Role of protein kinase C and carboxyl-terminal region in acute desensitization of vasopressin V1a receptor

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Abstract The role of protein kinase C activation and carboxyl-terminal region in rapid desensitization of the vasopressin V1a receptor was investigated in *Xenopus* oocytes. Preincubation of the oocytes with vasopressin or with the diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (OAG), or direct injection of active protein kinase C, all blunted the calcium response of the V1a receptor. Truncation of the 51 terminal amino acids (S374STOP) modified neither the intracellular calcium response to vasopressin nor its desensitization by vasopressin or OAG. These data suggest that desensitization of the V1a receptor is mediated by PKC activation and that its carboxyl-terminal domain is not required for signal transduction and rapid desensitization.

Key words: Vasopressin V1a receptor; Protein kinase C; Desensitization; Intracellular calcium; *Xenopus* oocyte

1. Introduction

Vasopressin V1a receptor is widely distributed in several tissues such as blood vessels, kidney, choroid plexus, liver or central nervous system [1]. It belongs to the superfamily of the G-protein-coupled receptors with seven transmembrane domains. Binding of vasopressin (AVP) to the V1a receptor activates phospholipase C and produces inositol tri-phosphate and diacylglycerol, which respectively increase intracellular calcium and activate protein kinase C (PKC). Like most G-protein-coupled receptors already studied [2], the V1a receptor undergoes desensitization when stimulated by its agonist. This homologous desensitization is characterized by the inability of the receptor to induce a further intracellular response when it is successively challenged by vasopressin. Such rapid desensitization is probably linked to uncoupling of the receptor from G proteins within seconds, whereas internalization would occur within minutes. Both rapid desensitization and internalization have been demonstrated for the V1a receptor in native tissue and in cells expressing the cloned receptor [3–6].

The most frequently invoked mechanism for rapid desensitization refers to phosphorylation of threonine and serine residues by protein kinase A or C and/or by G receptor kinase [7]. This phosphorylation may be consecutive to binding of the ligand to the receptor or to activation of protein kinase by second messengers [7–11]. The role of protein kinase A activation by cAMP on desensitization has been well demonstrated for adenylyl cyclase coupled receptor. On the other hand, the effect of protein kinase C activation by diacylglycerol on the desensitization process is less documented.

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The V1a receptor, like other transmembrane G protein receptors, has a number of threonine and serine residues which are putative sites for phosphorylation and may be involved in desensitization. These residues are located either on the intracellular loops or on the intracellular C-terminal tail of the receptor. Current experiments on adenylyl cyclase coupled receptors with a C-terminal tail which has been truncated or mutated indicate that phosphorylation of carboxyl-terminal residues is implicated in rapid desensitization [7]. Data on the role of C-terminal tail in phospholipase C coupled receptors are more conflicting. Carboxyl terminus truncation of the alpha-1b adrenergic receptor, for example, impairs agonist-dependent desensitization [12]. In contrast, experiments with angiotensin AT1a receptor brought evidence for a role of the carboxyl-terminal residues in the mechanisms of rapid desensitization [9], whereas other experiments did not [10]. It is in this respect that the roles of C-terminal tail and protein kinase C activation in desensitization of the V1a receptor was further investigated in this study.

2. Materials and methods

2.1. Construction of the mutant receptor cDNAs and expression vectors

Using the cloned rat V1a receptor [13,14], three different mutant stops were constructed by the Altered Site II in vitro mutagenesis System (Promega) as described by the manufacturer. Mutant oligonucleotides were as follows: S374STOP, 5′-CCATGCTGCACTGAATGG CGCAGAAATTCGCC-3′, Q367STOP, 5′-GCAAGACTGCGTTCTAAAGTTTCCCATG CTGC-3′, H360STOP, 5′-TACATGTTTTTCAG TGGCTAACTCCTGCAAGAC-3′. The first mutant was truncated at serine-374 (Fig. 1) of the carboxyl-terminal region of the receptor (S374STOP). This truncation removed the 51 last amino acids of the C-terminal tail. The second one was truncated at glutamine-367 (Q367STOP) and lacked the 58 C-terminal amino acids. The third one was truncated at histidine-360 (H360STOP) and lacked the 65 C-terminal amino acids.

In a second series, a stretch of nucleotides coding for a 9-amino acid epitope (TagHA) derived from the influenza virus hemagglutinin protein (YPYDVPDYA) was introduced in the three resulting cDNA and the wild type at their N-terminus *Csp*I restriction site [15]. This modification in the structure of the receptors did not alter the calcium response induced by vasopressin when expressed in oocytes (data not shown). All cDNA were sequenced before expression.

2.2. Oocyte expression and intracellular calcium detection by photoprotein aequorin

Female albino oocyte-positive *Xenopus laevis* were purchased from Xenopus One (Ann Arbor, MI, USA). Synthetic messenger RNA (cRNA) corresponding to the rat vasopressin V1a receptor (wild type or mutant) [13,14] was produced by in vitro transcription [16] using the mCAP mRNA Capping Kit from Stratagene. Two days after injection of the synthesized messenger cRNA (30 nl at 0.1 mg/ml) in defolliculated oocytes, the photoprotein aequorin (Friday Harbour Laboratory, USA) was injected (20 nl at 1 mg/ml). Changes in the intracellular calcium concentration following addition of vasopressin were detected by light emission [17] using a luminometer (Mini-Lumat LB9506, Berthold). Results were expressed as relative units of

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light emission (RLU). It corresponded to the percentage of the maximal response obtained on the day of the assays. Experiments were performed in a buffer solution (OR2) containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, 0.1% bovine serum albumin, pH 7.8. Each set of conditions was tested at least in five oocytes per animal and in three different animals. Means are given ± S.E.M. with *n* corresponding to the number of experiments.

2.3. Injection of purified protein kinase C

Thirty nl of 10 μg/ml (820 U/mg) catalytic fragment of protein kinase C from rat brain (Biomol, USA) were injected in expressing oocytes. Ten minutes after injection, oocytes were challenged with 10^{-7} M vasopressin and intracellular calcium mobilization was recorded.

2.4. Assay of desensitization by AVP

Receptors expressing oocytes were incubated for 2 min in OR2 buffer containing 0.1% BSA and 10^{-7} M AVP, then extensively washed with OR2 medium. 15 min later, a second challenge was made with 10^{-7} M AVP and light emission was monitored.

2.5. Assay of desensitization by OAG

Expressing oocytes were incubated in 50 μM of the diacylglycerol 1-oleoyl-2-acetyl-sn-glycerol (OAG) [18] (Calbiochem) for 10 min and then challenged with 10^{-7} M vasopressin.

2.6. Immunohistochemistry

Oocytes expressing TagHA receptors were equilibrated in OR2 30% saccharose, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), frozen on dry ice and cut into 10 μm thick sections. The sections were equilibrated with phosphate buffer saline (PBS) and treated with 3% BSA, 0.1% (v/v) Tween20 for 1 h at room temperature, then washed and incubated for 30 min with 25 μg/ml anti-HA (12CA5) monoclonal antiserum at room temperature in the blocking buffer. Sections were subsequently washed with PBS and incubated for 30 min with rhodamine-conjugated goat anti-mouse IgG (Pierce Chemicals) at a 1:160 dilution. After washing in PBS, the sections were mounted in 80% glycerol, 20% PBS and examined with an inverted microscope equipped with fluorescence optics.

3. Results and discussion

The addition of vasopressin to oocytes which had been previously injected with the full-length V1a cRNA receptor induced a rapid spike in intracellular calcium within seconds

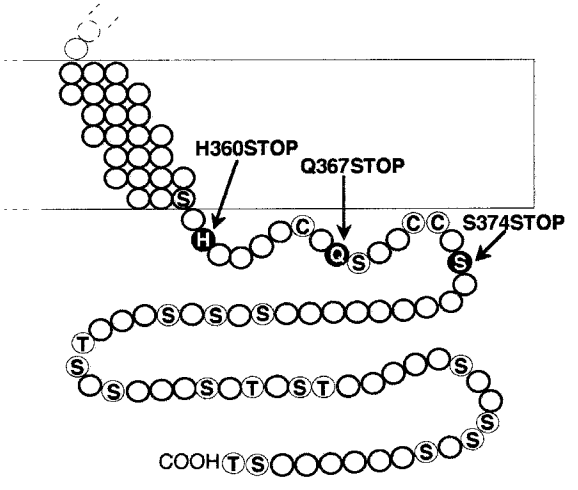

Fig. 1. Schematic representation of the carboxyl-terminal tail of the rat vasopressin V1a receptor. S corresponded to serine residue, T to threonine, C to cysteine, H to histidine and Q to glutamine. The three mutated receptors were truncated on position 360 (H360STOP), 367 (Q367STOP) and 374 (S374STOP), as indicated.

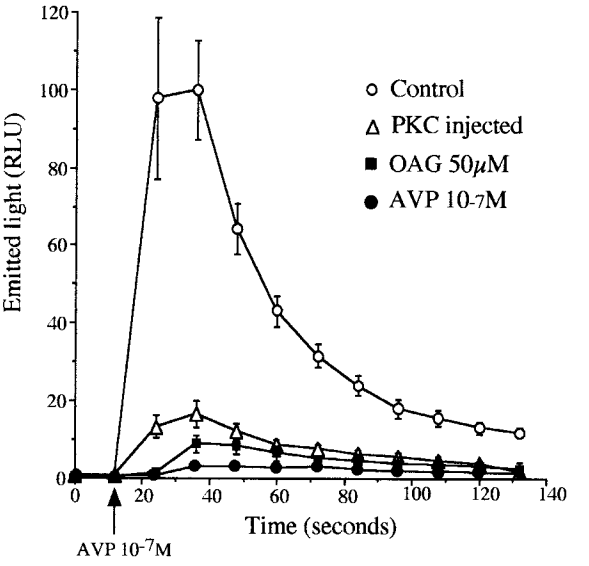

Fig. 2. Effect of PKC activation on V1a receptor expressed in *Xenopus* oocytes. Oocytes were injected with cRNA of the cloned V1a vasopressin receptor. Three days later they were stimulated with AVP, and intracellular calcium was detected by aequorin. (○) Control. (△) 0.3 ng catalytic fragment of human protein kinase C was injected 10 min before AVP stimulation. (■) Oocytes were preincubated in 50 μM of the diacylglycerol analog OAG for 10 min. (●) Oocytes were incubated for 2 min in 10^{-7} M AVP and then extensively washed before new stimulation. Mean of 3 different experiments.

following addition of the hormone (Fig. 2). This increase in intracellular calcium was transient. It reached a maximum in 12 s and returned to a plateau in 120 s. Addition of vasopressin to control oocytes which were injected with cRNA vehicle did not elicit any change in intracellular calcium.

Rapid desensitization of the V1a receptor was evidenced by successive stimulations. Addition of 10^{-7} M vasopressin to V1a-expressing oocyte for 2 min was followed by extensive wash of the incubation medium. A second challenge with vasopressin 15 min later failed to elicit a calcium spike (Fig. 2). This rapid desensitization of the V1a receptor is in good agreement with the data of Nathanson et al. in oocytes and of Cantau et al., Caramelo et al. and Grier et al. in liver or vascular cells [3–6]. The loss of vasopressin responsiveness of the oocyte was not attributable to impaired activity of the cell, since a second calcium spike could be induced by vasopressin 1 h after the first stimulation. This delay can be considered as the time necessary for full resensitization of the oocytes expressing the V1a receptor.

To test whether the observed desensitization involves the V1a receptor or its associated transduction pathways, G protein was directly activated by injection of the GTP analog GTPγS (20 pg) 10 min after AVP stimulation. In these conditions, GTPγS injection still induced an increase in intracellular calcium concentration (data not shown). This result suggests that desensitization, in these conditions, is essentially specific for the receptor.

The involvement of protein kinase C activation in desensitization of the V1a receptor was further investigated. The V1a-expressing oocytes were incubated for 10 min with 50 μM of the diacyl glycerol analog 1-oleyl-2-acetyl-sn-glycerol (OAG) [18]. As shown in Fig. 2, OAG attenuated the vasopressin-induced calcium peak by 80%. The role of PKC in this

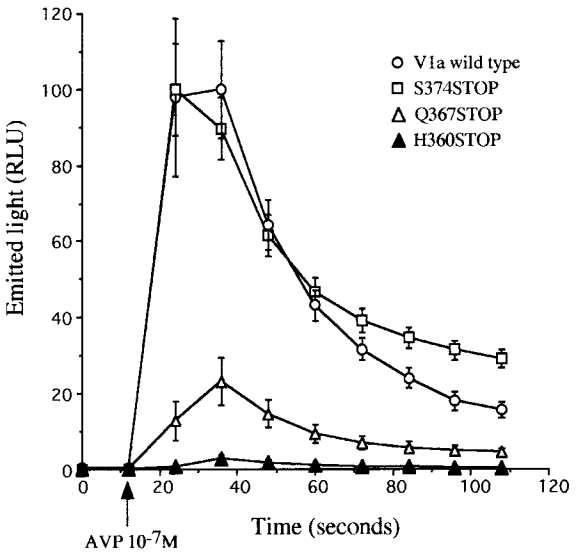

Fig. 3. AVP-dependent intracellular calcium signallisation of full length and truncated V1a receptors. Oocytes were injected with cRNA of full length V1a receptor (○), or receptor which C-terminal tail was truncated at serine-374 (□) or glutamine-367 (△) or histidine-360 (▲). Three days later they were stimulated with AVP, and intracellular calcium was detected by aequorin. Mean of 3 different experiments.

desensitization of the V1a receptor was subsequently addressed by direct injection within the oocyte of the catalytic fragment of the PKC. Increasing intracellular PKC activity through such a procedure blunted the response to vasopressin in V1a-expressing oocytes (Fig. 2). These experiments indicate that the activation of PKC by diacylglycerol following occupation of the V1a receptor by vasopressin contributes to its rapid desensitization.

The consequence of protein kinase C activation would be an increased phosphorylation of intracellular proteins. Among proteins which are putative targets for phosphorylation is the receptor itself, as evidenced for the vasopressin V2 receptor [8]. Although not yet demonstrated, it could be hypothesized that the parent V1a receptor is also a target for phosphorylation. In this respect, one may question which phosphorylated site of the receptor should be responsible for desensitization. A predominant role for phosphorylation of the intracellular C-terminal tail has been frequently invoked in these mechanisms of desensitization [8–12]. The strongest experimental evidence for such a role of the C-terminal tail is the lack of rapid desensitization of receptors which C-terminal tail is truncated [7,9,12,20]. The predominant role of the C-terminal region is also supported by the absence of rapid desensitization of receptors lacking the intracytoplasmic C-terminal domain, such as the gonadotropin-releasing hormone receptor [19]. However, other experiments indicated that the carboxyl-

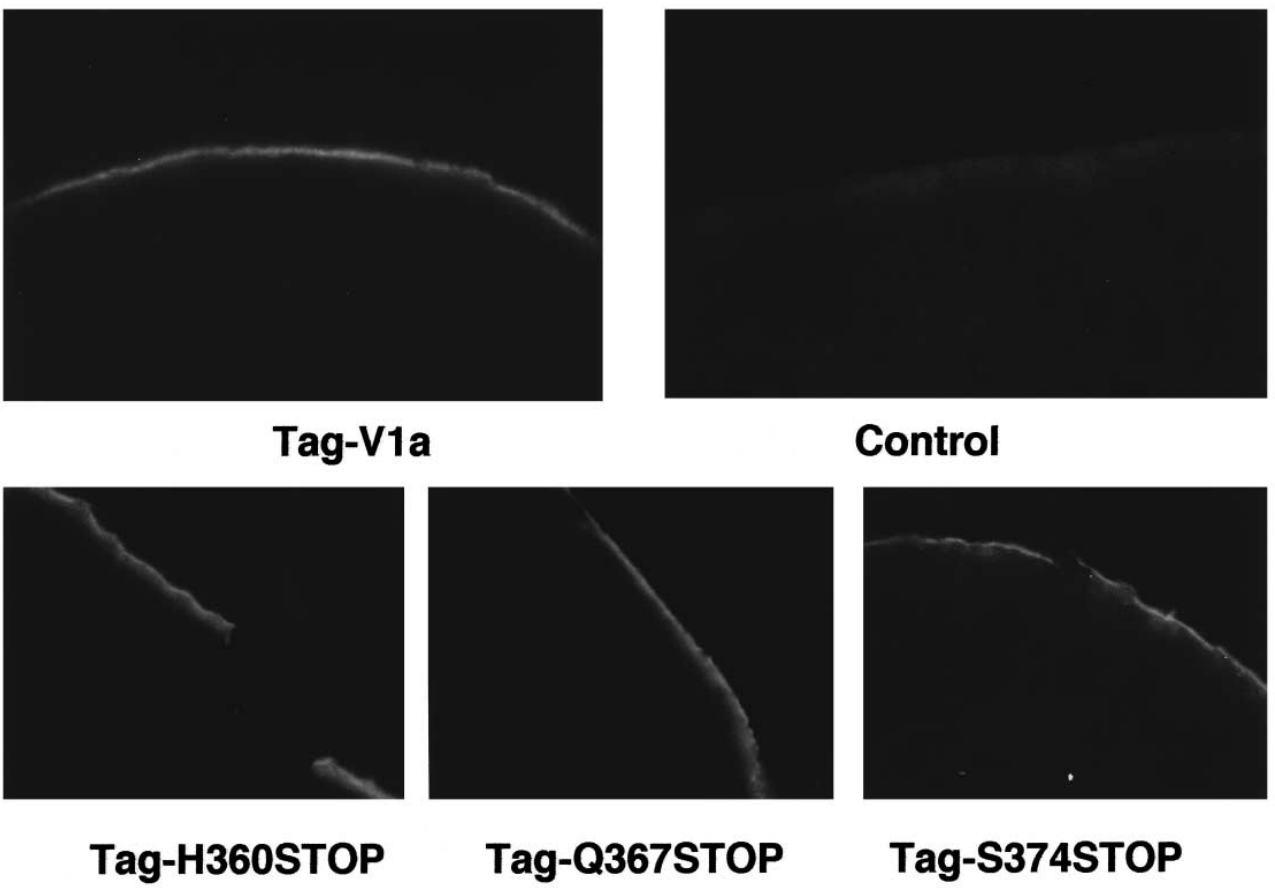

Fig. 4. Immunofluorescent localization of wild-type and carboxy-terminal truncated V1a receptor. Oocytes expressing epitope-tagged wild-type V1a receptor (Tag-V1a) or receptor which C-terminal tail was truncated at serine-374 (Tag-S374STOP), glutamine-367 (Tag-Q367STOP) or histidine-360 (Tag-H360STOP) was treated as described in Section 2. Controls was oocytes expressing non-tagged V1a receptor treated in same condition.

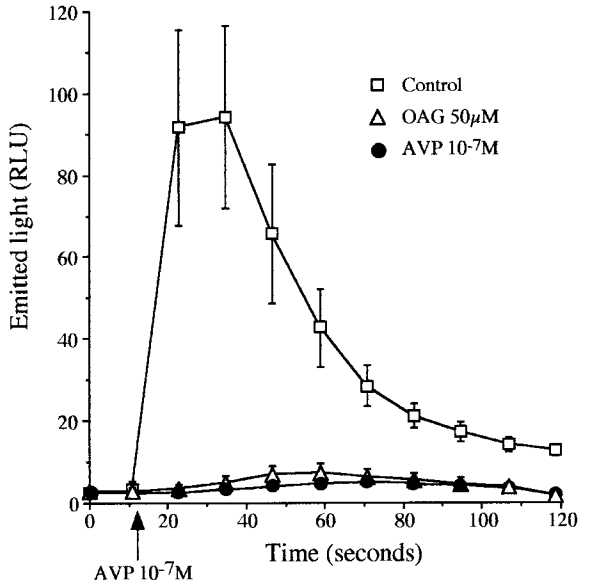

Fig. 5. Desensitization of the V1a receptor truncated on serine-374 (S374STOP). Oocytes were injected with cRNA of the mutated S374STOP vasopressin receptor. Three days later they were stimulated with AVP, and intracellular calcium was detected by aequorin. (□) Control. (△) Oocytes were preincubated in 50 μM of the cell permeable diacylglycerol analog OAG for 10 min. (●) Oocytes were incubated for 2 min in 10^{-7} M AVP and then extensively washed before new stimulation. Mean of 3 different experiments.

terminal region is not the only site of phosphorylation responsible for acute desensitization. Truncation of the terminal domain of the endothelin A receptor [20], for example, did not affect its rapid desensitization nor the agonist-dependent intracellular increase in calcium concentration.

In the present experiment, the contribution of the C-terminal domain of the V1a receptor to the diacyl glycerol-induced desensitization was investigated with different mutants. The first mutant (H360STOP) was truncated at histidine-360, which removed the last 65 C-terminal amino acids (Fig. 1). When expressed in oocytes, this H360STOP mutant did not elicit calcium response to vasopressin (Fig. 3). Oocytes expressing Q367STOP which lack the 58 C-terminal amino acids showed a small but significant intracellular calcium response after agonist stimulation. In contrast, stimulation of the S374STOP expressing oocyte with AVP elicited a calcium response which was comparable to that observed with the full-length V1a receptor (Fig. 3).

The differences in calcium response of the three mutants may be due to change in signalling capacity of the truncated receptors or to their expression at the membrane of the cell. The presence of the mutant receptors on the surface of the oocytes was evidenced by immunochemistry using epitope-tagged HA receptor. Comparable fluorescent signals at the surface of the oocytes were found for the epitope-tagged wild type and the three truncated receptors (Fig. 4). It indicates that all the tested receptors were well expressed at the cell surface. The difference in their calcium response to AVP is therefore likely attributable to the truncated 6 and 11 amino acids including the two adjoining cysteines in positions 372 and 371, and the single cysteine in position 365.

Desensitization of the functional S374STOP mutant deleted with the last 51 terminal amino acids was compared to that of the V1a wild type. This receptor retained the two cysteines residues in positions 371 and 372 which are suspected to be involved in the palmitoylation of the receptor and its anchorage to the membrane [21]. The AVP induced homologous desensitization performed on this truncated mutant was not different from that of the wild-type receptor (Fig. 5). Preincubation with OAG of the oocytes expressing the S374STOP mutant nearly abolished the calcium response to vasopressin, similarly to the full-length V1a receptor (Fig. 5). It indicates that the last 51 terminal amino acids are not necessary for acute desensitization of the V1a receptor.

In conclusion, V1a receptor undergoes rapid desensitization which can be mimicked by diacylglycerol analog and increased PKC activity. This desensitization is observed with truncated receptor which lacks most of its carboxyl-terminal domain, but which intracellular signalling was comparable to that of the wild-type receptor. It indicates that the C-terminal tail is not required for signal transduction and rapid desensitization of V1a receptor.

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