

Palmitoylation of the V₂ Vasopressin Receptor

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Received September 30, 1996; Accepted March 31, 1997

SUMMARY

Palmitoylation of the V₂ vasopressin receptor (V2R) and its functional role were investigated in transfected cells. Palmitoylation was assessed by incubating transfected cells with [³H]palmitic acid and immunoprecipitating the receptor with an antibody raised against a portion of the third intracellular loop of V2R. Wild-type and nonglycosylated V2R yielded tritium signals at 45–55 and 40 kDa, respectively, demonstrating that the V2R is palmitoylated and that receptor palmitoylation is independent of glycosylation. Substitution of CC341/342 for serines eliminated receptor palmitoylation, whereas replacement of a single amino acid, C341S or C342S, restored partial palmitoylation. Saturation binding assays revealed decreased cell surface expression of the nonpalmitoylated receptor com-

pared with the wild-type; this effect was more pronounced when a truncated form of V2R (G345ter) was studied. The presence of either cysteine residue (C341S or C342S) elevated receptor expression to normal levels, most likely due to the partial restoration of palmitoylation. Ligand binding affinity, hormone-induced stimulation of adenylyl cyclase activity, receptor internalization, and desensitization were not affected by the absence of palmitoylation. No increase but rather a slight decrease in the extent of receptor palmitoylation was detected after exposure to vasopressin. It was concluded that the V2R is palmitoylated in both cysteines, each cysteine is palmitoylated independently from the other, and palmitoylation enhances cell surface expression of the V2R.

Palmitoylation is a post-translational modification of proteins that contributes to their localization on the plasma membrane, as exemplified by p21ras and some of the α subunits of heterotrimeric G proteins (1, 2). The acylated amino acid is in most cases a cysteine that is linked to palmitate by a thioester. In the case of proteins that are intrinsic to the plasma membrane, the acylation is suggested to occur after they transverse the endoplasmic reticulum. This process may occur before and during the protein entry into the Golgi apparatus and/or in the cell membrane (1, 3). After the original report by Ovchinnikov *et al.* (4) describing the palmitoylation of bovine rhodopsin on two cysteines downstream of the seventh transmembrane region, comparison of the amino acid sequences of G protein-coupled receptors revealed the existence of cysteines in a similar location in many receptor proteins. Previously, only few G protein-coupled receptors have been shown to be palmitoylated (for a review, see Ref. 5). These studies demonstrated the incorporation of [³H]palmitic acid into wild-type receptors and the loss of incorporation after mutagenesis of the acceptor cysteine residues. A consensus sequence to identify the acceptor site has been proposed, but the sequence of the V2R deviates from it. Therefore, it was of interest to examine whether the V2R was

palmitoylated and in the process test the validity of the consensus signal.

It is unclear what role palmitoylation plays in the structure/function of G protein-coupled receptors. Ovchinnikov *et al.* (4) hypothesized that the palmitoyl group anchors the carboxyl terminal segment of the receptor to the plasma membrane, creating a fourth intracellular loop. Anchoring to the plasma membrane may play a role in determining the conformation of the intracellular loops involved in receptor/G protein coupling as well as in internalization and desensitization. For the β_2 -adrenoceptor, absence of palmitoylation increases the phosphorylation of the unliganded receptor by protein kinase A and reduces the ligand-induced increase in phosphorylation and the coupling to the G protein G_s (6, 7). On the contrary, lack of palmitoylation of the α_2 -adrenoceptors and the LH/hCG did not alter G protein coupling (8, 9). On the other hand, involvement of the palmitoyl group in receptor internalization has been shown for α_2 -adrenoceptors, β_2 -adrenoceptors, and LH/hCG receptors (8, 9). Hence, more data are required to generalize the role of palmitoylation in the function of G protein-coupled receptors.

The V2R is a member of the superfamily of G protein-coupled receptors that couples to the stimulatory G protein, G_s, and is located mainly in the kidney collecting ducts, in which it plays an important role in water homeostasis. In the

This work was supported in part by NIH Grant DK 41–244 to MB.

ABBREVIATIONS: LH, luteinizing hormone; hCG, human chorionic gonadotropin; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; D-PBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; AVP, arginine vasopressin; Iso, isoproterenol; VIP, vasoactive intestinal peptide; HEK, human embryonic kidney; V2R, V₂ vasopressin receptor; RIPA, radioimmunoprecipitation assay.

current study, we investigated the palmitoylation of the V2R and its possible functional role. Our results demonstrate that the V2R is indeed palmitoylated and that palmitoylation enhances the level of receptor expression at the plasma membrane but otherwise is not required for the receptor to be fully active.

Experimental Procedures

Materials. DMEM, HBSS, D-PBS, penicillin/streptomycin, 0.5% trypsin/5 mM EDTA, and FBS were from GIBCO (Grand Island, NY). Methionine/cysteine-free DMEM was from ICN Biochemicals (Costa Mesa, CA). Cell culture plasticware was from COSTAR (Cambridge, MA). AVP, (–)-isoproterenol, and 3-isobutyl-1-methylxanthine were from Sigma (St. Louis, MO). Forskolin was from Calbiochem (San Diego, CA). [³H]AVP (specific activity, 60–80 Ci/mmol), EXPRE³⁵S (³⁵S-Express Protein labeling Mix; specific activity, >1000 Ci/mmol), [³H]palmitic acid (specific activity, 30–60 Ci/mmol), and [α -³²P]ATP (specific activity, 3000 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). [³H]cAMP was from ICN Biochemicals (Irvine, CA). Amplify was from Amersham (Arlington Heights, IL). All other reagents were from Sigma.

Construction of mutant V2Rs. The N22Q, CC341/342SS, CC341/342GG, C341S, C342S, G345ter, and G345ter (CC341/342SS) mutations were introduced into the human V2R cDNA using a polymerase chain reaction-based approach (10). In the last two constructs, a stop codon was introduced at amino acid 345. The resulting constructs were sequenced fully according to the dideoxy chain termination method of Sanger *et al.* (11). For expression in eucaryotic cells, the cDNA-bearing mutants were cloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA).

Cell culture and transient expression of V2R. COS.M6 or HEK 293 cells were grown in DMEM-high glucose and supplemented with 10% heat-inactivated FBS, penicillin (50 units/ml), and streptomycin (50 μ g/ml). For transient transfection, COS.M6 cells, kept at <75% confluence, were plated at a density of 0.5×10^6 cells/100-mm dish and transfected the following day according to a modification of the method of Luthman and Magnusson (12). Briefly, after rinsing with HBSS, each plate with cells received 800 μ l of HBSS, pH 7.05, containing 3 μ g of plasmid DNA mixed with 0.5 mg/ml DEAE-Dextran. After 20 min at room temperature, 100 μ M chloroquine in DMEM containing 2% FBS was added. After 3 hr at 37°, the cells were exposed to 10% DMSO in HBSS for 2 min, rinsed twice with DMEM-high glucose without additives, and returned to growth medium at 37°.

Labeling with [³H]palmitic acid and immunoprecipitation. [³H]Palmitic acid incorporation was performed as described by Kennedy and Limbird (8). At 48 hr after transfection, 1 mCi [³H]palmitic acid in 1.0 ml of 10% FBS DMEM plus 1% DMSO was added to each 100-mm dish of transfected COS cells (two dishes were used for each experimental point). The [³H]palmitic acid was dried under N₂ before sequential dissolution in DMSO and DMEM. After 30 min at 37° in 5% CO₂, 2 ml of 10% FBS DMEM containing 1% DMSO was added to each dish, and the incubation was continued at 37° for a total of 2 or 8 hr. Cells were then rinsed, washed twice with ice-cold D-PBS, scraped from the plate, and collected by centrifugation in D-PBS. The cell pellet from each plate was disrupted in 500 μ l of 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 containing protease inhibitors 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml soybean trypsin inhibitor, and 0.5 μ g/ml leupeptin). Homogenization was achieved by drawing the cells through needles of decreasing gauge (20 gauge, 25 gauge) fit into a 3-ml plastic syringe. Cell extracts were then clarified by mixing them with 50 μ l of a 50% slurry of pre-washed Protein A/Sepharose in the same buffer. Pre-washed Protein A/Sepharose was prepared by the addition of 1.0 ml of 25 mg/ml BSA in RIPA buffer, mixing for 1 hr, and by two washes with RIPA buffer

alone. For immunoprecipitation, an antibody raised against a portion of third intracellular loop of human V2R (AntiV2 #2, peptide VPGPSERPGGRRRGR) was added to the clarified extracts at a concentration of 10 μ g/ml and incubated overnight at 4°. The antigen/antibody complexes were then separated by incubating the mixture with pre-washed Protein A/Sepharose for 2 hr at the same temperature. The beads were centrifuged, washed three times for 4 min on ice with RIPA buffer, and mixed with 80 μ l of 100 μ M peptide #2 in RIPA buffer for 30 min at room temperature to elute the receptor. Where indicated, 40 μ l of the eluates was mixed with 100 munits of PNGase F and incubated at room temperature for 1 hr. After mixing with an equal volume of 2 \times Laemmli sample buffer containing 50 mM β -mercaptoethanol, the samples were electrophoresed in 10% SDS-polyacrylamide gels. Radioactive bands were visualized by treating the gel with Amplify and exposing the dried gels to Kodak X-Omat film at –70° for the indicated times.

To examine the effect of AVP on palmitic acid turnover, the cells were labeled with palmitic acid for 100 min as described above, and AVP was added to the medium to a final concentration of 100 nM. Twenty minutes later, the reaction was stopped by chilling the cells, followed by receptor extraction and immunoprecipitation.

Metabolic labeling with ³⁵S-methionine/cysteine. Proteins were labeled in 100-mm dishes according to a modification of the method of Keefer and Limbird (13). At 48 hr after transfection, COS cells were fasted for 1 hr in methionine/cysteine-free DMEM and then were labeled for 1 hr 40 min or 8 hr with 2 ml of the same medium containing 100 μ Ci of ³⁵S-Express Protein Labeling Mix/plate. Cells were then rinsed, and the receptor proteins were extracted and immunoprecipitated as described above. The receptor protein was analyzed by SDS-PAGE and fluorography. The effect of AVP on the intensity of the labeled receptor band was tested by labeling the cells for 1 hr 40 min as described above and adding the hormone to the medium to a final concentration of 100 nM. Twenty minutes later, the reaction was stopped by chilling the cells, followed by receptor extraction and immunoprecipitation.

Phosphorylation of the V2R in intact cells. Transiently transfected COS cells were plated onto six-well plates at a density of 1.5×10^5 cells/well at 24 hr after transfection. After 18 hr, the cells were washed and incubated for 30 min with phosphate-free MEM, followed by the addition of 100 μ Ci/well of [³²P]H₃PO₄. After 2 hr at 37°, the cells were exposed to 100 nM vasopressin for 15 min at 37° and then chilled on ice and washed twice with PBS. The receptor proteins were extracted and immunoprecipitated as described above and analyzed by SDS-PAGE and autoradiography. The RIPA buffer used for the extraction contained, in addition to the protease inhibitors, 10 mM Na pyrophosphate, 10 mM NaF, and 300 mM okadaic acid as phosphatase inhibitors.

[³H]AVP binding to intact cells. Twenty four hours after transfection, cells were plated onto 24-well plates at a density of 0.5 – 1.0×10^5 cells/well. Binding assays were performed the following day. Cells were washed twice with ice-cold D-PBS, after which each well received 0.5 ml of ice-cold D-PBS with 2% BSA and the appropriate dilutions of [³H]AVP in the presence (nonspecific) or absence (total) of 10 μ M AVP (10). Plates were incubated for 2 hr on ice in the cold room before removal of the binding mixture by aspiration. After two quick rinses with ice-cold D-PBS, 0.5 ml of 0.1 N NaOH was added to each well to extract radioactivity. After 30 min at 37°, the fluid from the wells was transferred to scintillation vials containing 3.5 ml of ULTIMA-FLO M (Packard, Meriden, CT) scintillation fluid for radioassay. Experiments were repeated at least three times. The data in the text are expressed as mean \pm standard error.

Receptor-mediated internalization of [³H]AVP. COS cells were plated 24 hr after transfection at a density of 0.5 – 1.0×10^6 cells/plate onto 12-well plates. The next day, after two washes with D-PBS, we added to the wells 20 nM [³H]AVP in 0.5 ml of D-PBS containing 2% BSA. Cells were incubated at 37° for 20 min and chilled by placing the plates on ice. For measurement of total number of sites expressed per cell, the wells were washed with D-PBS and

lysed in 0.5 ml of 0.1 N NaOH. For determination of the number of internalized receptors, the wells were treated for 5 min with a solution of 50 mM sodium citrate, 90 mM NaCl, and 0.1 mM Na_2HPO_4 , pH 5.0 (acid wash), followed by two washes with ice-cold D-PBS. Radioactivity was extracted with 0.5 ml of 0.1 N NaOH as described above. Experiments were repeated five times. The data are expressed as mean \pm standard error.

Receptor desensitization. HEK 293 cells were plated 24 hr after transfection at a density of 3.0×10^6 cells/polylysine-treated 60-mm dish. The next day, after two washes with D-PBS at room temperature, the dishes received vehicle or 100 nM AVP in 2 ml of DMEM containing 10% FBS. Cells were incubated at 37° for 5 or 20 min and chilled by placing the dishes on ice. After two washes with ice-cold D-PBS, cells were exposed twice for 30 sec to 5 mM acetic acid/150 mM NaCl followed by three washes with ice-cold D-PBS. Cells were harvested and homogenates were prepared as previously described (10). Receptor stimulation of adenylyl cyclase activity was assessed by dose-response curves to AVP.

Adenylyl cyclase activity in cell homogenates. Adenylyl cyclase activity was assayed as previously described (10). In a final volume of 50 μl , the medium contained 0.1 mM [α - ^{32}P]ATP ($1-5 \times 10^6$ cpm), 4 mM MgCl_2 , 10 μM GTP, 1 mM EDTA, 1 mM [^3H]cAMP ($\sim 10,000$ cpm), 2 mM 3-isobutyl-1-methylxanthine, and a nucleoside triphosphate regenerating system composed of 20 mM creatine phosphate, 0.2 mg/ml (2000 units/mg) creatine phosphokinase, 0.02 mg/ml myokinase (448 units/mg), and 25 mM Tris-HCl, pH 7.4. Hormones (diluted in 1% BSA) were present at the concentrations indicated on the figures. Reactions were stopped by the addition of 100 μl of a solution containing 40 mM ATP, 10 mM cAMP, and 1% SDS. The cAMP formed was isolated by a modification of the standard double

chromatography over Dowex-50 and alumina columns (14, 15). Under these assay conditions, cAMP accumulations were linear with time of incubation for ≤ 40 min and proportional to the amount of homogenate. The activities were expressed as pmol of cAMP formed/min/mg of homogenate protein or percent maximal Iso or VIP response. Protein concentration was determined according to the method of Lowry (16) using BSA as standard. Experiments were repeated at least twice. The EC_{50} values are the average of the values obtained.

Results

Palmitoylation of V2R. The presence of cysteines within 15 amino acids after the seventh transmembrane region of the V2R predicted that the receptor could be palmitoylated, but as illustrated in Fig. 1, the sequence surrounding these cysteines deviated from that proposed by Bouvier *et al.* (5) as the acceptor site for receptor palmitoylation. The composition of the proposed consensus sequence is FXX L/I L/I (X) $_n$ C p , where F, L, and I represent phenylalanine, leucine, and isoleucine; the range for n is 0–4; and C p is palmitoylated cysteine. This sequence, which is derived from examination of the amino acid composition of the G protein-coupled receptors first reported as palmitoylated, is rather ambiguous. To test whether the V2R is palmitoylated, it was expressed transiently in COS.M6 cells and tested for incorporation of [^3H]palmitic acid as described in Experimental Procedures. Metabolic labeling and immunoprecipitation of the V2R ex-

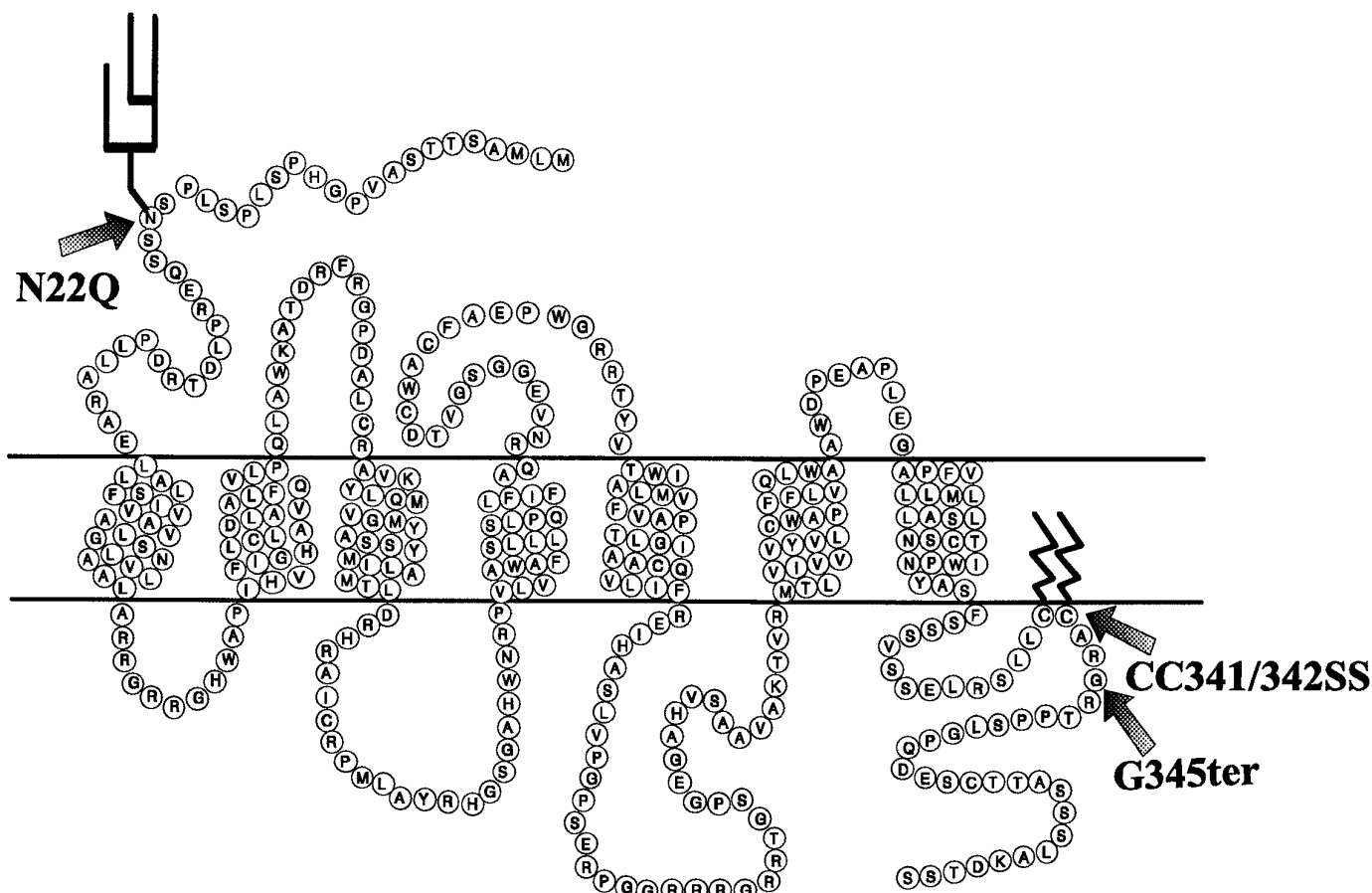


Fig. 1. Composition of the human V2R amino acid sequence and its topographic organization in the plasma membrane. Cys341 and Cys342 are shown anchored to the membrane by the aliphatic chain of palmitic acid; the single glycosylation site at Asn22 and codon 345, the site used to truncate the receptor, are also identified.

pressed in COS.M6 cells had been previously investigated in our laboratory (17). Expression of the glycosylated wild-type V2R produced two predominant bands of immature receptor and a weaker broad band of mature V2R. The intensity of these bands varied between experiments, but they were always present. Sensitivity to endoglycosidase treatments established that the broad band at 45–55 kDa corresponded to the mature glycosylated V2R, whereas the others represented a receptor precursor that was very prone to aggregation (17). Treatment with PNGase F resulted in the appearance of a 38–40 kDa band at the expense of the broad 45–55 kDa band. The different migration of the precursor and the mature proteins was not due only to a difference in the carbohydrate component because expression of the cDNA encoding the nonglycosylated mutant form of the receptor (N22Q) produced a sharp band at 38–40 kDa and an additional band that migrated faster than its glycosylated counterpart (17, 18). These data suggested that the receptor protein undergoes a refolding process to achieve its final conformation and that this “maturation” was independent of the processing of the sugar moiety.

As shown in Fig. 2, incubation of COS.M6 cells expressing the V2R with tritiated palmitic acid resulted in the appearance of a tritium band at 45–55 kDa for the glycosylated and a faster migrating tritium band for the nonglycosylated V2R. Treatment of the immunoprecipitated glycosylated receptor with PNGase F to cleave the asparagine sugar linkage produced a sharp band of 40 kDa with migration identical to the one produced by the nonglycosylated receptor. These results were consistent with the expected changes in migration due

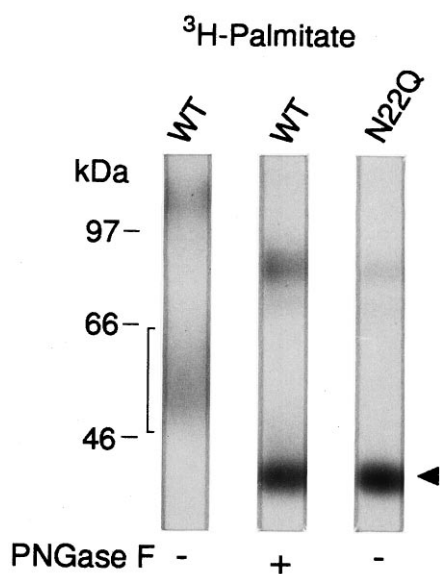


Fig. 2. Palmitoylation of the wild-type V2R. Transfected COS.M6 cells expressing the wild-type (WT) or the nonglycosylated V2R (N22Q) were treated with [3 H]palmitic acid for 8 hr at 37° as described in Experimental Procedures. The receptor was then extracted and immunoprecipitated using an antibody raised against the third intracellular loop (antibody #2). The proteins were eluted with 100 μ M epitope peptide in RIPA buffer, and an aliquot of the glycosylated receptor was treated with PNGase F as described in Experimental Procedures. The samples were mixed with 2 \times Laemmli buffer containing 50 mM β -mercaptoethanol and subjected to SDS-PAGE and fluorography. The dried gel was exposed to Kodak X-Omat film for 2 weeks at -70° to detect the tritium signal. Bracket, position of the glycosylated receptor. Arrowhead, deglycosylated and nonglycosylated receptor bands.

to the presence of sugar in the protein (see Ref. 17). These data indicated that palmitoylation of the receptor was not related to the presence of glycosylation. In all experiments assessing receptor palmitoylation, the immunoprecipitated proteins were mixed with 2 \times Laemmli's sample buffer containing 50 mM β -mercaptoethanol according to Magee *et al.* (19), who described the resistance of the palmitoyl bond to low concentrations of reducing agents. This concentration of β -mercaptoethanol incorporated into the Laemmli sample buffer provided a reducing environment that facilitated the entry and migration of the proteins in SDS-PAGE. As illustrated in Fig. 2, whether the receptor protein was glycosylated did not affect the incorporation of palmitic acid into the V2R. The experiments were carried out with the glycosylated and nonglycosylated receptor protein with identical results.

As shown in Figs. 3 (left), mutation of cysteine residues at codons 341 and 342 to serines eliminated palmitoylation of the mature form of the V2R. The same results were observed with mutagenesis of the cysteines to glycines. Replacement of only one cysteine by serine, at position 341 or 342, maintained palmitoylation of the receptor, but the reduction in the intensity of the tritium band was more pronounced than expected from halving of the wild-type signal. This raised the possibility that with only one of the cysteines present, the reactivity of the site, and thus the extent of palmitoylation, was reduced. To examine this possibility, the ratio between the incorporation of palmitic acid and the abundance of the mature proteins was determined. Abundance of the mature protein was assessed by metabolic labeling with 35 S-methionine/cysteine. As shown in Fig. 3 (right), it was possible to detect production of the mature form of the receptor for all the mutant cDNAs tested, but the intensity of the 35 S band was decreased when one or both cysteine residues were replaced by serines. The reduced intensity of these bands coincided with an increase in the intensity of the radioactive band at 33 kDa that corresponds to the immature receptor

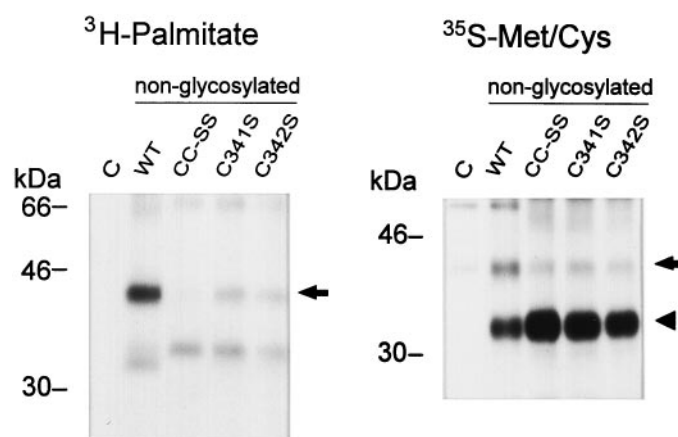


Fig. 3. Palmitoylation of mutant V2R. Nontransfected COS.M6 cells [control (C)] and transiently transfected cells expressing the nonglycosylated wild-type (WT) or mutant V2Rs were treated with [3 H]palmitic acid (left) or with 35 S-methionine/cysteine (right) for 8 hr at 37° as described in Experimental Procedures. The receptor was then extracted, immunoprecipitated, eluted, and analyzed in SDS-PAGE as described in Fig. 2 and detected by fluorography. To observe the tritium signal, the dried gel was exposed to Kodak X-Omat film for 3 weeks at -70°. To detect the 35 S-signal, the gel was exposed for 24 hr at -70°. Arrow, band corresponding to the mature receptor. Arrowhead, immature receptor.

protein (18). To evaluate the changes in palmitic acid incorporation, the receptor was labeled with ^{35}S and tritiated palmitic acid in parallel experiments. The receptor was extracted and analyzed as described in the same gel, and densitometric readings of the radioactivity incorporated into the bands of mature receptor were obtained. The values in each case were normalized as percent of the band detected for the protein that contained both cysteines, and they are illustrated in Fig. 4, A and B. Due to the reduction in the quantity of mature receptor detected by ^{35}S labeling, it was necessary to correlate the tritium signal with receptor protein abundance. The densitometric values obtained for the tritium signal were divided by the densitometric values obtained in the parallel experiment with ^{35}S labeling. This ratio was calculated for the mature band of each construct, and the values obtained were expressed as percent of the ratio of the wild-type protein. As shown in Fig. 4C, the presence of either cysteine reduced this ratio to about half, demonstrating that when cysteines are present both are palmitoylated and that a single cysteine at this location was sufficient to preserve palmitoylation. Unrelated experiments that examined the

expression and palmitoylation of the V2R protein truncated at Cys341 revealed that it was palmitoylated, ruling out the requirement of any sequence downstream of this cysteine as a recognition site for palmitoylation (data not shown).

Ligand binding activity of nonpalmitoylated V2R.

Cell surface expression and ligand binding affinity of the wild-type V2R and the receptors mutagenized at one or both acceptor cysteines were assessed by performing saturation binding assays in intact cells. The experiments were performed repeatedly with the glycosylated and nonglycosylated mutant proteins with similar results. Fig. 5 illustrates the results obtained in a representative experiment for saturation binding of the glycosylated V2R and Scatchard analysis of the data. Mutagenesis of both cysteines (CC341/342SS) reduced the number of receptors expressed in the plasma membrane. As shown in Table 1, the average receptor expression calculated from six experiments was $57 \pm 3\%$ of the wild-type V2R. On the other hand, mutation of only one of the cysteine residues, C341S or C342S, did not alter the level of expression of the receptor (Fig. 5 and Table 1).

Truncation of the carboxyl terminus upstream from the double cysteines abolishes expression of the V2R on the cell surface (20). As seen in Table 1, truncation of the carboxyl-terminal segment downstream from the cysteine residues by insertion of a termination codon at position 345 allowed for receptor expression, although it was at 50% the level of the wild-type, without alteration in hormone binding affinity. Replacement of Cys341 and Cys342 by serines in the G345ter mutant decreased by an additional 3-fold the cell surface expression of the truncated receptor without altering ligand binding affinity. These data demonstrate that inhibition of

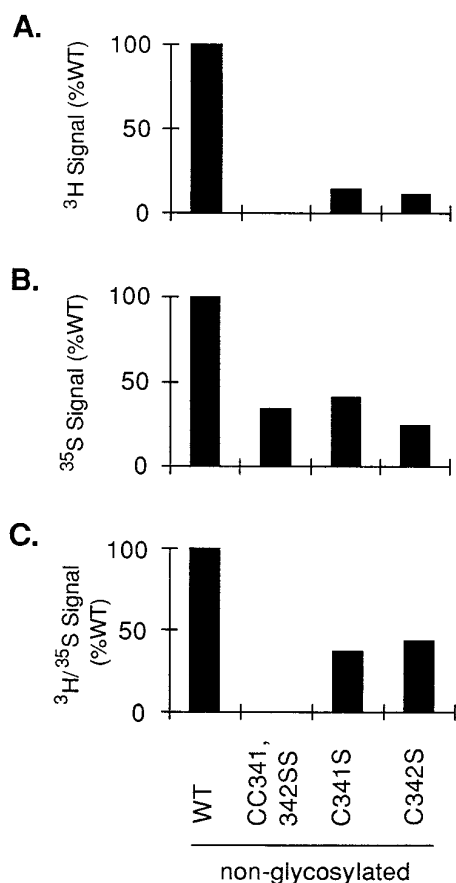


Fig. 4. Extent of palmitoylation of the mutant V2Rs. The ^{35}S and tritium bands corresponding to the mature form of the nonglycosylated wild-type (WT) and mutant V2Rs were quantified by densitometric readings. A, Densitometry of the intensity of ^3H labeling detected over the mature receptor bands in Fig. 3A. The data are expressed as percent of the nonglycosylated wild-type. B, Densitometry of the intensity of ^{35}S labeling of the mature receptor bands shown in Fig. 3B. The data are expressed as percent of the nonglycosylated wild-type. C, Ratio of the tritium signal, representing [^3H]palmitic acid incorporation, over the ^{35}S signal, indicative of the amount of receptor produced for each mutant V2R.

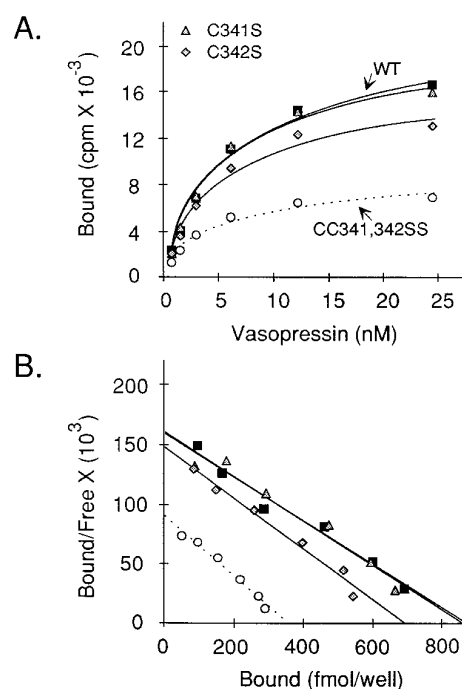


Fig. 5. Representative saturation binding assay in intact COS.M6 cells. A, Specific binding of [^3H]AVP to wild-type (WT) and mutant V2Rs lacking one (C341S or C342S) or both palmitoylation sites (CC341/342SS). Receptor proteins were expressed transiently in COS.M6 cells and saturation binding assays were carried out in 24-well plates containing $\sim 50,000$ cells/well as described in Experimental Procedures. B, Scatchard analysis of the data from A.

TABLE 1

Ligand binding affinity and cell surface expression of V2R in COS.M6 cells

K_D and number of sites per cell for the wild-type and mutant V2Rs expressed transiently in COS.M6 cells. The data are expressed as percent of wild-type V2R value. Expression level for the wild-type receptor was $6\text{--}10 \times 10^6$ sites/cell. Data are expressed as the mean \pm standard error from four to six experiments.

| V2R | Sites/cell | K_D |
|-----------------|--------------|---------------|
| | % wild-type | nM |
| Wild-type | 100 | 4.7 ± 0.4 |
| CC341,342SS | 57 ± 3 | 4.2 ± 0.3 |
| CC341,342GG | 63 ± 4 | 4.0 ± 0.1 |
| C341S | 110 ± 12 | 4.8 ± 0.5 |
| C342S | 91 ± 11 | 4.5 ± 0.4 |
| G345ter | 49 ± 5 | 4.5 ± 0.5 |
| G345ter (CC-SS) | 16 ± 2 | 3.9 ± 0.4 |

receptor palmitoylation has a pronounced effect on the abundance of receptor on the plasma membrane and that the effect is more pronounced for the truncated receptors. The glycosylated and nonglycosylated truncated receptors were equally affected in their level of expression by the absence of Cys341 and Cys342.

G protein coupling of nonpalmitoylated V2R. The ability of the nonpalmitoylated receptor to interact with G proteins was assessed by measuring AVP stimulation of adenylyl cyclase activity mediated by mutant receptors expressed in COS.M6 and HEK 293 cells. As seen in Fig. 6A, the CC341/342SS mutant V2R expressed in COS.M6 cells was able to stimulate adenylyl cyclase activity to the same extent as the wild-type receptor with an EC_{50} value of 90 pM for both. Similar results were obtained in HEK 293T cells (Fig. 6B). Truncation of the V2R at G345 did not alter the ability of the receptor protein to mediate AVP stimulation of adenylyl cyclase activity compared with the wild-type receptor. Lack of palmitoylation in the V2R G345ter reduced the maximal stimulation of adenylyl cyclase activity to 70% of the maximum response of the palmitoylated truncated receptor and produced a right shift in the EC_{50} value from 0.8 nM (G345ter) to 2 nM (G345ter-CC341/342SS). The observed rightward shift is most likely due to the reduction in the abundance of receptors per cell documented in Table 1 than to altered coupling to G_s . The coupling data were examined using the glycosylated nonpalmitoylated form of the receptor.

Internalization of nonpalmitoylated V2R. The impact of the absence of palmitoylation on receptor internalization was assessed by incubating COS.M6 cells expressing wild-type or either the CC341/342SS or CC341/342GG mutant receptors with 20 nM [3 H]AVP and measuring the efficiency of the different receptors to internalize the ligand. As illustrated in Fig. 7A, the results indicated that $54 \pm 2\%$, $58 \pm 1\%$, and $52 \pm 2\%$ (five experiments) of the receptors were internalized for the wild-type, CC341/342SS, and CC341/342GG V2R, respectively. For each experimental condition, the radioactivity remaining in the wells after the PBS wash (described in Experimental Procedures) was used as the 100% value. These results indicated that V2R internalization and G protein coupling were not affected by the lack of palmitoylation.

Effect of ligand on nonpalmitoylated V2R desensitization and phosphorylation. Ligand induced desensitization of nonpalmitoylated receptor was examined as described in Experimental Procedures. Cells expressing the wild-type

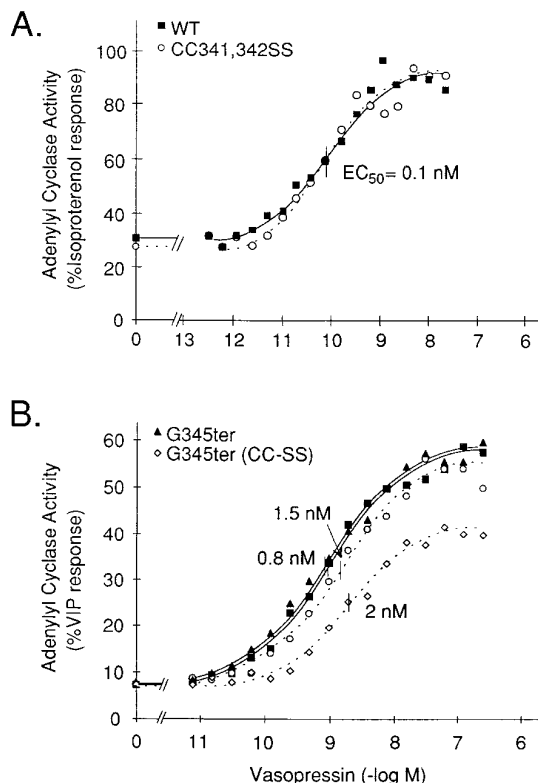


Fig. 6. Representative adenylyl cyclase assay in cell homogenates expressing the wild-type (WT) and nonpalmitoylated V2Rs. A, AVP-induced stimulation of adenylyl cyclase activity of COS.M6 cell homogenates, expressing the wild-type and nonpalmitoylated V2R. The results are expressed as maximal Iso response. Adenylyl cyclase activity in the absence of AVP (basal) was 46 and 27 pmol/mg/min for the wild-type (■) and CC341,342SS (○), respectively. B, AVP-induced stimulation of adenylyl cyclase activity of HEK 293 cells homogenates, expressing the wild-type (■), nonpalmitoylated wild-type (CC341/342SS) (○), truncated G345ter (▲), and nonpalmitoylated truncated G345ter (CC-SS) (◇) receptor. The results are expressed as maximal VIP response. Basal adenylyl cyclase activities were 6.0, 4.0, 4.0, and 4.0 pmol/mg/min for the WT, CC341/342SS, G345ter, and G345ter (CC/SS), respectively. The values for the maximal VIP response determined at 100 nM VIP were 74, 58, 51, and 53 pmol/mg/min, respectively.

or the mutant receptor were exposed to 100 nM AVP, subjected to acid washes, and homogenized. Adenylyl cyclase activity of the cell extracts was measured in the presence of various concentrations of AVP to examine whether receptor desensitization was changed by the absence of palmitoylation. As illustrated in Fig. 7B, the decrease in maximal response to AVP was the same for the mutant and the wild-type receptor after 5- or 20-min treatment with the hormone; the shift in EC_{50} value was also similar for both receptors (data not shown). Contrary to what has been described for the β_2 -adrenergic receptor, there was no change on the basal or ligand-stimulated phosphorylation of the V2R when both cysteines were eliminated, as shown in Fig. 8.

The possible effect of AVP on the turnover of palmitic acid was tested by exposing the cells containing the wild-type palmitoylated receptor to 100 nM AVP for 20 min after 2-hr labeling because it has been reported for the β_2 -adrenergic receptor that the effect of agonist on palmitic acid turnover was more apparent after shorter labeling times (22). As illustrated in Fig. 9, exposure to the hormone slowed the

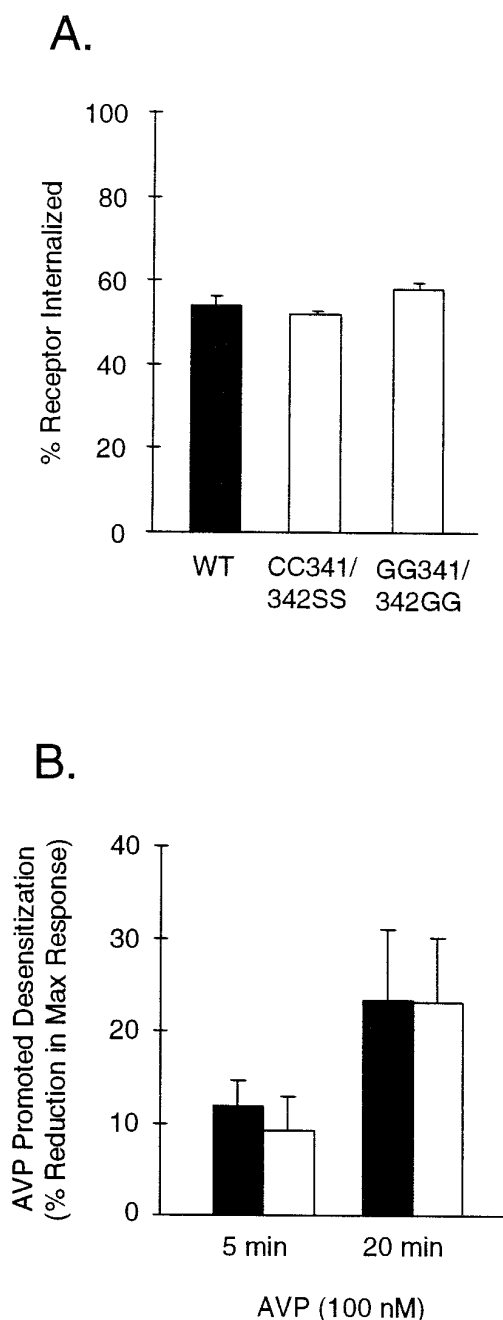


Fig. 7. AVP-induced changes of V2R in intact COS.M6 cells. **A**, Agonist-induced internalization of the wild-type (WT; ■) and nonpalmitoylated V2Rs (□) expressed in transfected in COS.M6 cells. Intact cells plated onto six-well plates were treated with 20 nM [³H]AVP at 37° for 20 min and washed three times with PBS (total bound) or with acid containing isotonic solutions (internalized). Radioactivity remaining in the wells was determined as described in Experimental Procedures. The data show percent of the total receptor internalized and are expressed as the mean ± standard error (five experiments). **B**, Desensitization of the wild-type (■) and the CC341/342SS mutant V2R (□) expressed transiently in HEK 293 cells. Cells were treated with 100 nM AVP, washed, and disrupted as described in Experimental Procedures. Dose-response curves of AVP stimulation of adenylyl cyclase activity were measured in cell homogenates, and the decreases in maximal adenylyl cyclase activities were used as an indication of receptor desensitization.

migration of the V2R in SDS-PAGE, probably due to ligand-induced phosphorylation of the receptor (21). Densitometric quantification of the tritiated palmitic acid signal revealed a

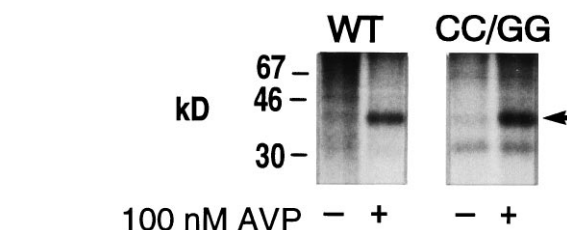


Fig. 8. Phosphorylation of the nonglycosylated wild-type (WT) and CC341/342SS V2R. COS.M6 cells transfected with each construct were labeled with ³²P orthophosphate 2 days after transfection and treated for 15 min at 37° with vehicle or 100 nM AVP. The V2R was immunoprecipitated and identified by SDS-PAGE and autoradiography as described in Experimental Procedures. Arrow, position of the phosphorylated receptor.

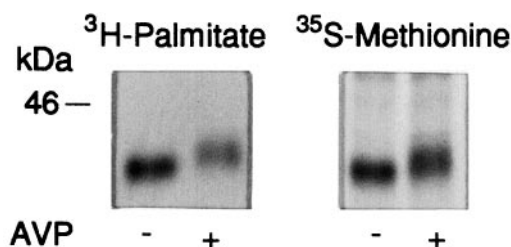


Fig. 9. Effect of AVP on V2R palmitic acid content. COS cells expressing the wild-type receptor were labeled with palmitic acid and treated with AVP, and the receptor protein was extracted and analyzed as described in Experimental Procedures. The effect of 20-min exposure to 100 nM AVP after 2-hr labeling with [³H]palmitic acid or with ³⁵S-methionine/cysteine is illustrated.

26% decrease in the amount of palmitic acid associated with the receptor. Receptor labeled for the same time with ³⁵S-methionine/cysteine and exposed to AVP under similar conditions showed the expected change in migration without a significant decrease in the intensity of the receptor band (8.3%). These results suggest that contrary to what was reported for the β_2 -adrenoceptor, exposure of the V₂ vasopressin receptor to ligand in the presence of labeled palmitic acid does not result in increased turnover but rather in loss of palmitic acid from a fraction of the receptor.

Discussion

Palmitoylation of V2R was investigated by immunoprecipitation of receptor proteins expressed transiently in COS.M6 cells and detection of incorporated [³H]palmitic acid. In cells expressing the wild-type glycosylated V2R, [³H]palmitic acid incorporation resulted in the appearance of a broad radioactive band at 45–55 kDa, which is the expected size for the mature receptor form. Deglycosylation with PNGase F produced a sharp band at 40 kDa. As seen in Fig. 2, this band was identical to the band obtained from cells expressing nonglycosylated receptor (17, 18). These results demonstrated that the V2R is palmitoylated and that protein glycosylation was not required to observe palmitoylation.

Analogous to other G protein-coupled receptors, the palmitoylation site was expected to be located 10–12 amino acids downstream of the seventh transmembrane region. Some receptors, such as the α_2 - and β_2 -adrenoceptors, contain only one palmitoylated cysteine; others, like rhodopsin and the LH/hCG receptor, contain two adjacent cysteine residues, both of which are palmitoylated. The two cysteine residues of the V2R at positions 341 and 342 were the candidate sites for

palmitoylation. Mutation of these cysteines to serines abolished the incorporation of [^3H] palmitic acid, identifying them as the palmitoylation site. Palmitoylation of the V2R challenged the validity of the consensus sequence proposed by Bouvier *et al.* (5) because the acceptor sequence for the V2R lacks a phenylalanine at the predicted location.

Mutants containing only one cysteine (C341S or C342S) were used to examine whether one or both are palmitoylated. Incorporation of palmitic acid was detected for both mutations at a level close to 50% of the wild-type protein, demonstrating that both cysteines are palmitoylated and that each one can be palmitoylated independently of the other. This is contrary to the observation by Karnik *et al.* (23), who established that in bovine opsin the palmitoylation of Cys323 is dependent on the palmitoylation of Cys322. In turn, our results are in agreement with those of Kawate and Menon (9), who demonstrated independent palmitoylation of Cys621 and Cys622 of the LH/hCG receptor.

In addition to the tritium signal coincident with the mature receptor, a tritium signal was also present at a location coincidental with the migration of the immature receptor. The lack of correlation of this band with the presence of Cys341/Cys342 suggests that it is due to a protein that coprecipitates with the receptor.

Assessment of the level of expression of the wild-type and the nonpalmitoylated V2R revealed a 40–50% reduction in the number of binding sites for the latter. The dependency of this effect on palmitoylation was demonstrated by the restoration of wild-type-like levels of expression for the single-cysteine mutant V2Rs. A similar reduction of cell surface expression of the CC341/342SS mutant V2R was reported by Schulein *et al.* (22), but these authors did not examine the effect of single amino acid mutations at the palmitoylation sites on receptor expression. A reduction in levels of receptor expression has been reported for the nonpalmitoylated LH/hCG receptor; however, this effect was not reversed by mutagenizing a single cysteine (9). Therefore, we are the first to report a direct correlation between cell surface expression of a G protein-coupled receptor and palmitoylation. Because the reduction in receptor expression was observed when either serine or glycine replaced cysteine, this change is most likely due to the absence of palmitoylation rather than to the identity of the amino acid present. Palmitoylation may affect the processing of the newly synthesized receptors because our results showed an increase in the quantity of immature nonpalmitoylated mutant V2R compared with the amount present for the wild-type receptor. After ligand-induced sequestration of receptors expressed in HEK 293 cells, it was not possible to detect the return of receptors to the cell surface after removal of the hormone; both wild-type and nonpalmitoylated proteins behave in the same manner (data not shown). Therefore, it was not possible to test experimentally whether there are differences in receptor recycling.

Despite the differences in cell surface expression between the nonpalmitoylated V2R and the single palmitoylated or wild-type V2R, ligand binding affinity and G protein coupling of the receptor were not affected in our experiments. Schulein *et al.* (24) did not observe changes in ligand binding affinity but described a 2–7-fold change in the EC_{50} value of AVP stimulation of adenyl cyclase activity for the mutants compared with the wild-type receptor. Because their study was performed in stably transfected cells expressing a varying

number of sites per cell, they proposed receptor abundance as the cause for the observed differences, a result that is in agreement with data we previously reported (10). The transfection conditions used in our experiments result in high levels of receptor expression such that only significant reductions in abundance observed in binding assays are accompanied by detectable changes in coupling to G_s .

The lack of interference with G protein coupling observed for the nonpalmitoylated V2R is in agreement with what has been reported for the α_2 -adrenoceptor and the LH/hCG receptor (8, 9). Thus, the data suggest that once the receptor is inserted properly into the plasma membrane, it does not require palmitoylation to interact successfully with G_s . In accord with these results, we observed that the CC341/342SS was as good as the wild-type V2R as substrate for ligand-induced receptor phosphorylation. In addition, ligand-induced desensitization of the V2R was not affected by lack of palmitoylation. The alteration in G protein coupling reported for the nonpalmitoylated β_2 -adrenoceptors has been recently ascribed to the modulation of phosphorylation of this protein by the presence or absence of palmitic acid (6, 7). The V2R does not contain such a site, and for the β_2 -adrenoceptor, once the adjacent cAMP-dependent protein kinase site is eliminated, there is no effect of palmitoylation in the coupling to G_s .

In apparent contrast with what Schulein *et al.* (24) reported, we did not observe a significant change in agonist-induced V2R internalization as a consequence of abolishing palmitoylation. Close examination of the reported differences in receptor internalization reported by these authors reveals that in their experiments, the internalization of the wild-type and nonpalmitoylated V2Rs occurring within the first 5 min of exposure to AVP was virtually identical, although they chose to focus their comments on the differences in the subsequent slower rate of internalization. The use of stably as opposed to transiently transfected cells could be argued as an explanation for the difference, but changes in internalization of the nonpalmitoylated LH/hCG receptor expressed in transiently transfected 293T cells have been observed (9). We have no explanation for the differences observed between these studies.

The turnover of palmitic acid attached to G protein α subunits and some G protein-coupled receptors has been found to be modified by occupancy of the receptor by ligand (25). The ligand-induced increases in the amount of radioactive palmitic acid associated with the β_2 -adrenoceptor was interpreted as a consequence of increased turnover of the fatty acid. Recently, Loisel *et al.* (26) reported that the extent of palmitoylation of the β_2 -adrenoceptor lacking the adjacent protein kinase A phosphorylation site was not altered by exposure to ligand, suggesting a correlation between phosphorylation and palmitoylation for this receptor. The V2R lacks acceptor sites for protein kinase A, and we have shown that phosphorylation is not altered by lack of palmitoylation. Therefore, it was not surprising that treatment with 100 nM AVP for 20 min during a 2-hr labeling period did not enhance the amount of tritiated palmitic acid associated with the receptor. As for the observed reduction of palmitic acid, it could be partially due to ligand-induced sequestration and subsequent degradation of the receptor, reflecting loss of protein, and partially to enhanced depalmitoylation.

In summary, the V2R is palmitoylated at the two cysteine

residues located toward the carboxyl terminus of the receptor. The two sites are palmitoylated independently of each other, and palmitoylation of these residues contributes to V2R expression in the plasma membrane. Once the receptor reaches the membrane, its function does not seem to be influenced by the presence of the fatty acids. These data suggest that receptor palmitoylation may play a role in maintaining the insertion of the V2R into the plasma membrane.

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