An expanded V2 receptor retention signal

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Abstract Following ligand-promoted internalization the human type 2 vasopressin receptor (hV2R) is not recycled to the cell surface after removal of the agonist. A retention motif consisting of a serine triplet present in the cytoplasmic tail was previously found to require for retention, but serine 357, and threonines 359, 360 located upstream were not examined. Evidence is now presented that substitution of these amino acids did not change V2 internalization although it reduced the levels of arginine vasopressin (AVP)-induced phosphorylation as compared to the wild type (WT). Contrary to the WT hV2R, these mutant receptors were recycled to the cell surface after a 2 h incubation in the absence of AVP identifying these changed residues as additional members of the retention motif of the hV2R.
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Key words: V2 receptor; Phosphorylation; Recycling; Dephosphorylation; Retention motif; Arrestin

1. Introduction

Arginine vasopressin (AVP) is a peptide hormone that regulates water homeostasis by binding to specific receptors of the G protein-coupled receptor (GPCR) superfamily [1]. As is the case for many GPCRs the type 2 vasopressin receptor (V2R) itself is rapidly phosphorylated after activation by AVP by a G protein-coupled receptor kinase (GRK) [2].

Receptor phosphorylation has been shown to accelerate receptor uncoupling from G proteins and initiate cell desensitization to subsequent stimulation by the same agonist [3]. For most receptors, this process is reversible. Resensitization involves internalization of the phosphorylated receptor followed by ligand dissociation and loss of the incorporated phosphates [4]. The dephosphorylated receptors return to the cell surface ready to commence another round of cellular response. A few members of the GPCR family, such as the endothelin B and the protease-activated receptors [5–8], are sorted to lysosomes for degradation instead of recycling to the cell surface. Although the human V2R (hV2R) is not recycled to the cell surface after ligand-induced internalization, it is not targeted to lysosomes as the protein remains intact and phosphorylated hours after removal of the agonist [9].

The hV2R has nine amino acids susceptible to phosphorylation downstream of the Asp-Glu motif recognized by GRKs [10]. Sequential deletion of the last residues led to a progressive decrease in the level of phosphorylation and conferred recycling to the truncated receptor. Site-directed mutagenesis of serines 362, 363, or 364 by alanine revealed the importance of this cluster in the retention phenomenon since all the mutant proteins recycled [11].

To verify whether the 'retention' motif is limited to these three serines or includes other residues susceptible to phosphorylation, serine 357 and threonines 359 and 360 were individually substituted by alanine (Fig. 1A) and the mutant hV2Rs examined for level of phosphorylation, internalization and recycling.

2. Materials and methods

2.1. Materials

2.2. Mutagenesis of the hV2R

A previously described polymerase chain reaction (PCR)-based methodology was used to introduce the mutations into the cDNA encoding for the wild-type (WT) hV2R [11].

2.3. Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose containing 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml of penicillin and 50 mg/ml of streptomycin. For transient expression of the V2R, cells were plated at 3.5×10^6/100 mm dish and transfected the following day as previously described [11]. Cells were assayed 2 days later.

2.4. Receptor phosphorylation

Cells seeded in six-well plates ($\sim 6 \times 10^5$ cells/well) previously coated with polylysine (Sigma) were labeled with 100 µCi/well of H_3 $^{32}PO_4$ for 2 h at 37°C. AVP was added to make the medium 100 nM AVP and the incubation continued at 37°C for the times indicated. Cells were then chilled on ice and processed as previously described for receptor immunoprecipitation with an antibody raised against the cytoplasmic tail of the hV2R [2]. The precipitated proteins were separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE), the gel was dried and the bands were visualized by autoradiography and quantified using the OptiQuant® software from the Cyclone® Storage Phosphor Scanner (Packard Instrument Company, Inc.) system. Gel loading was normalized for cell surface expression determined by [^{3}H]AVP binding.

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Abbreviations: hV2R, human type 2 vasopressin receptor; GRK, G protein-coupled receptor kinase

2.5. Binding assay

Transfected cells seeded in polylysine-treated 24-well plates at a density of 1.5×10^5 cells/well were washed twice with ice-cold D-phosphate-buffered saline (PBS) and exposed to 25 nM of [^{3}H]AVP in 250 μl of D-PBS containing 2% bovine serum albumin (BSA) to determine the number of binding sites/cell as previously described. Non-specific binding was determined in the presence of 10 μM unlabeled AVP. Binding experiments were performed in triplicate.

2.6. V2R sequestration and recycling

Cells were plated in polylysine-treated 24-well plates at a density of 1.5×10^5 cells/well 24 h after transfection. The following day the cells were challenged with 100 nM AVP in DMEM high glucose containing 1% BSA and 20 mM HEPES (pH 7.4) for 20 min at 37°C. Plates were then chilled on crushed ice and the cells washed twice with cold D-PBS, twice with ice-cold 150 mM NaCl/5 mM acetic acid and finally three times with D-PBS. 500 μl of DMEM/BSA/HEPES solution was added to each well and the cells were returned to 37°C for the indicated time. The number of receptors on the cell surface was then evaluated by [^{3}H]AVP binding as described above. Results are expressed as a percent of the number of receptor sites present before hormone treatment. Data are presented as means ± S.E.M. of at least three independent experiments.

3. Results

To determine whether the putative phosphorylation sites T360, T359 and S357 of the hV2R contribute to the retention motif, these residues were individually substituted by alanine and the internalization and recycling of the resulting mutant receptors were examined. The WT and S363A V2Rs were used as reference in these experiments. As it was the case for all mutations and truncations modifying the last 20 amino acids of the V2R, the mutant proteins were expressed at the same level as the wild-type receptor and its binding and coupling to Gs remained unchanged [2,11].

The impact of the mutations on internalization and recycling was examined first. Cells expressing the WT receptor lost 37% of the surface receptors after a 20 min exposure to 100 nM AVP at 37°C, and under the same conditions the same fraction of surface receptor was lost for all the hV2R mutants. Following the removal of the external AVP, acid wash, and the addition of fresh medium, plates were returned to the 37°C incubator for different times. As seen before, after a 2 h incubation there was no change on the abundance of WT V2R on the cell surface, whereas all the mutant V2Rs exhibited recycling after the agonist was removed. The S363A V2R receptor abundance returned to ∼95% of the original
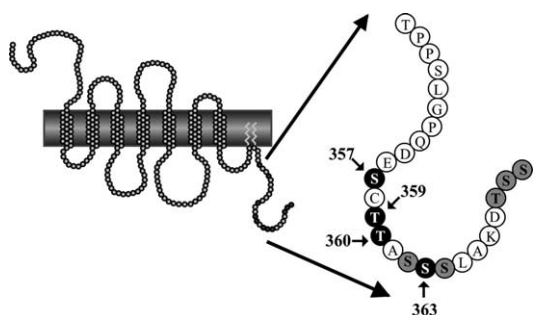

Fig. 1. Schematic representation of the hV2R. The amino composition of a section of the cytoplasmic tail containing the substituted residues (on a black background) is represented next to the receptor. The substitution S363A in the triple serine sequence has been described previously [10]. In this study, the residues 357, 359 and 360 were individually substituted by alanine and compared to the WT and S363A mutant V2Rs.
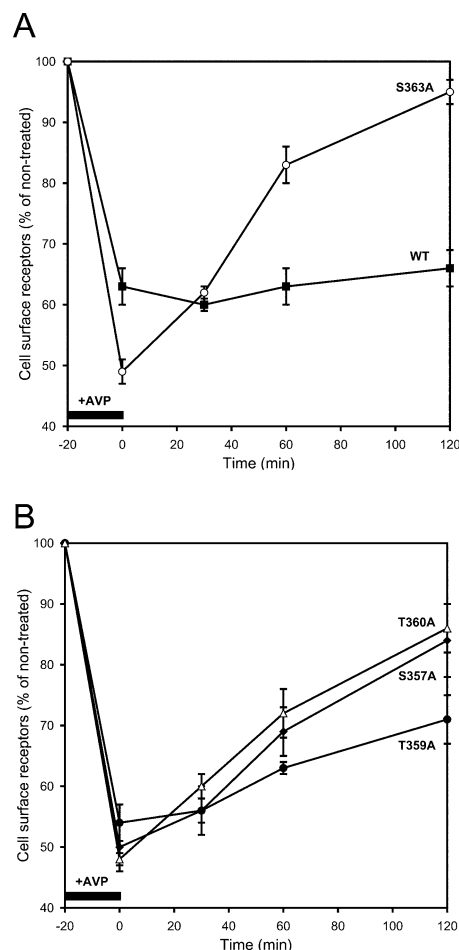

Fig. 2. AVP-induced sequestration and recovery of WT and mutant hV2Rs. Receptor density on the cell surface was evaluated by [^{3}H]AVP binding on HEK293T transiently expressing the WT (■, $n = 7$), S363A (○, $n = 5$), S357A (♦, $n = 4$), T359A (●, $n = 4$) or T360A (△, $n = 4$) V2Rs. After a treatment with 100 nM AVP for 20 min at 37°C, cells were washed and incubated at 37°C in complete medium for the indicated recovery time. [^{3}H]AVP binding was performed as described in Section 2. Results (mean ± S.D.) are expressed as percent of the number of receptor sites present before hormone treatment.

value, the S357A and T360A V2Rs recovered 84 ± 6% and 86 ± 4% of binding sites, respectively, whereas a lower recovery was observed for the T359A mutant receptor. For this protein, the abundance returned only to 71 ± 4% of the sites present on non-treated cells. Data from these experiments are illustrated in Fig. 2 and summarized in Table 1.

The extent and time course of phosphorylation of the recycling mutant proteins were examined next, the results are shown in Fig. 3. The data are expressed as percentage of the level of phosphorylation obtained for the WT receptor after 30 min of exposure to AVP. To compare the levels of phosphorylation of the proteins, the amount of immunoprecipitated labeled receptors loaded in the gels was normalized for differences in cell surface expression, as assessed by [^{3}H]AVP binding done in parallel for each transfection. The WT and mutant V2Rs were rapidly phosphorylated in the presence of AVP, the level of phosphorylation reaching a plateau between 5 and 15 min and remaining stable afterwards. The results suggested that the variation in the level of phosphorylation is dictated by the identity of the altered site; no evidence of
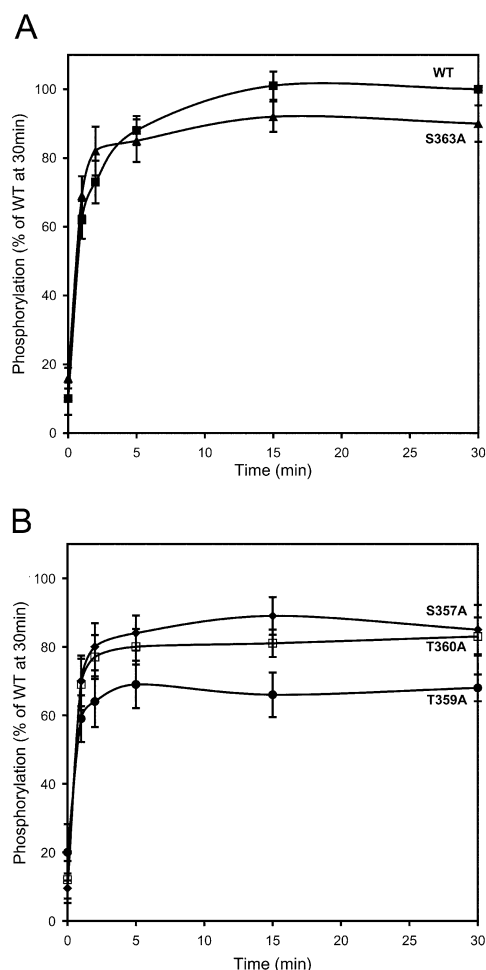
Fig. 3. AVP-induced phosphorylation of WT and mutant hV2R. Receptor phosphorylation was examined in HEK293T cells transiently expressing the WT (■, $n = 9$), S363A (▲, $n = 3$), S357A (♦, $n = 3$), T359A (●, $n = 4$) or T360A (□, $n = 5$) V2Rs. Cells were labeled with inorganic ^{32}P and stimulated for the indicated time with 100 nM AVP at 37°C. Receptor proteins were immunopurified as described in Section 2, resolved by SDS–PAGE, and analyzed for incorporated radioactivity using the OptiQuant™ software from the Cyclone™ Storage Phosphor Scanner system. The background was determined for each lane and subtracted from the value obtained from the receptor band. The results (mean ± S.D.) were normalized for cell surface expression and are expressed as a percentage of the value obtained at 30 min with the WT V2R.

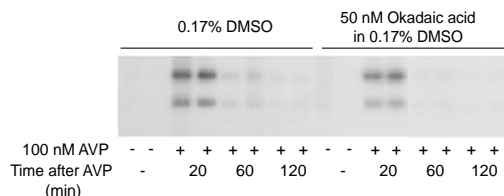Dephosphorylation of the S363A-V2R

Fig. 4. Dephosphorylation rate of S363A mutant V2R. Cell labeling and phosphorylation of the S363A V2R was carried out as described in the legend of Fig. 3 with the exception that throughout the experiment the cell medium contained 50 nM okadaic acid in 0.17% dimethylsulfoxide (DMSO). The control cells were exposed to 0.017% DMSO alone. After 20 min exposure to 100 nM AVP at 37°C, the cells were acid washed, neutralized and returned to the incubator with fresh medium containing either DMSO or okadaic acid/DMSO. At the indicated times cells were harvested and the receptor protein immunopurified and analyzed as described in the legend of Fig. 3.

rapid dephosphorylation induced by the mutations was detected (Table 1). The reduction in maximal phosphorylation for the mutant receptors reflected the loss of more than one phosphate acceptor site, suggesting that phosphorylation of those residues was required for the subsequent phosphorylation of other acceptor sites. Hierarchy of phosphorylation sites has also been reported for the A3 adenosine receptor and the N-formyl peptide receptor [12,13]. The T359A V2R showed the greatest reduction in phosphate incorporation although, as mentioned previously, its recycling to the cell surface was the least effective.

It has been shown that recycling of the human δ-opioid receptor is completely suppressed by treatment of the cells with 10 nM okadaic acid, a phosphatase 2A inhibitor, supporting the hypothesis that dephosphorylation is necessary for the recycling of certain GPCRs [14]. Similar treatments were carried out with cells expressing the S363A V2R mutant with 10 and 50 nM okadaic acid, but no alteration of recycling was observed (data not shown). As shown in Fig. 4 and consistent with the recycling data, the phosphatase inhibitor did not delay the cleavage of radioactive phosphate from the immunoprecipitated phosphorylated receptor, suggesting that phosphatases other than protein phosphatase 2A participate in this process.

Table 1
AVP-induced sequestration and phosphorylation of WT and mutant V2 receptors

V2 receptor	Cell surface receptors (%)		Phosphate incorporated (%)
	20 min+AVP	20 min+AVP 120 min−AVP	30 min+AVP
WT	63 ± 3 (7)	66 ± 3 (7)	100 (9)
S357A	50 ± 4 (4)	84 ± 6 (4)	85 ± 7 (3)
T359A	54 ± 3 (4)	71 ± 4 (4)	68 ± 4 (4)
T360A	48 ± 1 (4)	86 ± 4 (4)	83 ± 6 (5)
S363A	49 ± 2 (5)	95 ± 2 (5)	90 ± 5 (3)
WT, human	64 ± 5 (7)	63 ± 4 (4)	

Cell surface receptors are expressed as percentage of the number of AVP binding sites present on non-stimulated cells. Receptor sequestration and kinetics of recovery without AVP are presented in Figs. 2A, B and 4. The level of phosphorylation is expressed as percentage of ^{32}PO$_4$ incorporation observed for the WT receptor after a 30 min exposure to 100 nM AVP. Kinetics of phosphorylation are presented in Fig. 3A and B. The numbers between parentheses indicate the number of experiments performed.

4. Discussion

The phosphorylation acceptor sites of the hV2R were previously identified as the nine serines and threonines that follow the two negatively charged amino acids present at the carboxyl terminus [2,11]. Among these phosphorylated sites the serine triplet was found to be required for receptor retention inside the cell, evidence presented here identified the three amino acids susceptible to phosphorylation by GRK located upstream from the serine triplet as additional components of the hV2R retention signal. Substitution of serine 350 of the hV2R for alanine did not alter receptor recycling, indicating that this amino acid is not part of the motif (data not shown), and that only the phosphorylation acceptor sites immediately downstream from the two negatively charged amino acids are involved in receptor retention (see Fig. 1). The mutant T359A hV2R displayed the lowest level of phosphate incorporation and of return to the cell surface (Table 1) suggesting that the identity of the phosphorylated sites rather than their number determines the recycling behavior.

It has been suggested based on cell imaging data, that a prolonged interaction between the V2R and another protein, possibly of the arrestin family, could prevent dephosphorylation and block receptor recycling, but the biochemical proof for this hypothesis is still missing [15]. We recently reported that the non-recycling hV2R and the recycling S363A hV2R accumulate in the same perinuclear recycling compartment. In both cases arrestin(s) co-localized with the receptor in this organelle, indicating that neither the identity of the compartment, nor prolonged association or co-migration with arrestin, were responsible for the trapping of the WT hV2R [16]. This observation did not rule out the participation of arrestin in receptor trapping, but it clearly established that its prolonged interaction with the V2R did not by itself retain the receptor inside the cell. Studies carried out by other laboratories have assigned the trapping activity to the serine triplet [15], albeit the data presented indicate that additional amino acids play a role in the retention phenomenon.

It is difficult at the moment to speculate about the universality of the trapping factors or the biological implications of GPCR retention. The M2 acetylcholine muscarinic is another example of a receptor that does not recycle after ligand-induced internalization [17,18], although the amino acid composition of the third intracellular loop responsible for internalization reveals similarities but no significant identity with the hV2R retention signal. Whole cell binding assays performed before and after ligand-promoted internalization with [^{3}H]quinuclidyl benzylate, a cell permeable antagonist of the M2 receptor, indicated that the total number of receptors per cell did not change significantly after prolonged treatment with carbachol, although whole cell binding with [^{3}H]-*N*-methylscopolamine, a non-cell permeant antagonist of the M2 receptor, detected a 80% loss of cell surface receptors. This indirect assessment of the fate of the internalized M2 receptor protein suggests that the trapped receptor remains intact and able to bind its ligand inside the cell [18]. Similar results were also obtained with the acetylcholine M3 muscarinic receptor endogenously expressed in SH-SY5Y human neuroblastoma cells [19]. Further studies on the hV2R and the muscarinic receptors could lead to the discovery of a new family of GPCR-interacting proteins or ascribe receptor retention properties to an already characterized protein, a process that will be advanced by the accurate definition of the retention signal.

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