost completely blocked endocytosis of both wt- and C341A/C342A-V2R, whereas β-arrestin (319–418) and β-arrestin V53D inhibited the internalization of these receptors by 70 and 35%, respectively. These results confirm previous data indicating that V2R undergoes agonist-promoted endocytosis via a β-arrestin-dependent process presumably through clathrin-coated vesicles (18, 19). In contrast to its dramatic effect on receptor endocytosis, concanavalin A was without effect on the AVP-stimulated MAPK activation in cells expressing either wt- or C341A/C342A-V2R (Fig. 6*B*), clearly separating the two phenomena. However, both β-arrestin (319–418) and β-arrestin V53D significantly inhibited re-
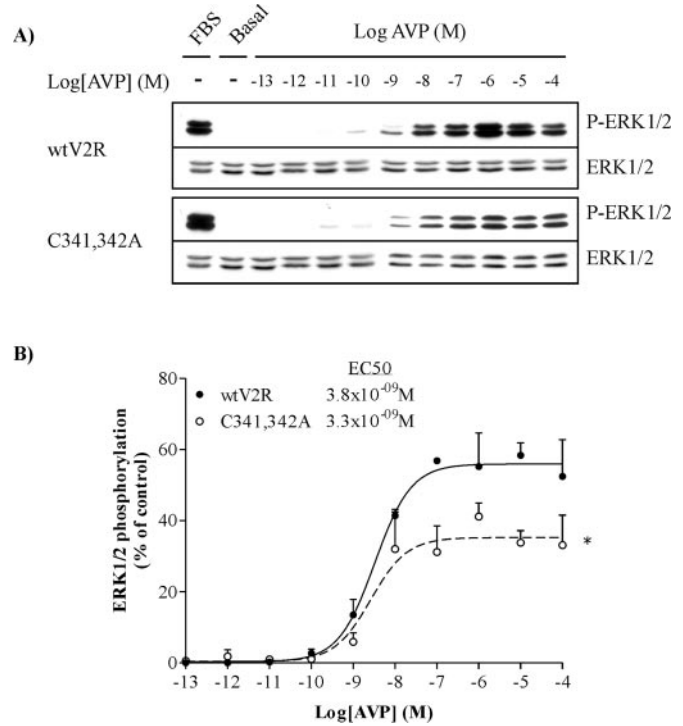
A)

B)

FIG. 4. **Concentration-dependent AVP-induced ERK1/2 activity.** *A*, HEK293 cells stably expressing myc-wtV2R or myc-C341A/C342A-V2R (*C341,342A*) were stimulated with increasing concentrations of AVP for 2 (wtV2R) or 4 min (C341A/C342A) at 37 °C. Reference stimulation was carried out using 10% FBS for 5 min. The MAPK activity was assessed as in Fig. 3. *B*, graphic representation of the data expressed as P-ERK1/2 over ERK1/2 in percentage of the levels observed with 10% FBS used as the reference control. The *curves* shown represent the mean ± S.E. of four independent experiments. *, indicates $p < 0.01$ between the asymptotes of the two *curves*.

ceptor-mediated ERK1/2 phosphorylation, suggesting an important role for β-arrestin in the AVP-stimulated ERK1/2 activation (Fig. 6*B*).

AVP-induced β-Arrestin Recruitment—The preceding data indicate that no causal link exists between V2R endocytosis and MAPK activation. However, they clearly place β-arrestin on the path of two processes affected by the palmitoylation state of the receptor. To test directly whether the decreased receptor endocytosis and reduced AVP-stimulated ERK1/2 activity of C341A/C342A-V2R could result from an altered β-arrestin interaction, the ability of AVP to promote β-arrestin recruitment to the wt- and C341A/C342A-V2R was first investigated in coimmunoprecipitation studies using the reversible membrane-permeable cross-linker DSP. As shown in Fig. 7*A*, β-arrestin2-GFP could be coimmunoprecipitated with both wt- and C341A/C342A-myc-tagged V2R. Although a single specific β-arrestin2-GFP band was observed in the whole cell lysates, the coimmunoprecipitated β-arrestin2-GFP appeared as a doublet including a less abundant species with a slower mobility. The nature of this β-arrestin species that is revealed in the coimmunoprecipitation conditions remains to be determined. In any case, AVP induced a significant increase of β-arrestin2-GFP in the myc-tagged receptor immunoprecipitates, reflecting the agonist-promoted recruitment of β-arrestin to both wt- and C341A/C342A-V2R after agonist exposure. Interestingly, however, less β-arrestin2-GFP was found to be associated with C341A/C342A-V2R (Fig. 7*B*), suggesting an altered interaction between β-arrestin and the V2R palmitoylation-less mutant.

To characterize further β-arrestin interaction with the wt- and C341A/C342A-V2R, we assessed the AVP-promoted β-arrestin recruitment to the receptors using a BRET-based assay
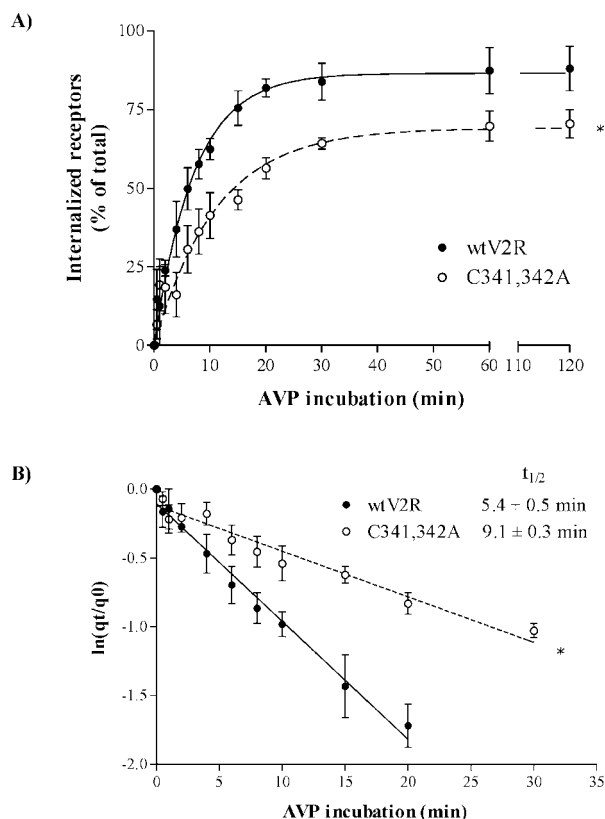
FIG. 5. **AVP-induced internalization of wt- and C341A/C342A-V2R.** HEK293 cells stably expressing myc-wtV2R or myc-C341A/C342A-V2R (*C341,342A*) were stimulated with 1 μM AVP for different periods of time at 37 °C. Cell surface receptor levels were measured by enzyme-linked immunosorbent assay using the monoclonal anti-myc 9E10 and anti-mouse horseradish peroxidase-conjugated antibodies. The immunoreactivity was revealed by colorimetry. *A*, internalization is defined as the loss of cell surface immunoreactivity and is expressed as percent of the total immunoreactivity measured under basal conditions. The data shown represent the mean ± S.D. of two independent experiments. *, indicates $p < 0.01$ between the asymptotes of the two *curves*. *B*, rate of receptor internalization determined using the initial portion of the *curves* presented in *A*. *, indicates $p < 0.01$ between the half-times.

(13). For this purpose, β-arrestin-*R*luc and wt- or C341A/C342A-V2R-GFP fusion proteins were transiently expressed in HEK293 cells. The AVP-promoted β-arrestin recruitment was then assessed by determining the agonist-dependent transfer of energy between β-arrestin-*R*luc and the receptor-GFP constructs. As shown in Fig. 8*A*, real time BRET measurements allow monitoring of time-dependent recruitment of β-arrestin to both wt- and C341A/C342A-V2R. The analysis of these kinetics revealed a significant ($p < 0.01$) decrease in the recruitment rate of β-arrestin by the C341A/C342A-V2R compared with that of the wild-type receptor (Fig. 8*B*; $t_{1/2}$: wtV2R, 3.0 ± 0.3 min *versus* C341A/C342A, 8.6 ± 0.5 min). Taken together, these results strongly suggest that mutation of the V2R palmitoylation sites significantly reduces its affinity for β-arrestin.

To confirm that the reduced interaction of β-arrestin with the C341A/C342A-V2R resulted from the lack of palmitoylation at cysteines 341 and 342 because of their mutation to alanines, we assessed the effect of the palmitoylation inhibitors 2-BrP (20) and tunicamycin (21) in the BRET-based β-arrestin recruitment assay. First, the ability of 2-BrP and tunicamycin to inhibit V2R palmitoylation was verified in metabolic labeling experiments. As shown in Fig. 9*A*, pretreatment of the cells with 2-BrP or tunicamycin greatly reduced [³H]palmitic acid incorporation in the V2R without significantly affecting receptor expression levels, as assessed by Western blot analysis.
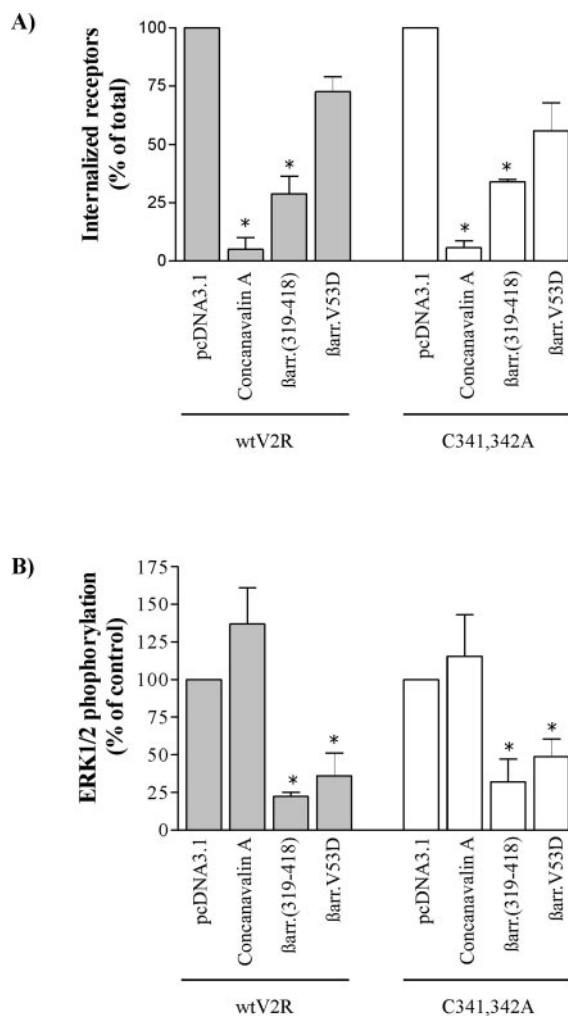

FIG. 6. **Role of V2R endocytosis and β-arrestin in the AVP-stimulated ERK1/2 activity.** HEK293 cells were transiently cotransfected with plasmids encoding myc-wtV2R or myc-C341A/C342A-V2R (*C341,342A*) along with the pcDNA3.1 plasmid either empty (negative control) or encoding dominant negative mutants of β-arrestin (βarr. (319–418) and βarr.V53D). Where indicated, cells were preincubated with 0.25 mg/ml concanavalin A for 1 h at 37 °C. *A*, cells were treated or not with 1 μM AVP for 30 min at 37 °C and receptor internalization measured as in Fig. 5. Data represent the mean ± S.E. of at least three independent experiments. *B*, quantification of ERK1/2 phosphorylation stimulated by 1 μM AVP for 2 (wtV2R) or 4 min (C341A/C342A) was carried out as in Fig. 3. The data shown represent the mean ± S.E. of at least three independent experiments. *, indicate $p < 0.05$ between treatment and control condition.

Inhibition of V2R palmitoylation by 2-BrP or tunicamycin decreased the rate of AVP-promoted β-arrestin recruitment in a manner similar to that observed with the C341A/C342A-V2R (Fig. 9*B*; $t_{1/2}$: wtV2R, 3.1 ± 0.6 min; C341A/C342A, 5.6 ± 0.6 min; wtV2R + 2-BrP, 5.0 ± 0.6 min; and wtV2R + tunicamycin, 5.5 ± 0.7 min). In addition, we did not observe any effect of 2-BrP or tunicamycin on the AVP-promoted β-arrestin recruitment to the C341A/C342A-V2R (data not shown), suggesting that the effects observed are specifically the result of palmitoylation inhibition.

Phosphorylation of V2Rs—Given the importance of receptor phosphorylation in the interaction between β-arrestin and GPCRs (22, 23), it could be hypothesized that palmitoylation regulates β-arrestin recruitment by affecting the receptor phosphorylation state. To test this hypothesis directly, basal and AVP-stimulated phosphorylation were assessed for wt- and C341A/C342A-V2R. Following metabolic labeling with ³²P$_i$, cells expressing either wt- or C341A/C342A-V2R were stimu-
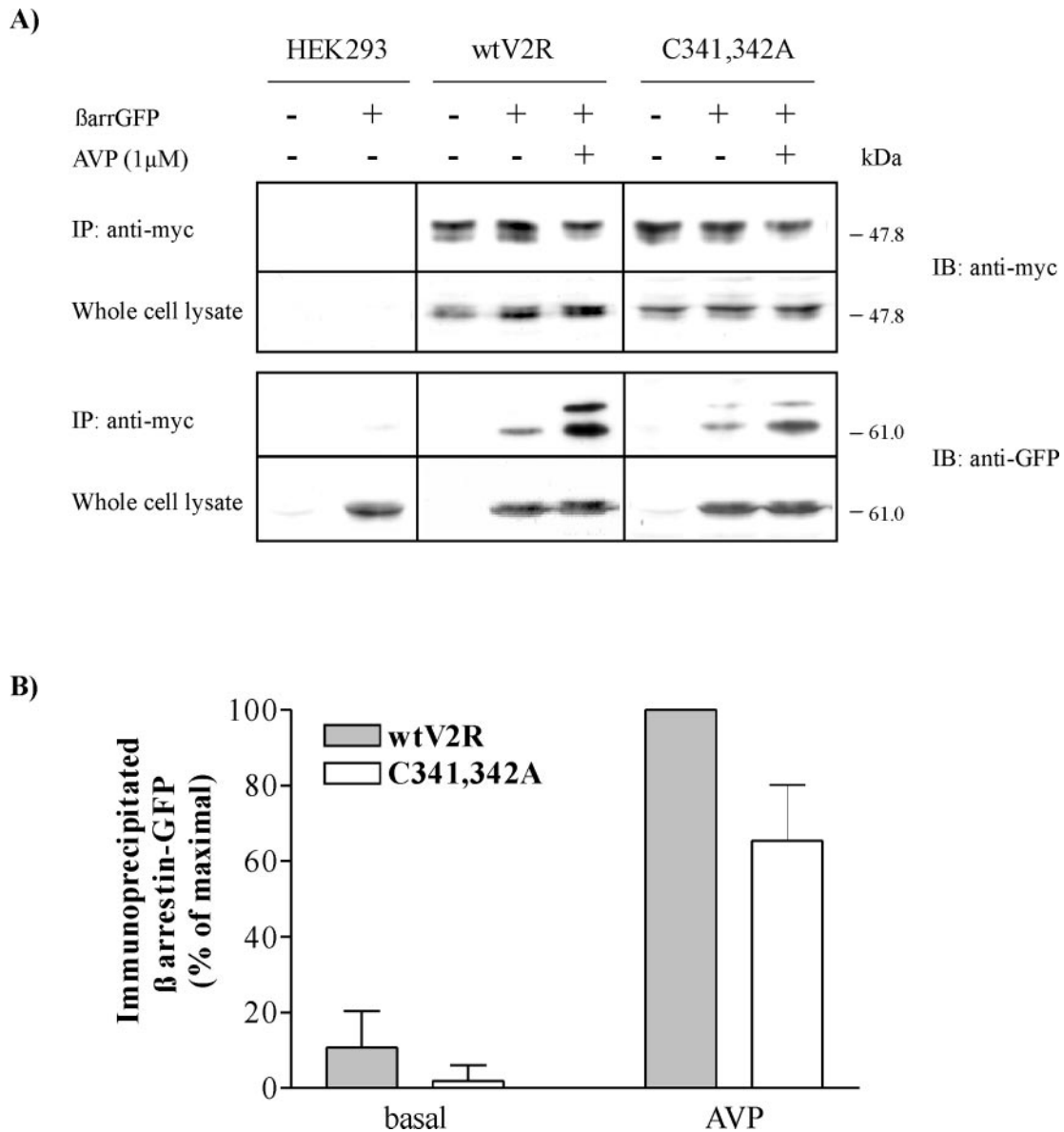
FIG. 7. **β-Arrestin coimmunoprecipitation with agonist-activated V2Rs.** Plasmids encoding myc-wtV2R or myc-C341A/C342A-V2R (*C341,342A*) were transiently cotransfected with β-arrestin2-GFP (*βarrGFP*) in HEK293 cells. Cells were treated or not with 1 μM AVP for 15 min at 37 °C before adding the cross-linking agent DSP. Myc-tagged receptors were then immunoprecipitated using the 9E10 anti-myc antibody and resolved on SDS-PAGE as described under "Experimental Procedures." *A*, the amount of myc-V2Rs and the presence of β-arrestin2-GFP in the immunoprecipitates were assessed by Western blot analysis using the A14 anti-myc and anti-GFP polyclonal antibodies, respectively. Blotting of the whole cell lysates was performed to control for the expression levels of myc receptors and β-arrestin2-GFP. *B*, quantification of coimmunoprecipitated β-arrestin2-GFP expressed as the ratio of anti-GFP over anti-myc immunoreactivities in the immunoprecipitates in percentage of the maximal obtained with the wtV2R. The data shown represent the mean ± S.E. of three independent experiments.

lated or not with AVP and the receptors purified by immunoprecipitation as described under "Experimental Procedures." As shown in Fig. 10 and in agreement with the previous report of Sadeghi *et al.* (4), comparable basal and AVP-promoted phosphorylation levels were observed for the wild-type and the palmitoylation-less receptor, excluding a role for phosphorylation in the reduced β-arrestin recruitment to C341A/C342A-V2R.

DISCUSSION

Taken together, the results of the present study demonstrate that the V2R palmitoylation state regulates distinct receptor functions differentially. Although mutation of the palmitoylation sites did not affect the receptor-stimulated adenylyl cyclase activity, it led to significantly delayed and decreased agonist-promoted ERK1/2 activation and receptor endocytosis. No causal link between the reduced MAPK activity and recep-

tor endocytosis was found, but both most likely resulted from the altered agonist-mediated β-arrestin recruitment observed for the palmitoylation-less mutant. Indeed, our results show that lack of palmitoylation of the V2R, either by mutation or pharmacological inhibition, slowed down and decreased its ability to recruit β-arrestin, suggesting a reduced affinity of the receptor for β-arrestin. Receptor palmitoylation can therefore be seen as a post-translational modification that favors β-arrestin-dependent processes.

Modulation of GPCR endocytosis efficiency by palmitoylation has been reported previously for several receptors. Although mutation of palmitoylated cysteines was found to increase the endocytosis of these receptors for the luteinizing hormone/human choriogonadotropin receptor (24) and the V1a vasopressin receptor (V1aR) (25), in most cases, it was found as in the present study to decrease their agonist-promoted endocytosis.
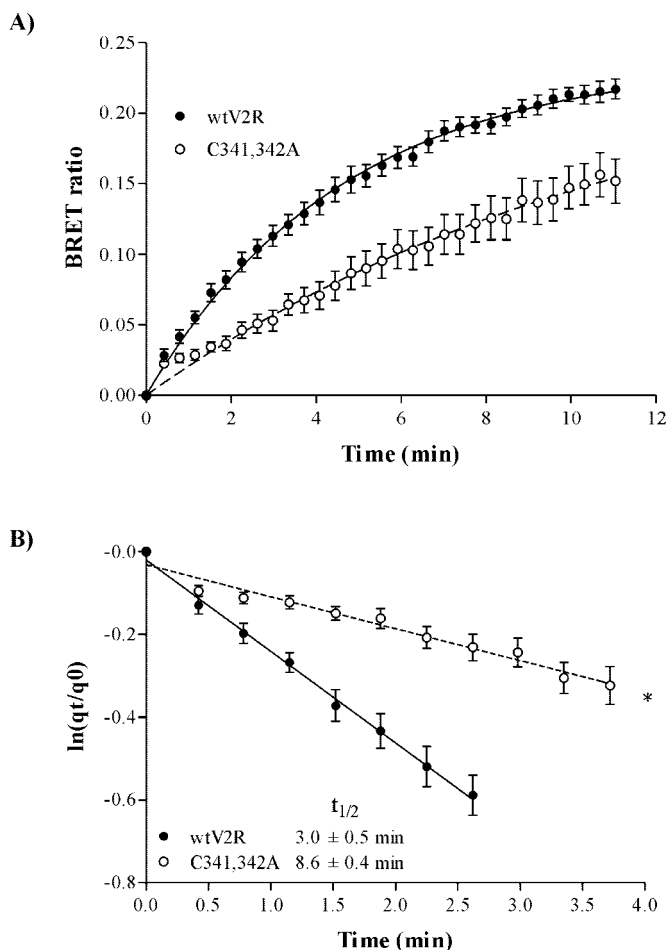
A)

B)

FIG. 8. **β-Arrestin recruitment to agonist-activated V2Rs measured by BRET.** Plasmids encoding wtV2R-GFP or C341A/C342A-GFP (*C341,342A*) were transiently cotransfected with β-arrestin2-*R*luc in HEK293 cells treated or not with 1 μM AVP at 25 °C in the presence of 5 μM coelanterazine. *A*, real time BRET measurements were taken at regular intervals for the indicated times. *B*, rate of β-arrestin recruitment determined using the initial portion of the curves presented in *A*. The data shown represent the mean ± S.E. of at least four independent experiments. * indicates $p < 0.01$ between the half-times.

Examples include the thyrotropin-releasing hormone receptor (26, 27), the somatostatin receptor SSTR5 (28), the CC chemokine receptor CCR5 (29), and the B2 bradykinin receptor (30). Interestingly, apparently opposite regulation of endocytic processes by receptor palmitoylation does not seem to be limited to GPCRs. Indeed, lack of palmitoylation of the transferrin receptor was found to increase transferrin-promoted receptor endocytosis (31), whereas a palmitoylation-less asialoglycoprotein receptor was less prone to ligand-induced receptor internalization (32). The reason for the apparently opposite roles of palmitoylation in the endocytosis of different receptors remains unclear.

Here, the observation that mutation of the V2R palmitoylation sites leads to comparable delays and reduction in both receptor endocytosis and β-arrestin recruitment coupled to the strong dependence of the V2R internalization on β-arrestin strongly suggests that the altered β-arrestin recruitment is responsible for the reduced receptor endocytosis. This is consistent with evidence from fluorescence microscopy suggesting that palmitoylation-deficient thyrotropin-releasing hormone receptor mutants, which showed reduced endocytosis, also failed to promote efficient β-arrestin translocation (27). However, whether modulation of the interaction between receptor and β-arrestin is the universal mechanism underlying the in-

fluence of the palmitoylation state on the receptor endocytosis remains to be investigated. Interestingly, such a mechanism could also apply to non-GPCRs because, in addition to its wide role in GPCR endocytosis, β-arrestin has been proposed to play a role in agonist-promoted internalization of a receptor belonging to the tyrosine kinase receptor family, the insulin-like growth factor I receptor (33).

Although our study provides the first direct evidence linking receptor palmitoylation to β-arrestin recruitment and subsequent MAPK activation, a growing number of studies suggest that, in addition to their function as endocytic adaptor proteins, β-arrestins play a central role in GPCR-mediated MAPK activation (2). The importance of endocytosis in GPCR-mediated MAPK activation has also been the subject of numerous studies. For example, using β-arrestin and dynamin dominant negative inhibitors of internalization, both m1 muscarinic acetylcholine receptor and β_2AR-mediated activation of MAPK was suggested to require clathrin-coated vesicle-mediated endocytosis (15, 34). However, as we found in the present study for the V2R, other receptors were shown to mediate MAPK activation in an internalization-independent manner (1). A potential explanation for this apparent contradiction was provided by subsequent papers. First, the observation that MAPK activation by an internalization-deficient μ-opioid receptor could still be inhibited by a dynamin-negative mutant (35) clearly distinguished the effect of the dynamin-negative mutant on the MAPK activation and the endocytosis of the receptor. Subsequently, α_2AR and β_2AR activation of MAPK was shown to require the transactivation as well as the internalization of the epidermal growth factor receptor (36), indicating that the effect of the dynamin dominant negative mutant resulted from the inhibition of the tyrosine kinase receptor internalization and not that of the GPCR. Although the need for clathrin-mediated endocytosis of targeted tyrosine kinase receptors has not been investigated in the present study, our data clearly show that V2R internalization is not required for AVP-mediated ERK1/2 activation because pharmacological inhibition of receptor endocytosis did not affect the MAPK activation. It follows that the altered ERK1/2 activation observed with the unpalmitoylated V2R cannot result from the reduced endocytosis of this mutant. Rather, our observation that two dominant negative mutants of β-arrestin significantly inhibited V2R-mediated ERK1/2 activation strongly suggests that the altered β-arrestin recruitment of the palmitoylation-less V2R is responsible for its delayed and reduced ability to activate MAPK. In addition, the more prolonged ERK1/2 activation observed with C341A/C342A-V2R could also be a consequence of altered β-arrestin recruitment because this leads to a slower receptor endocytosis that could result in a delayed signal termination. However, although the role of endocytosis in the desensitization of classical receptor-G protein activation is well characterized (1), whether β-arrestin-mediated endocytosis also leads to termination of the MAPK signaling remains to be determined.

Taken together, the above considerations suggests that palmitoylation affects β-arrestin-dependent events such as receptor endocytosis and MAPK activation but not the G_s-mediated adenylyl cyclase activation. Furthermore, given the strong dependence of the V2R-mediated ERK1/2 activation on β-arrestin, it is not surprising to find that the potency of AVP stimulation of MAPK (EC$_{50}$ of wtV2R = 3.8×10^{-09} M) is closest to the affinity of the receptor for the agonist (K_d of wtV2R = 0.8×10^{-09} M) than the potency of AVP-promoted cAMP accumulation (wtV2R = 6.7×10^{-11} M). Indeed, β-arrestin recruitment directly reflects agonist occupancy, whereas the amplification process taking place in the AVP-induced cAMP accumulation is expected to increase the apparent potency. Because the poten-
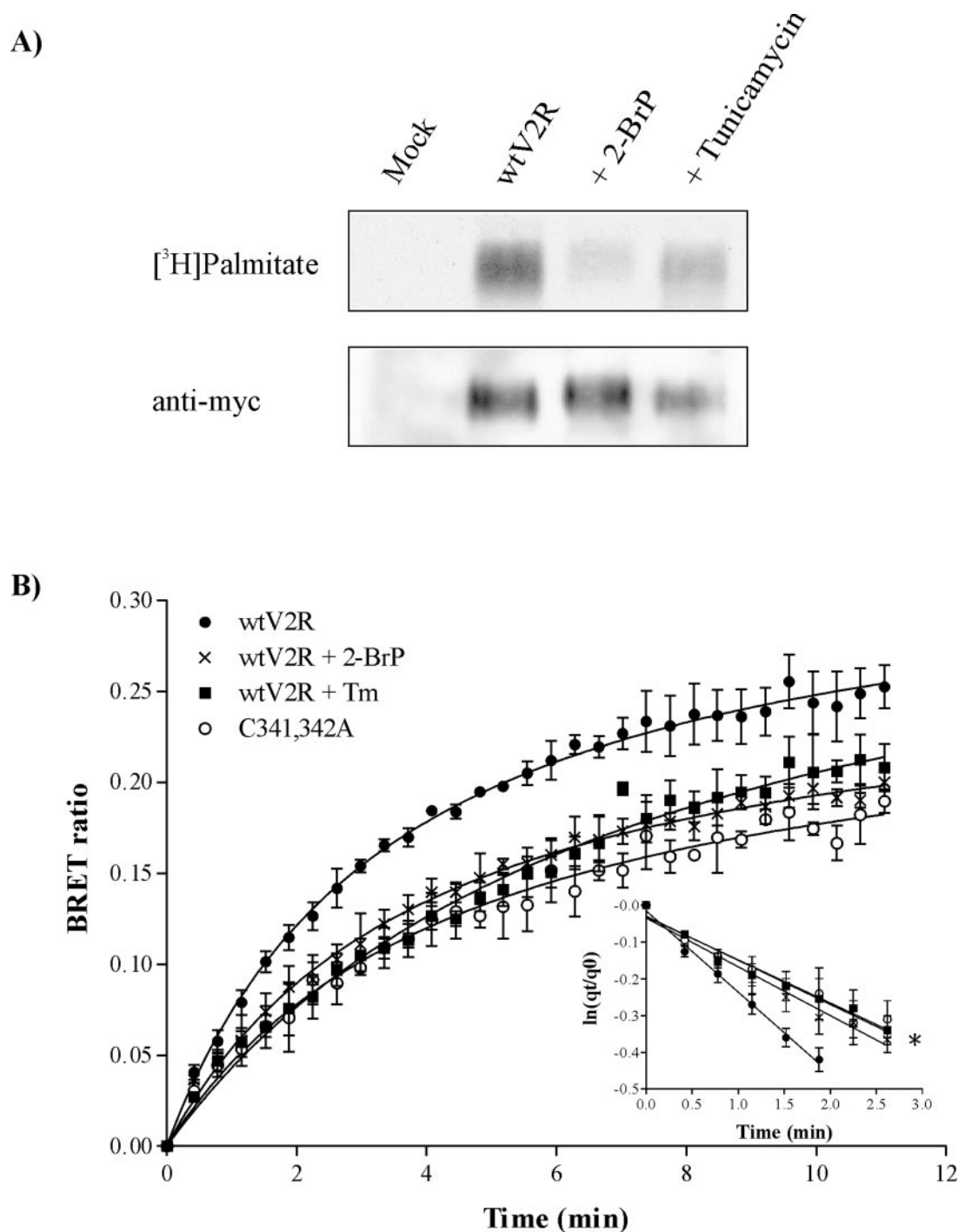
FIG. 9. **Effect of pharmacological inhibition of V2R palmitoylation on β-arrestin recruitment.** *A*, HEK293 cells stably expressing myc-wtV2R were pretreated or not with 100 μM 2-BrP for 16 h or 30 μM tunicamycin for 4 h at 37 °C followed by metabolic labeling with [^{3}H]palmitate as described under "Experimental Procedures." Receptors were then purified by immunoprecipitation and resolved on SDS-PAGE before being analyzed by autoradiography for [^{3}H]palmitate incorporation or Western blotting to control receptor expression. *B*, HEK293 cells were transiently cotransfected with wtV2R-GFP or C341A/C342A-GFP (*C341,342A*) along with β-arrestin2-*R*luc and treated or not with 2-BrP or tunicamycin (*Tm*) as described above, followed by 1 μM AVP at 25 °C in the presence of 5 μM coelanterazine. Real time BRET measurements were taken at regular intervals for the indicated times. *Inset*, rate of β-arrestin recruitment determined using the initial portion of the *curves* presented in *B*. The data shown represent the mean ± S.E. of three independent experiments. *, indicates $p < 0.01$ compared with wtV2R half-time.

cies were both determined in whole cell assays and are thus directly comparable, the difference then suggests that the AVP-stimulated ERK1/2 activation observed in the present study does not depend on cAMP production. This was supported further by the observation that a protein kinase A inhibitor (KT5720) did not affect AVP-promoted ERK1/2 activation.[2]

Other studies have suggested pathway-specific modulation of receptor functions by palmitoylation. For instance, absence of palmitoylation in the endothelin A receptor was shown to prevent its coupling to the phospholipase C and ERK1/2 signaling pathways without affecting the adenylyl cyclase activation (37, 38). In this case, the authors suggested that palmitoylation was required for G_q but not G_s coupling. Similarly, mutation of the three palmitoylated cysteines of CCR5 has

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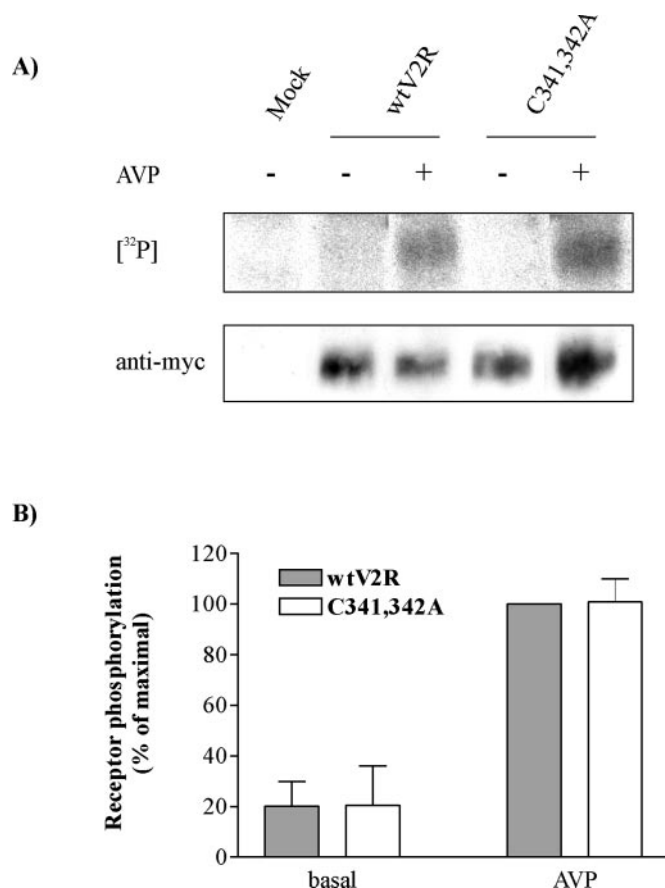[2] P. G. Charest and M. Bouvier, unpublished observations.

A)

B)

FIG. 10. **AVP-induced phosphorylation of wt- and C341A/C342A-V2R.** *A*, HEK293 cells stably expressing myc-wtV2R or myc-C341A/C342A-V2R (*C341,342A*) were metabolically labeled with $^{32}P_i$ and treated or not with 1 μM AVP for 15 min. The receptors were purified by immunoprecipitation using the 9E10 anti-myc antibody and resolved on SDS-PAGE. $^{32}P_i$ incorporation was revealed by autoradiography using a PhosphorImager after transfer to nitrocellulose. The expression level of each receptor was assessed by Western blot analysis of the same membrane using the A14 anti-myc antibody. Immunoprecipitation using untransfected HEK293 cells was carried out as a control (*Mock*). *B*, quantification of receptor phosphorylation is expressed as the ratio of ^{32}P labeling over anti-myc immunoreactivity in percentage of the maximal obtained with the wtV2R. The data shown represent the mean ± S.D. of two independent experiments.

been shown to affect efficient coupling to only a subset of its signaling repertoire (39). However, the potential role of β-arrestin in these pathway-specific effects of palmitoylation was not investigated. These observations and the findings reported herein suggest that different agonist-stimulated processes (such as selective G protein activation, β-arrestin recruitment, and receptor endocytosis) may involve distinct receptor conformations and/or domains that can be regulated differentially. Supporting this idea, a modified parathyroid hormone receptor uncoupled from its cognate G protein can still undergo agonist-induced β-arrestin-mediated endocytosis (40). Hence, because palmitoylation occurs mostly at the level of the carboxyl-terminal tail of GPCR, it will most likely affect processes involving this particular domain. Interestingly, it was shown that for several receptors, including the V2R, the carboxyl-terminal tail is implicated in the formation of stable receptor-β-arrestin interactions (18, 41).

How receptor palmitoylation could affect the coupling to β-arrestin is unknown. However, palmitoylation has been shown to play important roles in protein-protein interaction for a number of proteins. For example, palmitoylation of $G\alpha_s$ has been shown to favor interactions with $G\beta\gamma$ (42), whereas palmi-

toylation of $G\alpha_z$ was found to decrease the affinity of the G_z-GTPase activating protein for its GTP-bound form (43). Palmitoylation of Src family tyrosine kinases has also been shown to be required for their interaction with glycosylphosphatidylinositol-anchored proteins (44) as well as with their B cell substrate Igα (45). Finally, palmitoylation of caveolin-1 was found to be essential for its interaction with c-Src (46) and that of tetraspanin proteins important for their self-association (47).

Binding of β-arrestin to agonist-activated GPCR is thought to involve multiple interactions (48). A large region within the amino-terminal half of β-arrestin, termed the activation recognition domain, recognizes the activated state of GPCRs. This domain of β-arrestin appears to bind the third intracellular loop of several receptors, including the α_2AR, m2 and m3 muscarinic acetylcholine receptors (49). This is followed by the binding of a smaller positively charged region in the central portion of β-arrestin, termed the phosphorylation recognition domain, to the receptor carboxyl tail phosphorylated by GPCR kinase (50). Notably, agonist-induced phosphorylated clusters of serine or threonine residues, located downstream of the putative palmitoylation sites of several GPCR including the V2R, were identified as molecular determinants of the stability of receptor-β-arrestin complexes (18, 41). In fact, the absence of such phosphorylation clusters within the carboxyl tail of several receptors, including the β_2AR, has been invoked to explain the rapid dissociation of β-arrestin from these receptors.

Modulation of the phosphorylation state of the receptor by palmitoylation could thus be proposed as a potential mechanism regulating β-arrestin recruitment. Consistent with this hypothesis, GPCR palmitoylation sites are often located proximally to receptor phosphorylation sites and have been shown to affect GPCR phosphorylation for a number of receptors. For instance, mutation of palmitoylation sites in their carboxyl tail has been linked to an increased phosphorylation for the β_2AR (51), the GluR6 kainate receptor (52), and the A3 adenosine receptor (53), whereas it led to a decrease phosphorylation for bovine opsin (54), CCR5 (29), and V1aR (25). However, changes in the phosphorylation status of the V2R does not seem to account for the altered β-arrestin recruitment of the palmitoylation-less mutant because in agreement with the previous finding of Sadeghi *et al.* (4), no difference in either basal or AVP-induced phosphorylation levels was observed between wt- and C341A/C342A-V2R. Instead, we propose that the reduced affinity of β-arrestin for the palmitoylation-less mutant resides in the altered conformation of C341A/C342A-V2R carboxyl tail compared with the wtV2R.

Our proposition is in part based on the fact that the high affinity of β-arrestin for the receptor appears to depend on the position of the phosphorylated cluster of serines within the carboxyl tail (18). In addition, to show that the V2R cluster could not reintroduce the β-arrestin high affinity when added at the end of the carboxyl tail of the β_2AR, Oakley *et al.* (41) showed that the position of the clusters within the carboxyl tail of several receptor displaying high affinity for β-arrestin is relatively well conserved. This conservation is even more remarkable for receptors such as the V2R, the neurotensin-1 receptor, and the oxytocin receptor, with carboxyl-terminal tail of similar length and containing putative palmitoylation sites. Because receptor palmitate moieties are inserted in the plasma membrane where they limit the carboxyl side of the eighth α-helix (55), they also define the distance between the plasma membrane and downstream residues. Therefore, by controlling the positioning of the β-arrestin-interacting domains, palmitoylation could optimize its association with the regulatory protein. Lack of palmitoylation could then affect this conformation, leading to decreased affinity of the receptor for β-arrestin

and resulting in the slower and reduced recruitment to C341A/C342A-V2R observed in our study.

In conclusion, our study suggests that palmitoylation of V2R increases receptor affinity for β-arrestin thus allowing rapid agonist-promoted recruitment of the adaptor protein that leads to efficient endocytosis and ERK1/2 activation. Given the fact that palmitoylation is dynamically regulated during the receptor activation cycle (3), regulated changes in this post-translational modification could modulate receptor endocytosis and determine the relative contribution of different signaling pathways in response to receptor stimulation.

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