Role of the carboxyl-terminal region, di-leucine motif and cysteine residues in signalling and internalization of vasopressin V1a receptor

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Received 21 September 1999

Abstract The structural requirements for internalization and signalling of the vasopressin V1a receptor were investigated in stably transfected HEK-293 cells. Removal of the 51 C-terminal amino acids did not affect vasopressin binding, calcium signalling, heterologous desensitization or internalization of the receptor. Deletion of 14 additional amino acids reduced vasopressin-dependent calcium increase and impaired receptor internalization. Substitution of cysteines 371-372 did not affect intracellular signalling, but decreased endocytosis by 26%. Substitution of the 361-362 leucine by alanine residues reduced by 56% V1a receptor sequestration without affecting calcium signalling. These results indicate that di-cysteine and mostly di-leucine motifs present in the C-terminal region of the V1a receptor are involved in its internalization.

Key words: Vasopressin; V1a receptor; Internalization; Di-leucine motif; C-terminal region; Calcium signalling

1. Introduction

The vasopressin V1a receptor is a G protein-coupled receptor whose stimulation activates membrane phospholipases Cβ and generates inositol triphosphates and diacylglycerol. Inositol-1,4,5-triphosphates bind to endoplasmic reticulum receptors to induce a transient rise in intracellular calcium. Diacylglycerol increasing protein kinase C activity results in phosphorylation of intracellular proteins. Stimulation of the V1a receptor is followed by its homologous phosphorylation and desensitization [1–4]. Similarly, stimulation of the endogenous muscarinic receptor m3 or direct activation of protein kinase C induced heterologous phosphorylation and desensitization of the V1a receptor when transfected in *Xenopus laevis* or HEK-293 cells [1,2].

In addition to rapid desensitization, the V1a receptor is internalized within minutes following its stimulation by vasopressin. Different internalization pathways for membrane receptors have been described, including arrestin and caveolae-mediated endocytosis [5]. They involve recognition sequences between the membrane proteins and the endocytosis machinery which have been identified for some proteins. These are mostly related to serine and cysteine residues, a di-leucine motif or complex sequences such as NPX_iY, which may differ from one protein to another.

The C-terminal domain of the V1a receptor contains several motifs possibly involved in the internalization process (Fig. 1). The 14 serine and four threonine residues are putative phosphorylation sites which could be involved in sequestration of the receptor. The di-cysteine motif at positions 371 and 372 or the cysteine at position 365 may be palmitoylated/depalmitoylated and participate in internalization of the receptor through anchoring to the membrane [6]. The di-leucine motif at position 361-362 is also a candidate for internalization and targetting sequences [7].

To determine the possible role of these amino acids in internalization of the V1a receptor, several mutations were introduced in the C-terminal domain and the mutants were stably transfected in HEK-293 cells. These included partial or total deletion of the C-terminal domain and substitution of cysteine or di-leucine motifs by alanine residues. For each mutant, the receptors affinity and expression were determined by binding experiments, intracellular signalling was characterized from the transient change in intracellular calcium concentration and internalization was measured by an enzyme-linked immunosorbent assay (ELISA) of epitope-tagged receptors.

2. Materials and methods

2.1. Transfection of V1a mutants in HEK-293 cells

Influenza virus hemagglutinin epitope (Tag HA) was introduced in the extracellular N-terminal domain of the cloned rat V1a receptor as previously described [8]. Deletion and point mutation mutants (see Fig. 1) were constructed with the Altered Site II in vitro mutagenesis system (Promega) as described by the manufacturer. All cDNAs were sequenced before expression study. The S374STOP mutant, truncated at serine 374, lacked the last 51 amino acids of the C-terminal region. The H360STOP mutant truncated at histidine 360 lacked the last 65 C-terminal amino acids, including cysteines 365, 371, 372 and di-leucine 361-362. In the L361-362A mutant, the di-leucine motif was replaced by di-alanine and in the C365A, the C371-372A and the C365-371-372A mutants, cysteines were substituted by alanines.

Wild-type and mutated receptors were cloned in the expression vector pcDNA3.1(−) (Invitrogen), which contains a geneticin resistance gene, and were stably transfected in HEK-293 cells. The cells were cultured at 37°C under 95% air/5% CO_2 in DMEM medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Gibco). They were plated on 30 mm dishes and transfected with the superfect reagent method (Qiagen). The day after, cells were transferred to 100 mm dishes and clones were selected by addition of 1 mg/ml geneticin (Gibco).

2.2. Binding experiments

The number of vasopressin binding sites at the surface of the transfected cells was determined with [^{3}H]vasopressin (NEN) on 24 well plates. Cells were rinsed with D-PBS, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ (Gibco BRL) and 0.1% bovine serum albumin (BSA) and incubated with the same solution containing 10^{-8} M [^{3}H]vasopressin for 45 min at room temperature with gentle rocking. This concentration of vasopressin was found saturating for V1a vasopressin binding in prelimi-

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PII: S0014-5793(99)01360-5

nary experiments. Non-specific binding was determined by addition of 10^{-6} M vasopressin to the incubation medium. Cells were then rinsed with D-PBS, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, lysed by 0.5 ml of 0.2 N NaOH with 0.1% SDS and radioactivity was counted by liquid scintillation (Tri-carb 1500 Packard). All binding determinations were performed in duplicate.

K_d values of V1a wild-type and mutated receptors were determined on membrane suspension prepared from T75 flasks. Cells were grown to confluence, harvested by trypsinization and suspended in medium containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 3 mM $MgSO_4$. They were subsequently homogenized with a motor-driven Teflon potter, centrifuged for 30 min ($20\,000 \times g$) at 4°C and the pellet was resuspended in initial buffer. The pellet protein content was quantified according to Bradford's method with BSA (Sigma) as standard and aliquots were stored at −80°C. Binding experiments were performed on 50 μg of membrane proteins in 200 μl of initial buffer containing 0.1% BSA, 0.01% bacitracin and from 0.2 to 20 nM [^{3}H]vasopressin. Non-specific binding was determined by addition of 5×10^{-6} M AVP to the medium. Incubations were performed for 1 h at room temperature with gently rocking. The membrane suspension was thereafter diluted in 4 ml incubation medium free of vasopressin and filtered under vacuum through a GF/C filter previously coated in buffer medium with 1% BSA for 45 min. After filtration of the membrane suspension, the filter was rinsed with 4 ml of vasopressin-free incubation buffer, dried at 80°C for 10 min and the radioactivity was measured by liquid scintillation counting. K_d values were determined with the GraphPad PRISM software 2.0.

2.3. Intracellular calcium measurement

Changes in intracellular calcium were measured as previously described [13]. Confluent T25 flasks of the different cell lines were trypsinized and loaded at room temperature for 45 min with 2 μM Fura 2-AM (Molecular Probes) in culture medium. One million cells were introduced into a 25°C thermostated cuvette of an Amincon Bowman SLM 2 spectrofluorimeter. The excitation wavelengths were 340 and 380 nm and fluorescence emission was recorded at 512 nm. The ratio between the intensity of emission following excitation at 340 and 380 nm was calculated every 0.5 s and the calcium concentration was determined with the equation of Grynkiewicz et al. [9]. The minimum ratio R_{min} was obtained by addition of 5 mM EGTA and 20 μM digitonin and the maximum ratio R_{max} with 10 mM calcium.

2.4. Internalization measurements by ELISA

Stably transfected cells were cultured to confluence on 96 well plates. On the day of the experiment, they were rinsed with D-PBS, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, containing 1% BSA, and incubated with 10^{-7} M vasopressin for different times from 5 to 60 min. After incubation, the cells were fixed with 4% paraformaldehyde, in PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$ for 30 min, incubated with D-PBS containing 50 mM NH_4Cl for 15 min and rinsed with D-PBS, 1 mM $CaCl_2$, 0,5 mM $MgCl_2$ and 1% BSA. Cells were subsequently incubated overnight at 4°C with 1 μg/ml of the anti-Tag HA antibody (12CA5) followed by 30 min incubation at room temperature with mouse IgG antibody coupled to horseradish peroxidase (HRP) (Promega). The optical density resulting from 30 min reaction with OPD peroxidase substrate (Sigma) was measured at 450 nm on a multiscan Bichromatic apparatus (Labsystem). The specific optical density corresponding to the epitope-tagged receptors was obtained by subtraction in each experiment of the optical density of HEK-293 cells transfected with non-tagged wild-type V1a receptor and treated as previously described.

2.5. Immunofluorescence

The cells stably transfected with the epitope-tagged V1a receptor were plated at confluence on glass coverslips and treated as described for internalization measurement by ELISA until the paraformaldehyde fixation step. Cells were therefore permeabilized with 0.2% Triton X-100 for 30 min before incubation with 0.1 μg/ml anti-Tag HA antibody (12CA5) for 2 h at room temperature and with mouse IgG coupled to rhodamine for 1 h. The coverslips were mounted on slides for fluorescence microscopy.

Results are expressed as mean ± S.E.M. and statistical significance was determined by ANOVA. Significance was set at $P < 0.05$

3. Results

3.1. Binding experiments

Every transfected mutant generated clones which bound [^{3}H]vasopressin, indicating targetting of the receptors to the plasma membrane. As far as possible, clones with a receptor density close to 150 000 sites/cell (125 000–230 000) were identified and characterized (Table 1). This density corresponds to the number of vasopressin V1a receptors found in hepatocytes by Fishman et al. [10]. However, two mutated receptors, the one lacking its last 65 amino acids (H360STOP) and the other with three cysteines substituted by alanine (C365-371-372A), regularly exhibited lower expression than wild-type or other mutated V1a receptors. For these two mutations, cell clones

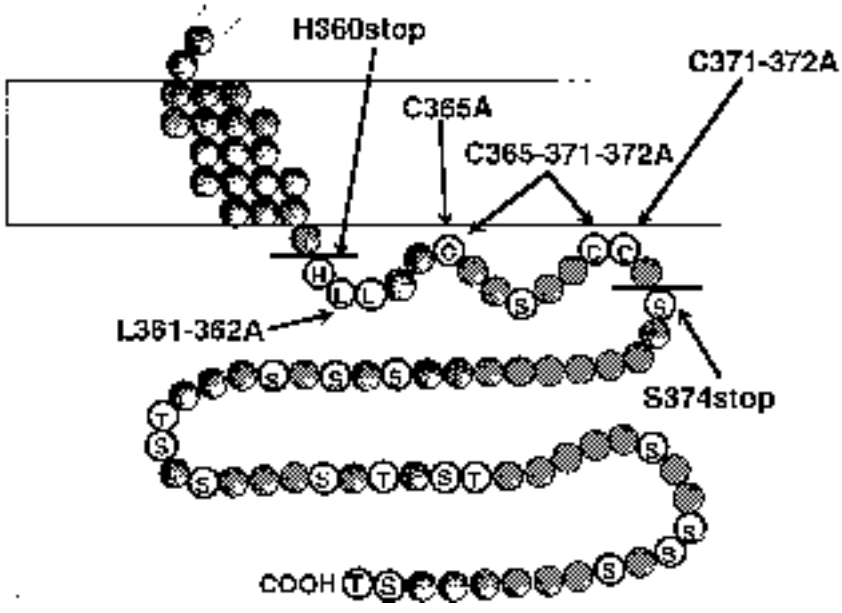

Fig. 1. Schematic representation of the carboxyl-terminal region of the rat vasopressin V1a receptor, with indications of the performed truncations (H360STOP, S374STOP) and substitutions (C365A, C371-372A, C365-371-372A and L361-362A).

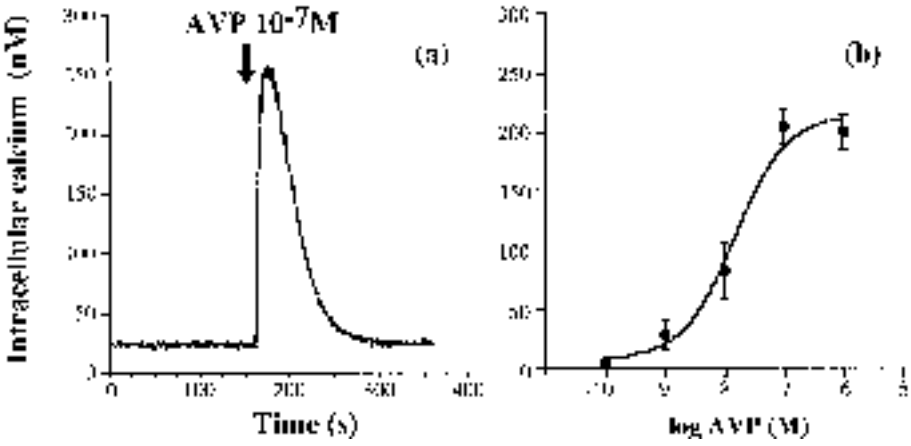

Fig. 2. Effect of vasopressin on intracellular calcium increase in HEK-293 cells transfected with the rat V1a receptor. (a) Representative experiment. (b) Dose-response curve of vasopressin-dependent intracellular calcium increase, mean ± S.E.M. of four experiments.

with the highest V1a receptor density were selected, corresponding to 35 000 sites for the H360STOP mutant and 69 000 sites for the C365-371-372A mutant (Table 1).

The K_d of epitope-tagged recombinant V1a receptor determined from binding experiments on membrane suspension was 0.44 ± 0.05 nM ($n = 7$). This value was not significantly different from the K_d of wild-type V1a receptor (0.57 ± 0.11 nM, $n = 4$), indicating that insertion of Tag HA at the N-terminal region of the receptor did not modify its binding properties. The K_d values of every tested mutant were comparable to that of the native receptor, with the exception of the di-leucine-substituted mutant whose K_d was slightly, but significantly, increased as compared to wild-type V1a receptor (Table 1).

3.2. Calcium signalling

A representative change in intracellular calcium induced by the addition of 10^{-7} M vasopressin to HEK-293 cells transfected with epitope-tagged V1a receptor is illustrated in Fig. 2a. Vasopressin induced a rapid and transient cytosolic calcium increase whose amplitude was dependent on the ligand concentration (Fig. 2b). Every mutant exhibited a comparable calcium increase when stimulated with 10^{-7} M vasopressin (Table 2), except the V1a receptor lacking 65 amino acids (H360STOP) (Table 2). Because receptor expression of this mutant was lower than that of the control V1a clone (Table 1), cells were incubated for 18 h with 10 mM butyrate to increase protein expression. Rising the wild-type V1a receptor density from 140 000 to 230 000 did not modify the calcium peak induced by 10^{-7} M vasopressin (213 ± 27 and 215 ± 17 nM, $n = 4$, respectively). Increasing the density of the H360STOP from 35 000 to 190 000 sites/cell was associated with a small increase in the vasopressin-dependent calcium peak from 40 ± 12 to 103 ± 29 nM ($n = 4$).

3.3. Heterologous desensitization

HEK-293 cells express a m3 phospholipase Cβ-linked muscarinic receptor for which stimulation by 10^{-4} M carbachol induced a calcium peak of mean amplitude 380 ± 45 nM ($n = 25$). In HEK-293 cells transfected with the Tag HA V1a receptor, this stimulation reduced the subsequent increase in cytosolic calcium by 40% consecutive to the addition of 10^{-7} M vasopressin as compared to control (Table 2). Heterologous desensitization of the different mutants was comparable to that recorded with the wild-type V1a receptor with the exception of the H360STOP mutant with a reduced calcium response, which was fully desensitized by carbachol (Table 2).

3.4. Receptor internalization

Taking advantage of the inserted Tag HA on the extracellular N-terminal region of the receptor, internalization of the wild-type V1a and mutated receptors was determined by ELISA. The lack of influence of this external epitope on the sequestration of the V1a receptor was first checked by binding and immunofluorescence experiments. Pre-incubation with 10^{-7} M unlabelled vasopressin for 30 min of HEK-293 cells transfected with wild-type V1a or epitope Tag HA V1a receptor reduced subsequent [^{3}H]vasopressin binding sites by 60% (data not shown). This ability of the epitope-tagged V1a receptor to be sequestrated was confirmed by immunofluorescence. As shown in Fig. 3, fluorescence labelling of the V1a receptor under control conditions was diffuse at the surface of the cell. Incubation with 10^{-7} M vasopressin induced intra-

Table 1
Binding characteristics of wild-type and mutated V1a receptors stably transfected in HEK-293 cells

Clones	K_d (nM)	n	Sites per cell	n
V1a wild-type	0.44 ± 0.05	7	143 000 ± 8 000	9
S374STOP	0.59 ± 0.09	3	125 000 ± 20 000	5
H360STOP	0.74 ± 0.06	4	35 000 ± 6 000	6
C365A	0.80 ± 0.10	3	190 000 ± 15 000	5
C371-372A	0.54 ± 0.05	3	137 000 ± 18 000	6
C365-371-372A	0.51 ± 0.04	4	69 000 ± 6 000	6
L361-362A	1.47 ± 0.19	6	214 000 ± 20 000	6

Results are mean ± S.E.M. of n experiments.

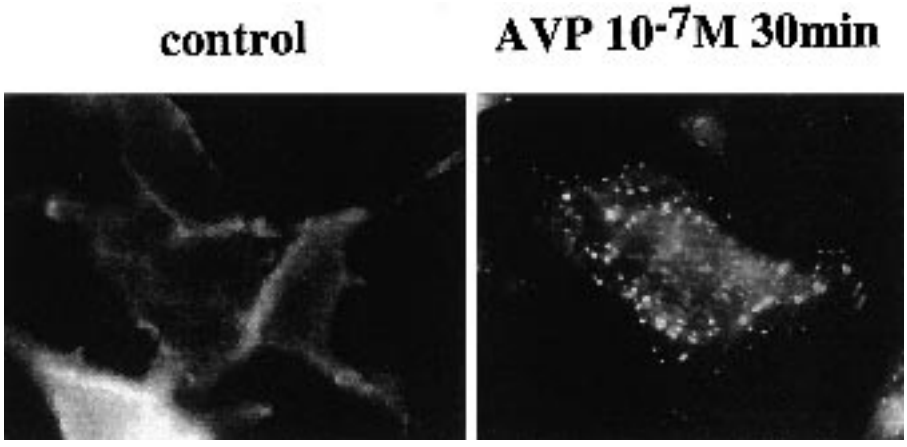

Fig. 3. Effect of 10^{-7} M vasopressin addition for 30 min on immunofluorescence labelling of Tag HA wild-type V1a receptors stably transfected in HEK-293 cells.

cellular punctate fluorescence, characteristic of receptor internalization in subapical vesicles [11].

The internalization kinetics of epitope-tagged V1a receptor, S374STOP and H360STOP mutants are presented in Fig. 4a. In the minutes following the addition of 10^{-7} M vasopressin, the wild-type V1a receptor was rapidly internalized from the cell surface. Within 30 min, 60% of the membrane receptors were sequestrated, a value comparable to that obtained in binding experiments. Removal of the last 51 amino acids of the C-terminal region did not significantly modify the kinetics and amplitude of internalization as compared to the wild-type receptor (Fig. 4a). In contrast, deletion of the last 65 amino acids reduced receptor internalization by 80% (Fig. 4a). Substitution of cysteine 365 by alanine did not significantly affect internalization of the receptor, whereas substitution of the dicysteine 371-372 motif or the three cysteines 365, 371 and 372 reduced the extent of receptor internalization by 26% (Table 2). Substitution of the di-leucine motif by alanine decreased sequestration of the mutated receptor by 56% (Table 2 and Fig. 4b). Because the receptor density of the L361-362A clone was slightly larger than that of the wild-type V1a receptor clone (230 000 versus 150 000), di-leucine mutant internalization was further determined in another L361-362A clone with 110 000 receptors/cell. Incubation of the cells with 10^{-7} M vasopressin for 30 or 60 min resulted in a comparable 30% internalization of the di-leucine-substituted V1a receptor in the clones with 110 000 receptors/cell or with 230 000 receptors/cell.

4. Discussion

Rat V1a receptor stably transfected in HEK-293 cells shared properties with those expressed in native tissues. The K_d of the transfected wild-type V1a receptor was comparable to that reported by Carnazzi et al. for liver plasma membrane [12]. Maximal internalization corresponded to a 65% decrease in the number of surface receptors in the transfected HEK-293 cells as in vascular smooth muscle or hepatocytes within minutes following addition of vasopressin [10,13]. Stimulation of the receptor with vasopressin elicited a dose-dependent transient increase in intracellular calcium, indicating functional coupling to phospholipase Cβ of the V1a receptor expressed in this heterologous expression system.

Deletion of the last 51 C-terminal amino acids of the V1a receptor removed 17 serine-threonine residues which are putative phosphorylation sites. The deletion did not affect targetting to the plasma membrane, the ability to increase intracellular calcium or sequestration of the mutated receptor following vasopressin stimulation. Previous experiments in oocytes indicated that homologous desensitization was not altered in the same deletion mutant nor was the heterologous desensitization in the present study [8]. This indicates that phosphorylation of the 17 serine and threonine residues present on the C-terminal tail is not essential for short-term homologous and heterologous desensitization, just as it is also not essential for sequestration of the V1a receptor. These results are consistent with those of Murray et al. who found unchanged internalization of opioid receptor with a truncated C-terminal tail [14]. Similarly, Innamorati et al. showed by deletion of the last 14 and 27 amino acids of the parent V2 receptor that phosphorylation of the C-terminal region is not required for its internalization [15]. On the other hand, deletion of the last 35 amino acids of the V2 receptor prevented expression of the protein at the cell surface [16].

Table 2
Calcium signalling, heterologous desensitization and internalization of wild-type and mutated V1a receptors stably transfected in HEK-293 cells

Clones	Calcium peak induced by AVP 10^{-7} M (nM)	% Desensitization induced by 10^{-4} M Cch	*n*	% Internalization at 30 min	*n*
V1a wild-type	206 ± 12	42 ± 3	22	64 ± 2	23
S374STOP	260 ± 21	54 ± 2	3	53 ± 4	7
H360STOP	40 ± 12	100 ± 0	4	14 ± 5	5
C365A	216 ± 10	44 ± 1	5	52 ± 7	5
C371-372A	205 ± 42	59 ± 5	3	48 ± 6	8
C365-371-372A	154 ± 28	60 ± 8	4	47 ± 5	6
L361-362A	195 ± 44	48 ± 13	4	28 ± 5	6

Results are mean ± S.E.M. of *n* experiments.

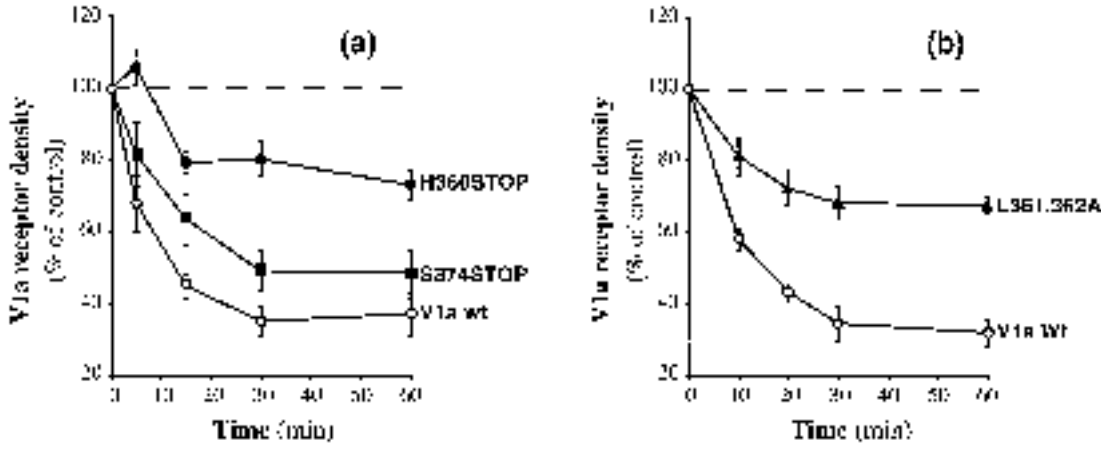

Fig. 4. Internalization of wild-type and mutated rat V1a receptors stably transfected in HEK-293 cells. Results are expressed in % of control, which corresponded for each clone to cells incubated in the absence of vasopressin. (a) Effect of truncations of the last 51 (S374STOP) or 65 (H360STOP) C-terminal amino acids. (b) Effect of substitution of the di-leucine motif 361-362 by alanine residues.

Deletion of the last 65 C-terminal amino acids of the V1a receptor, including three cysteines and a di-leucine motif, did not change the K_d of the receptor for vasopressin but reduced its expression at the cell surface. Similar K_d values indicated that the C-terminal region of the V1a receptor is not essential for appropriate folding of the protein, whereas the efficiency of its targetting to the plasma membrane is apparently affected. The stimulation of this truncated receptor by vasopressin elicited a small increase in intracellular calcium as compared to the wild-type receptor, whether or not its expression was enhanced by butyrate treatment. This suggests that the C-terminal domain of the V1a receptor is implicated in the coupling with Gq protein in addition to the second intracellular loop, as previously evidenced by Liu and Wess et al. [17]. Internalization of the V1a receptor lacking 65 C-terminal amino acids was also reduced. These data are consistent with the experiments on the angiotensin AT_{1A} receptor, indicating that deletion of the last 24–45 amino acids suppresses its internalization [18–20]. The implication of the C-terminal region in receptor sequestration is also supported by comparable data obtained with PTH, substance P, histamine, endothelin or neurokinin receptors [21–24].

The impaired sequestration of truncated receptor may be related to the loss of an internalization motif. At least two candidates for such motifs are present in the C-terminal domain of the V1a receptor, namely the cysteine and the leucine residues. Single or double cysteine residues in the C-terminal domain have been documented in several G protein-coupled receptors. These are mostly located near the cytoplasmic exit of the seventh transmembrane domain, within 20 amino acids [6]. Palmitoylation of the cysteines favors attachment of the C-terminal domain to the membrane, thus forming a fourth intracellular loop. Site-directed mutagenesis of cysteine abolished palmitoylation and Gs protein coupling of the β_2 adrenergic receptor [25–26], whereas it does not affect Gq coupling of the α_{2A} adrenergic receptor [27]. In the thyrotropin-releasing hormone, substitution of cysteine by glycine did not affect the accumulation of inositol but compromised its internalization [28]. In the V2 receptor, enhanced cell surface expression linked to cysteine palmitoylation has been documented by Sadhegi et al. [29]. Substitution of cysteine by serine did not affect vasopressin binding or adenylyl cyclase coupling, but impaired V2 receptor internalization, suggesting that the palmitoylation/depalmitoylation cycle is involved in endocytosis [30].

The functional characteristics of the receptor lacking the cysteine 365 were comparable to that of the wild-type, indicating that this amino acid alone is not essential for signalling and endocytosis of the V1a receptor. Substitution of cysteines 371-372 by alanine has no significant effect on binding properties, cell membrane expression or increase in intracellular calcium induced by vasopressin. In contrast, it significantly reduced the amplitude of internalization, indicating that the 371-372 cysteines are partly involved in the sequestration of the V1a receptor. This could be related to palmitoylation/depalmitoylation of these cysteines, as demonstrated for the V2 receptor [30]. Simultaneous substitution of cysteines 365, 371-372 resulted in an identical K_d, a lower density of receptors at the cell surface and a decreased internalization. Its shows that, in addition to its role in endocytosis, cysteines are required for full expression of the V1a receptor at the plasma membrane. However, such a reduced membrane expression influences binding properties, G protein coupling and heterologous desensitization of the V1a receptor.

The implication of a di-leucine motif in sequestration has been demonstrated for several membrane proteins including mannose 6-phosphate, T-cell, monoamine, IgFc, prolactin and insulin receptors [31–35]. It is also implicated in sorting of membrane proteins in the Golgi network [33,36]. In the present study, substitution of the di-leucine motif of the V1a receptor with alanine slightly increased its K_d, but did not affect cell surface expression of the receptor. Neither did it modify the increase in intracellular calcium elicited by vasopressin or heterologous desensitization of the receptor. In contrast, despite comparable membrane expression and calcium signalling, the sequestration of the V1a receptor was markedly reduced by removal of the di-leucine motif, as it was by truncation of the last 65 amino acids including this di-leucine motif.

In summary, neither deletion of the C-terminal region nor substitution of cysteine residues affects vasopressin binding to V1a receptor. The deletion of the last 51 amino acids did not alter signalling, internalization or heterologous desensitization of the V1a receptor, whereas deletion of 14 additional amino

acids impaired sequestration and calcium signalling. Substitution of individual amino acids indicated that cysteine residues which are putative sites of palmitoylation are not essential for G protein coupling and heterologous desensitization, but are partly implicated in internalization of the V1a receptor expressed in HEK-293 cells. The di-leucine motif present in the C-terminal region is not required for appropriate targetting to the cell membrane, for calcium signalling or for heterologous desensitization of the V1a receptor, but would be a major recognition sequence for its internalization.

Acknowledgements: We are grateful to Marion Lambert for her first class technical assistance.

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