Properties of the human arginine vasopressin V2 receptor after site-directed mutagenesis of its putative palmitoylation site

Ralf SCHÜLEIN,* Ursula LIEBENHOFF,* Helmut MÜLLER,* Mariel BIRNBAUMER† and Walter ROSENTHAL*‡

*Rudolf-Buchheim-Institut für Pharmakologie, Justus-Liebig-Universität Giessen, Frankfurter Str. 107, D-35392 Giessen, Federal Republic of Germany, and †Department of Anesthesiology, School of Medicine, UCLA, Los Angeles, CA 93324, U.S.A.

Most G-protein-coupled receptors have conserved cysteine residues in their C-terminal cytoplasmic domain that appear to be generally palmitoylated. An example is the human arginine vasopressin V2 receptor with cysteine residues at positions 341 and 342. Site-directed mutagenesis of the putative palmitoylation site was used to study the significance of palmitoylation for the V2 receptor. A multifunctional expression plasmid was constructed by cloning the V2 receptor cDNA into the vector pCDNA1.Neo. The resulting plasmid allowed site-directed mutagenesis experiments without subcloning, and stable and transient expression of the V2 receptor in *Ltk*[−] and COS.M6 cells respectively. The conserved cysteine residues Cys-341 and Cys-342 were replaced by serine residues, yielding the single mutants C-341S and C-342S and the double mutant C-341S/C-342S. Functional expression in stably transfected *Ltk*[−] cells showed that the affinity of the three mutant receptors for arginine vasopressin was not altered. In contrast with the activation of

adenylate cyclase through β_2 adrenergic receptors, arginine vasopressin stimulated adenylyl cyclase to the same extent and with similar EC_{50} values in both wild-type and mutant receptors. Transient expression of the C-341S/C-342S mutant receptor in COS.M6 cells confirmed an unaltered affinity of the mutant receptor for arginine vasopressin. However, the number of arginine vasopressin-binding sites on the cell surface was reduced by 30 %, suggesting that the transport of the mutant receptor to the cell surface was impaired. In addition, the decrease in detectable arginine vasopressin-binding sites on the cell surface following pre-exposure to hormone was reduced, indicating that the sequestration/internalization of the mutant receptor on the cell surface was affected. The present data indicate that palmitoylation of the V2 receptor is important for intracellular trafficking and/or sequestration/internalization but not for agonist binding or activation of the G_s /adenylate cyclase system.

INTRODUCTION

Binding of 8-arginine vasopressin (AVP, antidiuretic hormone, ADH) to V2 vasopressin receptors activates the G_s /adenylate cyclase system of renal collecting duct epithelial cells. The increase in cAMP leads to the insertion of water channels (aquaporins) into the cell membrane (for a review see [1]). As a consequence, the water permeability of the cell membrane is dramatically enhanced, allowing reabsorption of water (antidiuretic effect).

Molecular cloning and sequencing of the cDNAs encoding human and rat V2 receptors revealed structural features common to the large family of G-protein-coupled receptors, such as a heptahelical transmembrane organization, an extracellular N-terminus and an intracellular C-terminus [2,3]. The C-terminal cytoplasmic domain of the V2 receptor contains two adjacent cysteine residues (Cys-341 and Cys-342). The corresponding cysteine residues of the rat luteinizing hormone/human chorionadotropin receptor (LH/hCG receptor) [4] and of bovine rhodopsin [5,6] are known to be palmitoylated. In other receptors, the C-terminal domain possesses only one corresponding cysteine residue, which was found to be palmitoylated in the β_2 adrenergic receptor [7] and in the α_{2A} adrenergic receptor [8]. It is generally assumed that all G-protein-coupled receptors with cysteine residues at an appropriate distance from the seventh transmembrane helix are palmitoylated at these sites. Direct demonstration of V2 receptor palmitoylation is currently complicated

by the lack of specific antibodies and by the fact that insufficient amounts of recombinant receptor protein can be expressed.

It is believed that the palmitate tails serve to attach the C-termini of the receptors to the membrane, but conflicting results have been obtained regarding the functional consequences of such a modification. With the help of site-directed mutagenesis experiments, palmitoylation was shown to be necessary for both coupling of the β_2 adrenergic receptor to G_s [7] and for long-term down regulation of the α_{2A} adrenergic receptor [9]. Furthermore, palmitoylation-deficient LH/hCG receptors were internalized more rapidly than wild-type receptors following exposure to agonist [4], whereas non-palmitoylated thyrotropin-releasing hormone receptors (TRH receptors) were internalized at slower rates [10]. The data suggest a role for the fatty acids in sequestration/internalization of receptors. The present results indicate that palmitoylation may serve different functions in different receptor/G-protein systems. We have replaced the conserved cysteine residues of the human V2 receptor with serine residues and assessed the functional consequences.

MATERIAL AND METHODS

Materials

Escherichia coli strains, phages and eukaryotic cell lines are listed in Table 1, and nucleic acids are listed in Table 2. [3H]AVP

Abbreviations used: AVP, 8-arginine vasopressin; D-PBS, Dulbecco's PBS; DMEM, Dulbecco's modified Eagle's medium; G418, Geneticin 418; LH/hCG receptor, luteinizing hormone/human chorionadotropin receptor; TRH receptor, thyrotropin-releasing hormone receptor.

‡ To whom correspondence should be addressed.

Table 1 Bacterial strains, bacteriophages and eukaryotic cell lines

Name	Relevant characteristics	Source
<i>E. coli</i> SDM2	Φ80d/ <i>lacZ</i> ΔM15, <i>mrcA</i> Δ(<i>mrr</i> , <i>hsd</i> /RMS, <i>mcr</i> BC), Δ(<i>lac-proAB</i>), Δ(<i>recA</i> 1398), <i>deoR</i> , <i>rpsL</i> , <i>sri</i> , <i>thi</i> , <i>F'</i> (<i>proAB</i> ⁺ <i>lacI</i> ^q , <i>lacZ</i> ΔM15)	United States Biochemical
<i>E. coli</i> BMH 71-18	Δ(<i>lac-proAB</i>), <i>thi</i> , <i>supE</i> , <i>F'</i> (<i>proAB</i> ⁺ <i>lacI</i> ^q , <i>lacZ</i> ΔM15)	B. Müller-Hill (Köln)
<i>E. coli</i> MC1061/P3	<i>Ara</i> D139, D(<i>ara</i> , <i>leu</i>) 7696, <i>galU</i> , <i>galK</i> , D <i>lac</i> Y74, <i>hsmr</i> ⁺ , <i>strA</i> , <i>deoR</i> ⁺ , (p3: amber <i>ampR</i> , amber <i>terR</i> , <i>kmR</i>)	Invitrogen
M13K07 phage	Helper phage for plasmid ssDNA isolation, <i>KmR</i>	Pharmacia
COS.M6	African green monkey kidney cells, SV40-transformed	F. Fahrenholz (Frankfurt)
<i>Ltk</i> ⁻	Mouse fibroblasts	M. Birnbaumer (Los Angeles)

(64.8 Ci/mmol) was purchased from Du Pont (Bad Homburg, Germany) and [α -³²P]ATP (30 Ci/mmol) from Amersham (Braunschweig, Germany). Lipofectin and Genitacin 418 (G418) were obtained from Gibco BRL (Eggenstein, Germany), and penicillin and streptomycin were from Serva (Heidelberg, Germany). Fetal bovine serum was purchased from PAN-Systems (Aidenbach, Germany). Dulbecco's modified Eagle's medium (DMEM) and amino acids were from Sigma (München, Germany). All other reagents were from Merck (Darmstadt, Germany).

Cell culture

Ltk⁻ and COS.M6 cells were cultured at 5% CO₂ in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). For culture of stably transfected *Ltk*⁻ cells, the medium was supplemented with G418 (400 µg/ml).

DNA manipulations

Standard DNA preparations and manipulations were carried out according to the handbooks of Sambrook et al. [11] and Perbal [12]. The nucleotide sequences of DNA fragments were de-

Figure 1 Plasmid map of pRCDN2

Shown are the main features for site-directed mutagenesis and for stable and transient expression of the V2 receptor cDNA. Abbreviations used: V2R, V2 receptor cDNA; SV40 ori, origin of replication of the SV40 virus; M13 ori, origin of replication of the *E. coli* M13 virus; CMV-P, major intermediate-early promoter/enhancer region of the human cytomegalovirus; RSV-LTR, promoter of the Rous sarcoma virus long terminal repeats; Neo, neomycin resistance marker.

termined using the Sequenase plasmid sequencing kit from United States Biochemical (Cleveland, OH, U.S.A.).

Construction of an expression plasmid for the V2 receptor cDNA and site-directed mutagenesis

The original cDNA of the V2 receptor [13] was PCR amplified with primers introducing *Eco*RI (5') and *Bam*HI (3') sites respectively (see Table 2) and removing the bulk of the 5'- and 3'-non-coding regions. For reasons that are not relevant for this paper, the *Eco*RI/*Bam*HI-cut PCR fragment was first cloned into the *Eco*RI/*Bgl*II-cut baculovirus transfer vector pBacPAK9. From the resulting plasmid, the V2 receptor cDNA was excised as a *Bam*HI-*Xba*I fragment and cloned into the *Bam*HI/*Xba*I-cut vector pCDNAI.Neo, yielding pRCDN2 (Figure 1). Construction of pRCDN2 was carried out in the host strain *E. coli* MC1061/P3.

The entire V2 receptor cDNA insert was sequenced to verify its composition. The plasmid was used for site-directed mutagenesis and for transient and stable expression in COS.M6 and *Ltk*⁻ cells respectively (see below). For mutagenesis of the putative palmitoylation site, pRCDN2 was transformed into the *F'*-strain *E. coli* BMH71-18. Single-stranded DNA of pRCDN2 was isolated with M13K07 helper phage. Mutagenesis was carried

Table 2 Oligonucleotides and vector plasmids

Oligonucleotides for site-directed mutagenesis of the V2 receptor palmitoylation site		
Name	5'-3' Sequence	Mutation
P1S	GAAGCTTGCTCAGCTGTGCCCGGGGACG	C-341S
P2S	GAAGCTTGCTCTCAGTGCCCGGGGACG	C-342S
P12S	GAAGCTTGCTCAGCAGTGCCCGGGGACG	C-341S/C-342S
Oligonucleotides for PCR reamplification of the V2 receptor cDNA		
Name	5'-3' Sequence	Application
PEcoRI	CCTCCAGGCCCTCAGGAATTCCTGCCCCAGCCC	5'-Primer
PBamHI	GACACCAACGGATCCTCACGATGAAGTG	3'-Primer
Vector plasmids		
Name	Characteristics	Source
pCDNAI-Neo	Multifunctional eukaryotic expression vector	Invitrogen
pBacPAK9	Baculovirus transfer vector	Clontech

out with the T7-Gen *in vitro* mutagenesis kit (United States Biochemical, Cleveland, OH, U.S.A.) using *E. coli* SDM2 as host strain. In the mutagenesis experiments, cysteine residues 341 and 342 were replaced by serine residues, yielding the single mutants C-341S and C-342S and the double mutant C-341S/C-342S. The mutant plasmids were identified by both their novel restriction sites (*EspI* for C-341S and C-341S/C-342S, *PstI* for C-342S; for primer sequences see Table 2) and by DNA sequencing.

Transient expression of wild-type and mutant V2 receptors in COS.M6 cells

For transfection of COS.M6 cells, the lipid-mediated DNA-transfection procedure [14] was used. Lipofectin (7.5 μ l) was diluted in 100 μ l of serum-free medium and mixed with 0.5 μ g of plasmid DNA in 100 μ l of serum-free medium. The mixture was incubated for 15 min at room temperature and diluted with 0.8 ml of serum-free medium. COS.M6 cells (9×10^4) in a 35-mm-diam. dish were washed twice with serum-free medium and subsequently incubated with the DNA/liposome mixture. After 20 h of incubation, the serum-free medium was exchanged for 2 ml of complete medium. Cells were incubated for 3 days and then assayed for [3 H]AVP binding.

Stable expression of wild-type and mutant V2 receptors in *Ltk*⁻ cells

Ltk⁻ cells (2×10^5) were transfected with 2 μ g of plasmid DNA in a 60-mm-diam. dish as described above. After 2 days of incubation in complete medium, cells were diluted 5-fold with medium containing G418 (400 μ g/ml) and spread on 60-mm-diam. dishes to select for resistant cells. For the wild-type and each mutant receptor, 20 individual clones were isolated, expanded and screened for functional expression of the V2 receptor on the basis of specific [3 H]AVP binding to intact cells. Only those clones expressing more than 50 000 receptor sites per cell were used for further studies.

[3 H]AVP binding assay

The procedure described previously [13,15] was essentially followed. Transfected COS.M6 or *Ltk*⁻ cells in 35-mm-diam. dishes were washed twice with 2 ml of binding buffer consisting of Dulbecco's PBS (D-PBS) (0.9 mM CaCl_2 /0.5 mM MgCl_2 /2.7 mM KCl /136.9 mM NaCl /1.5 mM KH_2PO_4 /8.1 mM Na_2HPO_4 , pH 7.4) supplemented with 180 ng/ml tyrosine, 165 ng/ml phenylalanine, 1 mg/ml glucose and 20 mg/ml BSA. [3 H]AVP, diluted in 1 ml of the same buffer, was added to achieve the indicated concentrations. Non-specific binding was determined in the presence of 10 μ M unlabelled AVP. Cells were incubated with the hormone for 2 h on ice (to avoid receptor internalization) and washed quickly three times with ice-cold D-PBS, lysed with 1 ml of 0.1 M NaOH and held at room temperature for 30 min. The fluid was transferred to scintillation vials containing 3.5 ml of scintillant. Radioactivity was determined with a liquid scintillation counter.

For desensitization studies, COS.M6 cells in 35-mm-diam. dishes were transfected with plasmid DNA as described above. After 3 days of incubation in complete medium, AVP (100 nM) was added, and the cells were further incubated for different times at 37 °C. Desensitizing treatments were terminated by washing the cells with 2 ml of ice-cold 5 mM acetic acid in isotonic saline solution [13]. Neutral pH was restored by three rinses with D-PBS supplemented with 1 mg/ml glucose and 10 mg/ml BSA. The cells were then tested for [3 H]AVP-binding as described above. The fraction of receptors internalized/

sequestered was calculated by comparison with [3 H]AVP binding to cells incubated with AVP-free medium.

Adenylate cyclase activity assay

Preparation of nuclei-free crude membrane fractions and the adenylate cyclase assay were as described previously [13]. [32 P]cAMP was isolated according to the two-column method [16].

RESULTS

Elimination of the putative palmitoylation site does not alter the affinity of the V2 receptor for AVP

Cell lines derived from *Ltk*⁻ cells expressing wild-type and mutant receptors were tested for agonist binding. Representative [3 H]AVP-binding profiles and the corresponding Scatchard transformations are shown in Figure 2. The K_D values for

Figure 2 [3 H]AVP binding profiles of *Ltk*⁻ cell-derived clones stably expressing the wild-type V2 receptor and the mutants C-341S/C-342S, C-341S and C-342S

Data points for specific and unspecific binding of [3 H]AVP to intact cells represent means $\pm$ S.D. ($n = 3$). The results of four individual experiments with representative clones are depicted. Shown are binding isotherms (left) and the derived Scatchard transformations (right). The calculated K_D values for binding of [3 H]AVP to intact cells and the number of receptors per cell are indicated.

Figure 3 Adenylate cyclase assay of *Ltk⁻* cell-derived clones stably expressing the wild-type V2 receptor and the mutants C-341S/C-342S, C-341S and C-342S

Adenylate cyclase activity was determined in a crude membrane preparation. Data are calculated as the percentage of activity following maximal prostaglandin E_1 (PGE_1) stimulation. The results of representative clones are shown. Data points represent mean values of duplicates. ■, Wild type ($EC_{50} = 2$ nM; 286 000 receptors/cell); ○, C-341S ($EC_{50} = 10$ nM; 80 000 receptors/cell); ◇, C-342S ($EC_{50} = 4$ nM; 227 000 receptors/cell); ▼, C-341S/C-342S ($EC_{50} = 14$ nM; 62 000 receptors/cell). Basal (b) and PGE_1 -stimulated (s) adenylate cyclase activities (pmol of cyclic AMP/mg of protein per min) were: wild-type, 3.2 (b) and 24.3 (s); C-341S/C-342S, 3.0 (b) and 23.9 (s); C-341S, 4.8 (b) and 18.2 (s); C-342S, 4.3 (b) and 21.9 (s).

[3H]AVP binding to intact cells expressing the wild-type and the three mutant receptors were indistinguishable within the experimental error, ranging from 1.0 to 1.6 nM. These results indicate that palmitoylation of the V2 receptor is not relevant for agonist binding. For all clones, the number of AVP receptors was well above the critical value (28 000 per cell) required for

maximal activation of the G_s /adenylate cyclase system of *Ltk⁻* cells [15].

The putative palmitoylation site is not required for efficient coupling to the G_s /adenylate cyclase system

Previous studies have revealed that non-palmitoylated β_2 adrenergic receptors lose guanyl nucleotide sensitivity of agonist binding and show a 70% reduced ability to stimulate adenylate cyclase [7]. To examine whether the latter holds true for the V2 receptor, membranes of cell lines expressing wild-type and mutant receptors were prepared and AVP-sensitive adenylate cyclase activity was measured (Figure 3). For comparison of different cell clones, enzyme activity was expressed as the percentage of maximal response to prostaglandin E_1 , which activates the G_s /adenylate cyclase system of *Ltk⁻* cells through endogenous prostaglandin E_1 receptors [15]. The extent of adenylate cyclase stimulation was similar in all clones. The EC_{50} values of the mutants C-341S (10 nM) and C-341S/C-342S (14 nM) were slightly higher than those of the wild-type receptor (2 nM) and the mutant C-342S (4 nM) (see Figure 3). These minor differences may reflect differences in the number of receptors per cell. The results suggest that, in contrast with the palmitate modifications of the β_2 adrenergic receptor, the putative palmitoylation of the V2 receptor does not have a major effect on the activation of the G_s /adenylate cyclase system.

Elimination of the putative palmitoylation site decreases the number of detectable cell-surface receptors

The selection procedure for identification of V2 receptor-expressing clones precludes the possibility of assessing the effect of a given mutation on cell-surface expression of receptors. To avoid clonal influence, the presence of V2 receptor mutants on the cell surface was examined in COS.M6 cells transiently expressing V2 receptors after transfection with recombinant plasmids. To this end, COS.M6 cells transfected with equal amounts of wild-type and C-341S/C-342S plasmid DNA were assayed for their capacity to bind [3H]AVP (Figure 4). Similar to

Figure 4 Comparison of specific [3H]AVP binding to intact COS.M6 cells expressing the wild-type V2 receptor or the double mutant C-341S/C-342S

Data points for specific binding of [3H]AVP to intact cells represent single values. Similar results were obtained in another independent experiment. The left panel shows binding isotherms: ■, wild type; □, C-341S/C-342S. The right panel shows the derived Scatchard transformations: ■, wild type ($K_D = 2.9$ nM; 1.95×10^{11} receptors/cell); □, C-341S/C-342S ($K_D = 2.6$ nM; 1.38×10^{11} receptors/cell). The K_D values were calculated for binding of [3H]AVP to intact cells. Unspecific binding contributed up to 30% of total binding.

Figure 5 Agonist-induced loss of specific [^3H]AVP-binding sites on COS.M6 cells expressing the wild-type V2 receptor or the double mutant C-341S/C-342S

Cells were preincubated for the indicated times with 100 nM AVP at 37 °C, and residual binding capacity for [^3H]AVP was determined following acid washes. Shown is specific [^3H]AVP binding to intact cells as the percentage of binding to cells unexposed to the hormone. ■ and ○, wild type; ▼ and △, C-341S/C-342S. The experiments were performed in duplicate (corresponding to the two symbols for each cell type). Similar results were obtained in two independent experiments. There was no detectable loss of cells with up to 24 h of preincubation with AVP.

data obtained with *Ltk*⁻ cells, the K_D values for [^3H]AVP binding to intact cells expressing the wild-type and the mutant receptors were essentially indistinguishable (2.9 and 2.6 nM respectively). The number of mutant receptors expressed on the cell surface, however, was reduced by 30% at saturating concentrations of [^3H]AVP. Similar results were obtained in another independent experiment (results not shown). In four other experiments, in which only a single concentration of [^3H]AVP (10 nM) was employed, the number of binding sites on cells expressing the double mutant was reduced to a similar degree (results not shown).

The data are consistent with a decreased delivery of the double-mutant receptor to the cell membrane or an increased rate of sequestration/internalization.

Elimination of the putative palmitoylation site delays agonist-induced loss of detectable cell-surface receptors

Previous studies have shown that agonist-induced desensitization of the V2 receptor is accompanied by a rapid loss of detectable AVP binding sites on the cell surface, presumably resulting exclusively from internalization [13]. To examine whether the reduced number of double-mutant receptors can be explained by an increased rate of sequestration/internalization, AVP-induced loss of [^3H]AVP binding to COS.M6 cells expressing wild-type and mutant receptors was measured. Cells were preincubated with non-labelled AVP (100 nM) for 5 min–24 h at 37 °C, hormone was removed by acid washes, and residual binding capacity was determined with [^3H]AVP to estimate the number of receptors remaining on the cell surface (Figure 5). The experiment confirmed previously published data that the apparent loss of wild-type receptor from the cell surface is a rapid process that is more than 80% complete within 5 min [13]. After 30 min of preincubation with AVP, only 15% of initially measured wild-type receptors were detectable. Surprisingly, the rate of loss of the mutant receptor was reduced over a period of at least 4 h to approx. 50% of that of the wild type. For example,

after 30 min of preincubation with AVP, about 35% of the initially determined cell-surface receptors were still present. No difference between wild-type and mutant receptors was found after preincubation with AVP for 24 h (9–10% and 6–9% of the initially detected binding sites were still present on cells expressing wild-type and mutant receptors respectively). These results suggest that the double-mutant receptor cannot be internalized/sequestered as efficiently as the wild-type receptor, when occupied by agonist.

DISCUSSION

We have eliminated the putative palmitoylation site of the human V2 vasopressin receptor to assess the functional consequences of this post-translational modification. Our results indicate that palmitoylation does not influence agonist binding. Except for the β_2 adrenergic receptor [7], the same was observed for all G-protein-coupled receptors so far examined. Furthermore, palmitoylation of the V2 receptor is apparently not necessary for full activation of the G_s /adenylate cyclase system. The minimal right-shift of the dose–response curves of mutants C-341S and C-341S/C-342S may reflect the relatively low number of receptors per cell or a slight aberration in overall receptor folding rather than specific impairment of coupling to G_s in the absence of palmitate side chains. Our results are by no means comparable with the dramatic decrease in adenylyl cyclase stimulation observed with the non-palmitoylated β_2 adrenergic receptor [7]. Instead, they agree with previous work that demonstrated the independence from palmitoylation of the signal-transducing properties of the m_2 muscarinic receptor [17], α_{2A} adrenergic receptor [8,9], rhodopsin [18], TRH receptor [10] and LH/hCG receptor [4].

In COS.M6 cells expressing the double mutant C-341S/C-342S, the number of receptors detectable on the cell surface reaches only 70% of the wild-type level (Figure 4), as previously observed for the non-palmitoylated LH/hCG receptor [4]. An impaired membrane insertion can be excluded because palmitoylation takes place in the *cis*-Golgi cisternae after insertion in the membrane of the endoplasmic reticulum [19]. Therefore the reduced number of detectable binding sites could result from a decreased transport from the *trans*-Golgi cisternae to the surface or an increased sequestration/internalization of mutant receptors. Since changes in transcription or translation efficiency cannot be excluded, it also remains possible that the total amount of receptor is lower in cells expressing the mutant as opposed to cells expressing the wild-type receptor.

Recently published data show that palmitoylation-deficient LH/hCG receptors are internalized at higher rates than wild-type receptors [4]. In contrast with these data, the loss of detectable AVP-binding sites following agonist incubation was decreased in COS.M6 cells transiently expressing the mutant C-341S/C-342S, suggesting that sequestration/internalization of the mutant V2 receptor was delayed rather than accelerated. Our results agree with the reduced internalization rate of the palmitoylation-defective TRH receptor, which was only 50% of that of the wild-type receptor [10].

In summary, V2 receptors with an eliminated putative palmitoylation site appear to be impaired in two related respects: those of transport to and rate of agonist-induced removal from the cell surface. A plausible explanation for this phenomenon would be that palmitate modifications are a signal for the vesicle-forming machinery or vesicle budding [19]. Alternatively, the fatty acids could be necessary to promote C-terminal conformations of G-protein-coupled receptors that interact with other components of the vesicular transport system.

Recent work has demonstrated the existence of palmitoylation-independent positive and negative endocytosis signals in the C-terminal cytoplasmic tail of the parathyroid hormone/parathyroid hormone-related protein receptor [20]. This finding may provide an explanation for the receptor-specific inhibitory [4] or stimulatory influences ([10]; this study) of palmitoylation on receptor endocytosis.

We thank Petra Kronich for excellent technical assistance. We also thank John Dickson for critical reading of the manuscript and many helpful suggestions during the course of this study. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 249).

REFERENCES

- 1 Breyer, M. and Ando, Y. (1994) *Annu. Rev. Physiol.* **56**, 711–739
- 2 Birnbaumer, M., Seibold, A., Gilbert, S., Ishido, M., Barberis, C., Antaramian, A., Brabet, P. and Rosenthal, W. (1992) *Nature (London)* **357**, 333–335
- 3 Lolait, S. J., O'Carroll, A.-M., McBride, O. W., König, M., Morel, A. and Brownstein, M. J. (1992) *Nature (London)* **357**, 336–339
- 4 Kawate, N. and Menon, K. M. J. (1994) *J. Biol. Chem.* **269**, 30651–30658
- 5 Ovchinnikov, Y. A., Abdulaev, N. G. and Bogachuk A. S. (1988) *FEBS Lett.* **230**, 1–5
- 6 Papac, D. I., Thornburg, K. R., Büllsbach, E. E., Crouch, R. K. and Knapp, D. R. (1992) *J. Biol. Chem.* **267**, 16889–16894
- 7 O'Dowd, B. F., Hnatowich, M., Caron, M. G. C., Lefkowitz, R. J. and Bouvier, M. (1989) *J. Biol. Chem.* **264**, 7564–7569
- 8 Kennedy, M. E. and Limbird, L. L. (1993) *J. Biol. Chem.* **268**, 8003–8011
- 9 Eason, M. G., Jacinto, M. T., Theiss, C. T. and Liggett, S. B. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11178–11182
- 10 Nussenzveig, D. R., Heinfinkel, M. and Gershengorn, M. C. (1993) *J. Biol. Chem.* **268**, 2389–2392
- 11 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor
- 12 Perbal, B. (1988) *A Practical Guide to Molecular Cloning*, 2nd edn., John Wiley & Sons, New York
- 13 Birnbaumer, M., Antaramian, A., Themmen, A. P. N. and Gilbert, S. (1992) *J. Biol. Chem.* **267**, 11783–11788
- 14 Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7413–7417
- 15 Birnbaumer, M., Gilbert, S. and Rosenthal, W. (1994) *Mol. Endocrinol.* **8**, 886–894
- 16 Salomon, Y., Londos, C. and Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548
- 17 van Koppen, C. J. and Nathanson, N. M. (1991) *J. Neurochem.* **57**, 1873–1877
- 18 Karnik, S. S., Ridge, K. D., Bhattacharya, S. and Khorana, G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 40–44
- 19 Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V. and Rothman, J. E. (1989) *Cell* **59**, 95–102
- 20 Huang, Z., Chen, C. and Nissenson, R. A. (1995) *J. Biol. Chem.* **270**, 151–156