Phosphorylation of the V2 Vasopressin Receptor*

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The V2 vasopressin receptor undergoes ligand-induced sequestration and desensitization (Birnbaumer, M., Antaramian, A., Themmen, A. P. N., and Gilbert, S. (1992) J. Biol. Chem. 267, 11783–11788). The V2 receptor expressed in transfected cells labeled with [32P]orthophosphate was phosphorylated following the addition of 100 nM arginine vasopressin (AVP). Phosphorylation was complete 5 min after addition of AVP, and was not stimulated by increased levels of Ca2+ or cAMP. The half-maximal dose of AVP that stimulated phosphorylation was 2.4 ± 0.4 nM, similar to the receptor Kd of 4.5 ± 0.4 nM. The role of phosphorylation on receptor desensitization was investigated by studying two vasopressin receptors 14 and 27 amino acids shorter than the wild type receptor. The missing segments were not needed for normal ligand binding or coupling to Gsa, but the last 14 amino acids were required for phosphorylation. The truncated receptors exposed to 100 nM AVP were sequestered and desensitized. The R137HV2R mutant receptor that binds vasopressin with wild type-like affinity and does not couple to Gsa (Rosenthal, W., Antaramian, A., Gilbert, S., and Birnbaumer, M. (1993) J. Biol. Chem. 268, 13030–13033) was phosphorylated and subjected to ligand-induced sequestration. These results established that phosphorylation is not essential for sequestration and desensitization of the V2 vasopressin receptor. Furthermore, they revealed that the conformation acquired after ligand occupancy is necessary for receptor phosphorylation and sequestration, while coupling to Gsa is not.

Exposure of receptors to ligands triggers not only their activation but also a chain of events, termed desensitization, that result in a reduction of cellular response to the agonist. Two major components have been identified for G protein-coupled receptors desensitization: sequestration/internalization of the occupied receptor (probably into vesicles physically separated from the plasma membrane), and phosphorylation of the intracellular domains of the receptor (1, 2). The first mechanism reduces the number of receptors available for signal transduction, while the second may reduce the activity of the receptor, either directly or by promoting binding of the receptor to inhibitory proteins that block signaling through G proteins (arrestin-like) (see Bennett and Sitaramayya (3) and Attramadal et al. (4)). Receptor phosphorylation can be catalyzed by cytosolic kinases activated by the increase in second messenger concentrations (such as protein kinase A or protein kinase C), or by any of the receptor kinases that can phosphorylate G protein-coupled receptors following their activation and interaction with G proteins (GRKs) (5–8). Some receptors, such as the β2-adrenergic and the angiotensin receptors, are phosphorylated by both mechanisms. The specific consequences of these phenomena on receptor activity have not yet been completely clarified, and it is still difficult to attribute the reduction in receptor function to one mechanism as opposed to the other (9).

The human V2 vasopressin receptor (V2R), is a member of the superfamily of G protein-coupled receptors that mediates the antidiuretic effects of vasopressin in the kidney. The V2R activated by arginine vasopressin (AVP) stimulates adenyl cyclase activity, and the now elevated levels of cAMP initiate a phosphorylation cascade that increases the water permeability of the collecting duct, thus facilitating water reabsorption (10). Our previous studies, utilizing stably transfected L cells expressing the human V2R encoded by its gene, characterized the agonist-induced desensitization and sequestration of this receptor (11). The data demonstrated that the human V2R was not desensitized by protein kinase A, a result later confirmed by our observation that the cDNA encoding the protein does not predict the existence of a protein kinase A consensus site for phosphorylation (12). Since it was still unknown whether the human V2R was subjected to ligand-induced phosphorylation, this possibility was examined when it became possible to isolate the V2R from transfected cells by immunoprecipitation. To carry out these studies, we expressed in COS and HEK 293 cells both the wild type V2R and a mutated vasopressin receptor cDNA in which asparagine 22 has been replaced by glutamine, thus destroying the only N-glycosylation acceptor site present in the protein. The levels of expression, ligand binding affinity and coupling to Gs for the nonglycosylated receptor (V2Rg) are identical to these parameters for the wild type glycosylated protein (13). While the wild type receptor migrates in SDS-PAGE as a broad band, the nonglycosylated receptor migrates as a discrete band, thus facilitating the quantification of radioactivity incorporated into the protein. Rabbit polyclonal antibodies, raised against peptides corresponding to a segment of the third intracellular loop and the carboxyl-terminal segment of the V2R, were used to immunoprecipitate the receptor from cell extracts.

Here we report that the human V2R is subjected to ligand-induced phosphorylation and describe the characteristics of this reaction. Two receptor truncations were also analyzed for their ability to undergo phosphorylation. The V2RgQ358t receptor retains the DE motif, a possible consensus site for GRK phosphorylation (14), and lacks the last 14 amino acids that

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1 The abbreviations used are: GRK, G protein receptor kinase; V2R, V2 vasopressin receptor; AVP, arginine vasopressin; PAG, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hanks’ balanced salt solution; PBS, phosphate-buffered saline; D-PBS, Dulbecco’s PBS; VIP, vasoactive intestinal peptide; RIPA, radiimmune precipitation buffer.
Phosphorylation of an Uncoupled V2R Mutant

10–100 μl of plasmid DNA in 1 ml EDTA, 10 mM Tris-HCl, pH 7.5, sterile H2O to bring the volume to 900 μl; 1 ml of 250 mM CaCl2 and 100 μl of 15 mM NaH2PO4, 50 mM HEPES, 150 mM NaCl, and 5 mM KCl, adjusted to pH 7.05 with NaOH. All reagents were added slowly dropwise, with gentle mixing after each addition.

10 min at room temperature, half the wash suspension was added dropwise to each plate containing cells and mixed by gentle swirling.

After 18 h in the incubator, the medium was removed and cells were treated with 2 ml of 25% glycerol in HBSS at 37 °C. After 1 min, the glycerol/HBSS mixture was diluted with 10 ml of HBSS, added slowly with continuous mixing.

The solution was aspirated, and the cells were rinsed once with HBSS. Fresh medium was then added, and the plates were returned to the incubator. The next day, the cells were trypsinized and diluted with the selection medium containing 400 μg/ml G418. Cells were then distributed into the wells of two 96-well microtiter plates (2,000–4,000 cells/well), using a Costar translate device. G418-resistant clones were picked after 16–18 days, and expanded in 6-well plates to assay for stimulation of adenyl cyclase activity, as described previously.

Phosphorylation of the V2R in Intact Cells—Transiently or stably transfected cells were plated in 6-well plates (6–8 × 105 cells/well for HEK, 1.5 × 106 cells/well for COS). After 18 h, the cells were washed and incubated for 30 min with phosphate-free minimal essential medium, followed by the addition of 100 μCi/well [32P]H3PO4. After 2 h at 37 °C, the cells were exposed to vasopressin or other agents for the times and concentrations described in the text. Following treatment, the plates were chilled on ice and washed twice with phosphate-buffered saline, and the cells were solubilized for 1 h at 4 °C in 300 μl of RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1% Nonidet P40, 0.1% sodium deoxycholate, 0.1% SDS) with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin) and phosphatase inhibitors (10 mM sodium pyrophosphate, 10 mM NaF, 300 mM okadaic acid). Solubilization was helped by drawing the cells through needles of decreasing gauge (20 G, 25 G) fitted to a 1-ml plastic syringe. Cell extracts were then clarified by addition of 50 μl of a 50% slurry of prewashed protein A-Sepharose in RIPA and centrifugation. Prewashed protein A-Sepharose was pre pared by treating for 1 h with 25 mg/ml bovine serum albumin in RIPA, followed by two washes with RIPA alone. Two rabbit polyclonal antibodies purified by affinity column were used for immunoprecipitation.

The antibodies were raised against peptides corresponding to the third intracellular loop (peptide VGPSERPGGRRGR, antibody no. 2), or the carboxyl terminus (peptide ARGRTPPSLGPNDES, antibody no. 3) of the human V2R. The anti-peptide polyclonal antibodies and the 12CA5 monoclonal (anti-HA epitope) antibody immunoprecipitated the same proportionally labeled protein from transfected cells (13, 18). The clarified extracts were incubated overnight at 4 °C with 9 μg/ml peptide purified antibody, and the formed antigen/antibody complexes separated by incubating with prewashed protein A-Sepharose for 2 h at 4 °C.

The beads were centrifuged and washed five times with 600 μl of RIPA (4 min), recovering them each time by centrifugation. The proteins were eluted for 20 min at room temperature with 80 μl of 2 × Laemmli buffer containing 10% (β-mercaptoethanol), and samples were dried in 10% polyacrylamide gels and visualized by exposing the dried gels to Kodak-Omat film at −70 °C. Quantification of the 32P incorporated into proteins was performed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Identical rectangles containing the bands of interest were drawn to circumscribe the areas to be integrated. The values obtained were normalized for each sample, using a 36-kDa nonspecific band present in precipitated extracts from naive HEK cells when antibody no. 2 was used. Variation in the intensity of the 36-kDa band was due to differences in the manipulation of the Sepharose beads, not to the extent of phosphorylation of the receptor. Background measured at the level of migration of the receptor in lanes containing samples of nontransfected cells was subtracted from these values. The results are expressed as percent of the phosphorylation detected at 30 min (Fig. 4A), or as percent of the phosphorylation detected with 100 mM AVP (Fig. 4B). The data in the graph are the means of three independent experiments ± S.E.

Hormone Binding to Intact Cells—HEK 293 cells were plated in 24-well plates previously treated for 1 h with polylysine at a density of 1.5–2.5 × 105 cells/well. Binding assays were performed the following day. Cells were washed twice with ice-cold D-PBS, after which the wells received 0.5 ml of ice-cold D-PBS with 1 mM tyrosine, 1 mM phenylalanine, 0.2% glucose, 2% bovine serum albumin, and the appropriate dilution of [3H]AVP. Plates were incubated for 2 h on top of crushed ice in the cold room before removal of the binding mixture by aspiration. After quickly rinsing twice with ice-cold D-PBS, 0.5 ml of 0.1 N NaOH was added to each well to extract bound radioactivity. After 30 min at
37 °C, the fluid from the wells was transferred to scintillation vials containing 3.5 ml of Beckman ULTIMA-FLO M (Packard, Meriden, CT) scintillation fluid for radioassay. Non specific binding was determined under the same conditions in the presence of 10 µM unlabeled AVP (13). Binding experiments were performed at least three times. The results are reported as averages ± S.E.

Hormonal Treatment of Intact Cells—Desensitization treatments with 100 nM AVP were carried out in DMEM-high glucose at 37 °C and terminated by rinsing the cells sequentially with 2 ml of ice-cold D-PBS, followed by two 40-s washes with 2 ml of an ice-cold solution of 5 mM acetic acid in Tris-buffered saline, and three rinses with normal D-PBS to ensure restoration of neutral pH. Cells were harvested to prepare homogenates, or subjected to saturation binding assays for receptor sequestration experiments.

Homogenate Preparation—Cells rinsed with D-PBS plus Ca2+ and Mg2+ were scraped off the dishes with a rubber policeman and were centrifuged. The supernatant was discarded. The cell pellet was resuspended in approximately 10 volumes of ice-cold 27% (w/v) sucrose, 1 mM EDTA, and 20 mM Na-HEPES, pH 7.8 (homogenization buffer) and homogenized in a Dounce homogenizer with 10 strokes of a tight-fitting pestle. Aliquots of the homogenate were used within 30 min to test for adenyl cyclase activities.

Adenyl Cyclase Activity in Cell Homogenates—Adenyl cyclase activity was assayed as described previously (11). The incubation medium contained, in a final volume of 50 µl, 0.1 mM [α-32P]ATP (1–5 × 106 cpm), 4.0 mM MgCl2, 10 µM GTP, 1 mM EDTA, 1 mM [3H]cAMP (~10,000 cpm), 2 mM isobutylmethylxanthine, a nucleoside triphosphate regenerating system composed of 20 µM creatine phosphate, 0.2 mg/ml (2,000 units/mg) creatine phosphokinase, 0.02 mg/ml myokinase (448 units/mg), and 25 mM Tris-HCl, pH 7.4. Incubations were at 32 °C for 20 min. Hormones (diluted in 1% bovine serum albumin) were present at concentrations indicated in the figures. Reactions were stopped by the addition of 100 µl of a solution containing 40 mM ATP, 10 mM cAMP, and 1% sodium dodecyl sulfate. The cAMP formed was isolated by a modification of the standard double chromatography over Dowex-50 and alumina columns (19, 20). Under these assay conditions, cAMP accumulation was linear with time of incubation, for up to 40 min, and proportional to the amount of homogenate. The activities were expressed as picomoles of cAMP formed per min per mg of homogenate protein. Protein concentration of the samples was determined by the method of Lowry (21) using bovine serum albumin as standard.

Statistical Analysis of the Data—The values obtained with the PhosphorImager, the AVP binding data, and the adenyl cyclase activity determinations, shown in Figs. 4–6, were fitted by the least square method using logistic functions. The minimization of the square differences was calculated with the function Solver in Microsoft Excel 5.0 C.

The values reported in these figures were obtained from the fitting curves. A paired sample t test was applied when mentioned; differences were considered statistically significant if p < 0.005.

RESULTS

Ligand-dependent V2R Phosphorylation—To assess whether the V2 receptor undergoes ligand-induced phosphorylation, two different recombinant cDNAs were transfected into COS cells, one encoding the wild type V2R, the other encoding a mutant V2R cDNA in which asparagine in the glycosylation consensus sequence had been substituted by glutamine (V2RQ). Cells previously incubated with 32P were exposed to 100 nM AVP for 15 min at 37 °C. Extraction and immunoprecipitation of the V2 receptor was performed as described under “Experimental Procedures” using rabbit polyclonal antibody no. 2. As illustrated in Fig. 2, the treatment with AVP promoted phosphorylation of the receptor. Cells expressing the wild type V2R yielded a phosphorylated broad band at 45–55 kDa that coincides with the migration observed for the glycosylated receptor (13). Cells expressing the nonglycosylated receptor treated under similar conditions yielded a radioactive band of approximately 40 kDa that had been previously identified as the nonglycosylated form of the V2R. In both cases, the phosphorylation of the receptor protein was AVP-dependent. Since this result proved that the absence of sugar did not interfere with the incorporation of phosphate into the receptor protein, and we had previously demonstrated the nonglycosylated receptor to be fully active (13), subsequent experiments to characterize V2R phosphorylation were performed with cells transiently or stably transfected with a plasmid encoding the nonglycosylated receptor (V2RQ) to improve the quantitation of the radioactive band.

To examine the specificity of the reaction, and whether the kinase(s) responsible for the phosphorylation of the V2R could be activated by second messengers, HEK 293 clonal cells expressing the V2RQ were treated with 100 µM ATP or 100 nM VIP to stimulate receptors intrinsic to these cells. In addition, cells were treated with 100 µM forskolin to increase intracellular levels of cAMP, or with 400 nM phorbol 12-myristate 13-acetate to test for the possible involvement of protein kinase C in receptor phosphorylation. As shown in Fig. 3, all these treatments failed to stimulate phosphorylation of the V2R, although they induced incorporation of radioactive activity in several of the proteins that coprecipitated with the receptor, thus modifying the radioactive background in all lanes. Treatment of transiently transfected COS cells expressing the V2R with radioactive phosphate and 10 µM isoproterenol, or with 100 µM ATP to stimulate receptors intrinsic to the COS cells also failed to promote phosphorylation of the V2R (data not shown).

Exposure of the cells to 100 nM AVP at 37 °C induced a fast rate of receptor phosphorylation. As illustrated in Fig. 4A, the incorporation of radioactive phosphate into the receptor protein was already detectable 30 s after AVP addition, the shortest time analyzed. Receptor phosphorylation reached apparent saturation in less than 10 min, and the level of radioactivity in the receptor band remained unchanged for at least 30 min in the presence of AVP. Experiments were performed to correlate phosphorylation and the extent of receptor occupancy by AVP. As shown in Fig. 4B, treatment with increasing concentrations of AVP for 15 min resulted in a concomitant increase in receptor phosphorylation, with an EC50 of approximately 2.4 ± 0.4 nM. This value is similar to the Kd of binding to vasopressin for...
the V2R, 4.5 ± 0.4 nM (see Innamorati et al. (13) and Fig. 6). Thus, the experiments established that, as described for other receptors, the activity of the kinases involved depends on occupancy of the binding site by the agonist, strongly suggesting that a form of GRK was responsible for V2R phosphorylation.

The R137H mutant V2R, previously characterized by Rosenthal et al. (23) was examined next. This mutation, found in patients suffering the X-linked form of nephrogenic diabetes insipidus, encodes a full-length receptor that binds AVP with the same KD as the wild type receptor but fails to stimulate adenylyl cyclase activity (23). Experiments to test whether this mutant receptor could be phosphorylated by receptor kinases were performed. As previously reported, the R137H V2R is expressed at a lower level than the wild type receptor, 0.7 compared to 6.0 × 10⁶ sites/cell in our latest experiments. Immunoprecipitation of the receptor with antibody no. 3 helped to reduce the radioactive background in the vicinity of the receptor band and thus increased the sensitivity of the assay. Quantification of ligand-dependent phosphorylation with the PhosphorImager detected 7.5 ± 0.4% phosphorylation in the R137H mutant compared to 100% for the wild type V2R. In the experiment shown in Fig. 5, the usual volume of extract containing the mutant receptor was loaded along with 0.1 volume of the extract containing wild type V2R to compensate for the reduced level of expression. As illustrated, it was evident that that the R137H V2R was phosphorylated in an AVP-dependent manner. A phosphorylated protein was detected at the same location prior to vasopressin treatment. COS.M6 cells not expressing the V2R were labeled with [32P]orthophosphate and incubated at 37 °C to 10 μM isoproterenol to stimulate their cAMP production, and then subjected to extraction and immunoprecipitation with antibody no. 3. No radioactive bands were detected at the position where the receptor migrates, suggesting that the band seen in transfected cells was the mutant protein. Unable to identify a transfection-independent phosphorylated protein at this location, it was concluded that the mutant V2R has a detectable level of basal phosphorylation.

Role of the Carboxyl Terminus in V2R Phosphorylation—As in many other G protein-coupled receptors, the carboxyl-terminal portion of the V2R is rich in serines and threonines. To explore whether this segment played a role in phosphorylation, we mutagenized the V2R cDNA, introducing two premature stop codons at Cys-358 and Gly-345 to direct the synthesis of truncated receptor proteins. As shown in a representative experiment illustrated in Fig. 6, the truncations did not alter the binding affinity of the mutant receptors for AVP. The average KD values calculated from three independent experiments were: 4.8 ± 0.7, 6.1 ± 1.3, and 4.7 ± 0.6 nM for the V2R, the V2RQ358t, and the V2RQ345t receptors, respectively. The level of expression of the truncated receptors, assessed by performing saturation binding assays in transiently transfected cells, was compared to that of the wild type receptor. Whereas the Bmax value obtained with the V2RQ358t protein was similar to that obtained with the full-length V2RQ (136 ± 6%), the Bmax value for the V2RQ345t receptor was 45 ± 8% lower than that of the V2RQ. The mutant receptors were next tested for their ability to mediate AVP stimulation of adenylyl cyclase activity by assaying homogenates prepared from transiently transfected cells. As illustrated in Fig. 7, no significant differences were detected either in the extent of stimulation of adenylyl cyclase activity, nor in the EC50 values of these stimulations among the V2RQ358t, V2RQ345t, and full-length receptor. Thus, reduced expression of the V2RQ345t receptor did not result in a detectable difference in its ability to stimulate the adenylyl cyclase system.

Although, according to the saturation binding data, both truncated proteins were present at levels comparable to those of the full-length receptor when tested in phosphorylation experiments, it was not possible to detect AVP-dependent incorporation of phosphate into either truncated receptor protein, revealing that the last segment of the carboxyl terminus is

**Fig. 4. Time and dose dependence of V2R phosphorylation.** Stably transfected HEK 293 cells expressing the V2RQ were labeled with [32P]orthophosphate and incubated at 37 °C in the presence of 100 nM AVP for the times indicated in the abscissa (panel A), or incubated for 15 min at 37 °C in the presence of the indicated concentrations of AVP (panel B). Immunoprecipitation and electrophoresis of the labeled proteins were performed as described in the legend of Fig. 2. The radioactive bands corresponding to the V2RQ were quantified with a Molecular Dynamics PhosphorImager. The experiments were performed three times, and the data are expressed as means ± S.E. Half-maximal phosphorylation was obtained after 1.8 ± 0.15 min.

**Fig. 5. Agonist effect on V2RQ and V2RQ-R137H receptor phosphorylation.** COS M6 cells transfected with each construct were labeled with [32P]orthophosphate 2 days after transfection and treated for 15 min at 37 °C with vehicle or 100 nM AVP. The receptors were extracted as described under "Experimental Procedures," immunoprecipitated with antibody no. 3, and identified by SDS-PAGE and autoradiography. The usual volume of extract containing the V2R R137H samples, 20 μl, was loaded along 2 μl of extract containing wild type receptor.
required for phosphorylation of the V2R. As illustrated in Fig.
8, no ligand-induced phosphorylation was detected for the
V2R358t immunoprecipitated with antibody no. 3 and ana-
yzed along the R137H and the wild type V2R. The suscepti-
bility of the truncated receptors to AVP-induced desensitiza-
tion was examined next. Transiently transfected cells
expressing the full-length and the truncated V2Rs were ex-
posed to 100 nM AVP, and the ability of the receptors to mediate
AVP stimulation of adenylyl cyclase activity was assessed in
homogenates of those cells. The maximal extent of desensitiza-
tion was evaluated for each construct in several independent
experiments. As shown in Fig. 9, the truncated forms of the
receptor were desensitized by exposure to 100 nM AVP. The
V2R358t showed only 15 ± 5% desensitization, while the
V2R345t showed 26 ± 3% desensitization compared to 35 ±
3% desensitization of the V2R4. Impaired desensitization of
the truncated forms might be due to the absence of phosphoryla-
tion; the difference observed between the truncated receptors
revealed the presence of segments in the carboxyl terminus
capable of modulating desensitization.

Sequestration of Nonphosphorylated Receptors—To examine
the role of phosphorylation in the sequestration/internalization
of the V2R, the extent of sequestration that could be achieved
with the fully active truncated receptors was compared to that
of the full-length receptor. As shown in Fig. 10, when exposed
to 100 nM AVP the truncated receptors disappeared from the
cell surface, although at a lower rate than the full-length V2R4.
Thus, the last 25 amino acids of the V2R were not required for
sequestration to occur. Deleting the last 14 amino acids from
the V2R impaired sequestration to a greater extent than delet-
ing the last 27 amino acids of the carboxyl-terminal segment.
A similar finding has been reported for the calcitonin receptor
(22). These data indicated that, for the V2R protein, phos-
phorylation was not required for receptor sequestration. Neither
was sequestration blocked by the absence of coupling activity.
As shown in Fig. 11, the R137H V2R mutant expressed in
stably transfected L cells can be sequestered in 10-min expo-
sure to 100 nM AVP to a similar extent as the wild type recep-
tor. Furthermore, the wild type and the mutant receptor bind-
ing sites returned to the cell surface with the same time course
following ligand-induced sequestration.

DISCUSSION

Similar to what has been described for other G protein-
coupled receptors, binding of agonist to the V2 vasopressin
receptor caused phosphorylation of the protein. Unlike what
has been reported for the β2-adrenergic or parathyroid hor-
monereceptors(6,7), therewas minimal phosphorylation of
the wild type V2R in the absence of ligand. While characteriz-
ing the pattern of expression of the V2R, Sadeghi et al. (18)
demonstrated that only a 35S-labeled broad band migrating
between 45 and 55 kDa represented properly processed glyco-
sylated vasopressin receptor, the other receptor bands present
consisting mostly of immature receptor, as revealed by the
composition of the sugar moiety. This immature protein was
also apparent when the nonglycosylated receptor was ex-
pressed and represented 50% or more of the V2R molecules
isolated from transiently transfected cells. Those experiments
provided no information as to the location of the immature
protein in the cell, nor did they address the issue of whether
this protein was functional to any extent. The observation that
the immature receptor protein was not phosphorylated in re-
sponse to AVP suggested that this protein may not reach the
plasma membrane, or if present on the cell surface, it cannot be
a substrate for phosphorylation. The data indicate that only the
mature receptor present on the plasma membrane (migrating
at 40 kDa when the V2R is not glycosylated), can interact with
the ligand and promote its own phosphorylation.

Because binding of AVP was a requirement for V2R phos-
phorylation and the extent of radioactive phosphate incorpora-
tion was proportional to the degree of occupancy of the receptor
by agonist, the involvement of one of the kinases able to rec-
ognize G protein-coupled receptors as substrate was suggested
(5). Incorporation of phosphate into the receptor came to com-
pletion within a few minutes, and it was sustained for as long
as 30 min after addition of 100 nM AVP to the cells. As Fig. 3
illustrates, there was no detectable decay in the amount of
phosphorylated receptor that could be isolated during this pe-
riod. Probably the majority of the receptors had been removed
from the cell surface in 30 min, thus these results suggest that
the phosphorylated protein does not suffer significant degra-
dation after sequestration/internalization. It is possible that the
phosphorylated receptor, once sequestered, might recycle
quickly back to the cell surface after losing its phosphate, to be
phosphorylated again once occupied by AVP, or that after phos-
Phosphorylation of an Uncoupled V2R Mutant

100 nM AVP – ++ – – – – –

FIG. 8. Agonist effect on V2R0, V2R-R358t, and V2R-R137H receptor phosphorylation. COS-M6 cells transfected with each construct were labeled with [32P]orthophosphate 2 days after transfection and treated for 15 min at 37°C with vehicle or 100 nM AVP. The V2R immunoprecipitated with antibody no. 3 was identified by SDS-PAGE and autoradiography. Twenty microliters of extract were loaded per lane.

FIG. 9. Desensitization of V2R0, V2R-R358t, and V2R-R345t receptors. HEK 293 cells transfected with the three different constructs were exposed to vehicle or to 100 nM AVP for 15 min at 37°C. After an acid wash to remove surface ligand, as described under “Experimental Procedures,” cell homogenates were prepared, and the ability of the receptors to mediate vasopressin stimulation of adenylyl cyclase activity was assessed in the presence of 100 nM AVP. Percent desensitization was calculated by dividing the difference in maximal activity between the vehicle and AVP treated samples by the maximal activity measured in the vehicle-treated sample. The results are presented as means ± S.E. For V2R4 and V2R-R345, n = 7, for V2R-R358t, n = 4. The average of the adenylyl cyclase activities at 100 nM VIP expressed as picomoles of cAMP/min/mg in these experiments were: 59.0 and 54.0 for V2R4; 59.0 and 56.0 for V2R-R345t; and 50.0 and 49.0 for V2R-R358t, for the vehicle and the 100 nM AVP-treated cells, respectively.

FIG. 10. Time course of AVP-induced sequestration of V2R0, V2R-R358t, and V2R-R345t receptors in transiently transfected HEK 293 cells. HEK 293 cells transfected with the three different constructs were exposed to vehicle or to 100 nM AVP at 37°C for the times indicated to stimulate hormone-dependent sequestration. Cells were then chilled, washed with PBS, subjected to an acid wash to remove bound ligand, and neutralized as described under “Experimental Procedures.” Saturation binding assays were performed to determine the number of high affinity binding sites remaining on the cell surface.

FIG. 11. Agonist effect on V2R0 and V2R-R137H receptor sequestration. Stably transfected L cells expressing the wild type V2R (wt) or the V2R-R137H (Q2–3) were treated for 10 min at 37°C with vehicle or 100 nM AVP. Cells were chilled, washed with PBS, subjected to acid wash to remove bound ligand, and neutralized as described under “Experimental Procedures.” Growth medium was added, and the cells were returned to the incubator for the times indicated. Binding assays at 20 nM [3H]AVP in the presence and absence of 10 μM cold AVP were performed in replicate at the indicated times to determine the number of binding sites present on the cell surface.

The observation that the truncated receptors were able to fully stimulate adenylyl cyclase activity, revealed that the carboxyl terminus, although required for phosphorylation, did not play a significant role in coupling the receptor to Gα. These data are consistent with the activity shown for the V1a/V2 chimeras reported by Liu and Wess (24), in particular the chimera where the whole segment of the V2R after the seventh transmembrane domain had been replaced by the equivalent segment of the V1a receptor.

When cells expressing the truncated receptors were exposed for 20 min to 100 nM AVP, the extent of sequestration observed was less pronounced for the V2R-R358t than for the V2R-R345t protein. Deletion of the last 14 amino acids resulted in a significant reduction in receptor sequestration, while deletion of the next 13 amino acids restored the ability of the receptor to be removed from the cell surface. These data suggested not a requirement but instead a modulatory role for this distal segment in the sequestration of the V2R, locating the sequestration signal itself elsewhere in the protein. The modulatory role of the terminal segment could depend on its interaction with arrestin-like molecules, known to participate in this process (25, 26). Because the missing protein fragments are rich in serines and threonines, a correlation between absence of phosphorylation and the reduction in sequestration of the truncated receptors is likely.

As originally described for rhodopsin, activated G protein-coupled receptors are phosphorylated by GRK, and as a consequence are able to bind β-arrestin with increased affinity. Recently, Ferguson et al. (26) reported that deletion of the COOH terminus at Cys-341, or substitution of the phosphorylation sites of the β2-adrenergic receptor resulted in reduction but not loss of internalization. As a matter of fact, these mutant β2-adrenergic receptors were sequestered 50% as well as the wild type, a result similar to what has been observed with the V2 receptor. In the same report, these authors describe a role...
for β-arrestin in promoting agonist-induced sequestration of nonphosphorylated β2-adrenergic receptor. If arrestin binding promotes sequestration, the existence of residual sequestration for the nonphosphorylated β2-adrenergic and the V2 receptors suggests that the intervention of the GRKs enhances but does not cause removal of receptors from the cell surface.

The structure of the occupied receptor, not the activation of a G protein, seems crucial for activating the receptor kinases. The observation that the unoccupied R137H mutant V2R is phosphorylated in response to agonist, indicates that it is possible to dissociate G protein activation from GRK activation as different functions of an activated receptor (27). Despite its functional flaws, the R137H V2R is recognized by the sequestration apparatus with only a small reduction in efficiency, and it can recycle to the cell surface after removal of the ligand in a manner similar to the wild type receptor. The phosphorylation in the absence of ligand detected for the R137H V2R is probably a unique property of the mutant receptor. Since the wild type receptor is present at 10-fold higher levels, it should be easy to detect a similar basal level of phosphorylation if it was present.

Although it is widely accepted that phosphorylation plays an important role in the desensitization of G protein-coupled receptors, it has not yet been clarified how or to what extent the presence of phosphate groups reduces coupling to G proteins. For other G protein-coupled receptors, the effect of second messenger dependent kinases can complicate the analysis of the data, while in the case of the V2R, there is no such interference. Nevertheless, a ligand-induced reduction in truncated receptors activity was observed in the absence of detectable phosphorylation. These data have revealed that, for the V2R, eliminating GRK phosphorylation reduces the extent of desensitization but does not block it.

In conclusion, we have demonstrated that the V2R is phosphorylated when occupied by AVP and that the last 14 amino acids of the protein are required for this effect. We also have demonstrated that abolishing phosphorylation by GRK reduced the extent of desensitization but did not block it. Thus, as observed for receptor sequestration, we concluded that GRK-catalyzed phosphorylation plays an enhancing, not a permissive, role in these phenomena.

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