Transient Phosphorylation of the V1a Vasopressin Receptor*

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The V1a arginine vasopressin receptor (V1aR) expressed in HEK 293 cells was phosphorylated after binding to arginine vasopressin (AVP). The phosphate was incorporated very rapidly into the protein but remained attached for a very short time despite the continuous presence of hormone. The extent of phosphorylation depended upon the concentration of AVP suggesting the involvement of G-protein-coupled receptor kinases. Protein kinase C (PKC) contributed to V1aR phosphorylation as demonstrated by the fact that inhibition of the kinase decreased the amount of phosphate incorporated into the receptor. However, PKC activity was not responsible for the transient nature of V1aR phosphorylation. The hormone-free receptor could be phosphorylated by phorbol ester-activated PKC. Although the phosphorylation was transient, the phosphate groups incorporated remained on the receptor protein longer than those incorporated after AVP treatment. PKC phosphorylation of unoccupied V1aR was not sufficient to promote sequestration. Vasopressin also promoted sequestration of about 80% of the surface receptor, but measurements of the rate of accumulation of inositol phosphates in the sustained presence of the ligand did not reveal a significant desensitization of coupling to phospholipase C activity. The addition of a V1aR antagonist inhibited the sustained accumulation of inositol phosphates establishing that the sustained stimulation of PLC was mediated by receptors located on the cell surface. The transient character of V1aR phosphorylation seemed intrinsic to the receptor protein rather than a consequence of signaling within the cell, and receptor sequestration appeared to be responsible for the desensitization observed in HEK 293 cells.

Agonist binding and activation of many G-protein-coupled receptors is followed by desensitization, an attenuation of the cellular response to the ligand that prevents sustained stimulation. Studies with rhodopsin and the β2-adrenergic receptor have implicated phosphorylation in receptor desensitization (1, 2) Receptor phosphorylation promotes the binding of arrestin, which in turn uncouples the receptor from the G-protein and enhances sequestration. In the particular case of the β2-adrenergic receptor, it has been postulated that sequestration of the phosphorylated receptor facilitates cleavage of the phosphate groups by phosphatases that associate with the endosomes and allows the recycling of the de-phosphorylated active protein to the cell surface (3). Data obtained with other G-protein-coupled receptors have indicated that phosphorylation is not required to observe receptor sequestration and desensitization. For example, a truncated form of the V2 vasopressin receptor lacking the phosphorylation acceptor sites at the carboxyl-terminal end was sequestered and desensitized, although to a lesser extent than the wild type receptor (4). Similarly, a mutant angiotensin II receptor missing a portion of the carboxyl terminus did not exhibit ligand-induced sequestration although it contained a putative GRK phosphorylation site (5).

Different kinds of protein kinases can be responsible for receptor phosphorylation. Those that phosphorylate the receptor occupied by agonist are termed G-protein-coupled receptor kinases (GRKs), of which six have been identified up to now (2). With the exception of GRK1 and GRK4, found in the retina and in testis, respectively, the other enzymes are present in most tissues and seem to catalyze in an agonist-dependent manner the phosphorylation of all the receptors of this family that have been tested until now. Receptors can also be phosphorylated by other kinases such as the cAMP-dependent and the Ca2⁺-dependent (PKC) protein kinases. These enzymes can be activated by an increase in second messengers and may contribute to reduce receptor activity. Some receptors can be substrates to multiple kinases, while others, exemplified by the V2 vasopressin receptor, serve as substrates to a single type of kinases, the GRKs (4).

Three vasopressin receptor subtypes are known to date: V1a, V1b, and V2 (6). Whereas the V2 receptor stimulates adenyl cyclase activity, the V1a and V1b receptor subtypes activate phospholipases A2, C, and D. Previous studies in hepatocytes and in smooth muscle vascular cells have described ligand-induced desensitization and sequestration of the V1a vasopressin receptor (V1aR), as well as its recycling to the cell surface following removal of the ligand (7, 8). However, data regarding V1aR phosphorylation and its relationship to receptor desensitization are lacking. The availability of the V1aR cDNA (9, 10) has enabled the examination of whether the V1aR is phosphorylated and the characteristics of this process.

EXPERIMENTAL PROCEDURES

Materials— Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium without sodium phosphate, Dulbecco’s PBS (D-PBS), penicillin/streptomycin, Geneticin (G418), 0.5% trypsin, 5 mM EDTA, and fetal bovine serum were from Life Technologies, Inc.; methionine/cysteine-free DMEM was from ICN, Costa Mesa, CA; cell culture plastics were from Costar, Cambridge, MA. [3H]Arginine vasopressin (specific activity 60–80 Ci/mmol), Expire 35S phosphate labeling mix (specific activity >1000 Ci/mmol), and [32P]Pi/PO₄, in water (pH 5–7) were purchased from NEN Life Science Products. Amplify was from...

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1 The abbreviations used are: GRKs, G-protein-coupled receptor kinases; V1aR, V1a arginine vasopressin receptor; AVP, arginine vasopressin; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; D-PBS, Dulbecco’s PBS; HBS, Hank’s buffered saline solution; IP, inositol phosphates; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PLC, phospholipase C; TyrPhaa, phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH₂; r, receptor; CCK, cholecystokinin.
Amorski acid was from RBI (Natick, MA). Phenylacetyltyr-O-Tyr(Et)-Ph-eVal-asLys-Pro-Tyr-NH2 (TyrPhaa) was from Bachem Bioscience (King of Prussia, PA); AVP, staurosporine and all other reagents were from Sigma.

Construction of Epitope-tagged V1aR—The hemagglutinin epitope (YPYDVPDYA) was introduced using a polymerase chain reaction-based approach (11). The resulting construct was sequenced fully by the deoxy chain termination method of Sanger et al. (12). For expression in eucaryotic cells, the cDNAs were cloned into the expression vector pCDN3 (Invitrogen, Boston, MA).

Transient Expression of V1aR—HEK 293 cells were grown in DME high-glucose medium supplemented with 10% heat-inactivated bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and trypsin. Cells were plated at a density of 2.5 × 10⁶ cells/100-mm dish and transfected the following day by a modification of the method of Luthman and Magnusson (13). Growth medium was replaced with 6.7 ml of a mixture of 100 μM chlordecone and 0.25 mg/ml DEAE-dextran in DMEM with 10% fetal bovine serum containing 3 μg of DNA. After 2 h at 37 °C, the solution was removed, and the cells were treated for 1 min at room temperature with 10% dimethyl sulfoxide in PBS. The cells were rinsed with PBS and returned to growth medium at 37 °C. Measurements were done 48 h after transfection.

Stable Expression of V1aR—Cells were transfected by the calcium phosphate precipitation technique of Graham and Van der Eb (14) as described previously. The day before transfection, 1.2 × 10⁶ cells/100-mm dish were plated into each of two 100-mm dishes. The DNA-calcium phosphate co-precipitate, containing 10 μg of DNA, was prepared immediately before use; all reagents were at 37 °C. The reagents were mixed in a 15-ml sterile polystyrene tube in the following order: 10–100 μl of DNA in 1 ml EDTA, 10 μM Tris-HCl, pH 7.5; sterile H2O to bring the volume to 900 μl, 1 ml of 250 mM CaCl2, 100 μl of 15 mM Na2HPO4, 150 mM NaCl, 5 mM KCl, 50 mM HEPES, adjusted to pH 7.05 with NaOH. All reagents were added dropwise slowly, with gentle mixing after each addition. After 10 min at room temperature, half of the whitish 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, and 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, diluted with the selection medium containing 400 μg/ml G418, and distributed into two 96-well microtiter plates (2000–4000 cells/well), using a Costar transplate device. G418-resistant clones were picked after 16–18 days of growth on top of the selection medium containing 400 μg/ml G418. The day following, the cells were washed and incubated for 30 min with phosphate-free minimal essential medium, followed by the addition of 100 μl/well [32P]H3PO4. After 1 h at 37 °C, the cells were exposed to vasopressin or other agents for the times and concentrations described in the text. The cells were then chilled, washed twice with phosphate-buffered saline, and solubilized for 1 h at 4 °C in 300 μl of RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with protease inhibitors (0.1 μM phenylmethylsulfonyl fluoride, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin) and phosphatase inhibitors (10 μM sodium pyrophosphate, 10 mM NaF, 300 mM okadaic acid). Solubilization was helped by sonication with an XL2020 Misonix Inc (Farmingdale, NY) sonicator at an output power of 440 watts. Three 30-s pulses were applied at intervals of 30 s each. Cell extracts were clarified by yeast aminopeptidase and the antigen-antibody complexes were separated by incubating with prewashed protein A-Sepharose® (Pharmacia Biotech Inc.) in RIPA for 30 min and centrifugation. Pre-washed protein A-Sepharose was prepared by treating for 1 h with 25 μg/ml bovine serum albumin in RIPA, followed by two washes in RIPA alone. The clarified extracts were incubated overnight at 4 °C with 9 μg/ml monoclonal antibody 12CA5, and the antigen-antibody complexes were separated by incubating with prewashed protein A-Sepharose alone for 2 h at 4 °C. The beads were centrifuged and washed 5 times with 800 μl of RIPA, recovering them after each wash by centrifugation. The proteins were eluted from the beads for 20 min at room temperature with 80 μl of 2% Laemmli buffer containing 10% β-mercaptoethanol. The samples were electrophoresed in 10% SDS-polyacrylamide gels and visualized by staining the dried gels to Kodak DNR. 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. Unless otherwise indicated, the values were expressed as percent of the phosphorylation obtained by treating for 1 min with 100 nM AVP; each experiment was performed with duplicate samples.

Metabolic Labeling with [35S]Methionine/Cysteine—The day after plating, the cells in 100-mm dishes were starved for 1 h in methionine/cysteine-free DMEM high glucose, followed by 2 h labeling in the same medium containing 100 μCi of Expre35S Protein Labeling Mix/plate. Cells were then rinsed, and the receptor was extracted and immunoprecipitated as described above. The bands were visualized by treating the gels with Amplify® followed by fluorography of the dried gels.

Statistical Analysis of the Data—The values obtained with the PhosphorImager, the binding data, and the IP accumulation data were fitted by the least square method using logistic function. The minimization of the square differences was calculated with the function Solver in Microsoft Excel 5.0. The values of t1, Kd, Bmax, and EC50 were obtained from the fitting curves.

RESULTS

Characterization of the Hemagglutinin-tagged V1a Vasopressin Receptor—Stably transfected HEK-293 cells expressing the tagged V1aR were selected by measuring AVP binding. Metabolic labeling with [35S]Met/Cys of one of the positive clones,
Role of PKC and GRKs in V1aR Phosphorylation

Followed by extraction and immunoprecipitation of the receptor with the 12CA5 monoclonal antibody as described under “Experimental Procedures,” allowed the characterization of the migration of the V1aR protein in SDS-PAGE. As shown in Fig. 1, a single broad band migrating between 62 and 76 kDa (V1aR) was detected by autoradiography in samples immunoprecipitated from transfected HEK 293 cells. The band was not present in naive HEK 293 cells (C). The V1aR has a predicted molecular mass of 45,500 daltons and contains three consensus sites for N-linked glycosylation (9); thus, the apparent larger mass of the receptor was consistent with the presence of glycosylated sites. To examine whether addition of the epitope had altered receptor function, saturation binding assays were performed on intact cells. The ligand binding affinity obtained was 8.5 ± 2 nm, similar to the value reported by Fishman et al. (7) for the V1aR present in cultured hepatocytes. The B∞ for clone HEK-V1aR-40 was estimated at 527,000 ± 76,000 sites/cell, and the EC₅₀ of vasopressin-stimulated inositol phosphate accumulation was 4 nm, similar to values reported for the rat V1aR present in WRK1 cells (17).

**Phosphorylation of the V1a Vasopressin Receptor**—To test whether activation by ligand resulted in receptor phosphorylation, clonal cells expressing the V1aR were incubated with 32P to label their nucleotide pools and then exposed to hormone. Cell membrane proteins were extracted at different times following addition of 100 nM AVP, and the receptor was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Fig. 2A illustrates the time course of phosphorylation of the V1aR. A significant amount of 32P was found incorporated into the receptor prior to the addition of hormone; this radioactivity accounted for as much as 36 ± 2.5% of the phosphorylation observed after a 1-min treatment with AVP, and it will be referred to as basal phosphorylation. Following a very fast increase in 32P incorporation, already maximal at the shortest time analyzed (15 s), the receptor became rapidly dephosphorylated despite the continuous presence of AVP. The estimated t₁/₂ of dephosphorylation was 6 min. After approximately 30 min, the amount of 32P incorporated into the receptor had returned to basal levels. Equilibration of the cells with 300 mM okadaic acid 10 min prior to the addition of AVP did not prevent the dephosphorylation of the receptor (data not shown).

As shown by the dose-response curve in Fig. 2B, the extent of V1aR phosphorylation was dependent on the amount of AVP present. The dependence of phosphorylation on hormone concentration was examined after 1-min exposure of the cells to different concentrations of AVP. Ligand-promoted phosphorylation not only increased the amount of 32P in the V1aR, it also changed the migration of the receptor protein in SDS-PAGE as seen with the receptor protein labeled with [35S]methionine, as shown in Fig. 1. Addition of 100 nM AVP promoted the rapid loss of 80% of the binding sites from the cell surface with a t₁/₂ = 1 min, as illustrated in Fig. 3. This rapid internalization is comparable to what had been reported in rat hepatocytes (7), and it reveals a temporal correlation between phosphorylation and ligand-induced internalization of the V1aR.

**Role of Protein Kinase C**—The V1aR contains several consensus sites for PKC phosphorylation, and receptor signaling activates this enzyme by increasing diacylglycerol and intracellular Ca²⁺ in the cell. Thus, it was relevant to investigate the involvement of PKC in the phosphorylation of the receptor promoted by AVP. Treatment of 32P-labeled cells with 100 nM PMA for 1 min, to stimulate PKC activity, doubled the level of receptor phosphorylation demonstrating that PKC could phosphorylate the unoccupied receptor. After 1 min of PMA treatment the phosphorylation obtained was 46 ± 1% of the response stimulated by AVP, and it could be completely blocked by treating the cells with 1 μM of the PKC inhibitor staurosporine. As illustrated in Fig. 4A, following the addition of PMA there was a slow increase in receptor phosphorylation that reached values similar to those obtained with AVP 10 min after addition of the reagent. This was followed by a slow decrease of the radioactivity incorporated into the protein. Treatment of the cells with the Ca²⁺ ionophore ionomycin also resulted in the transient phosphorylation of the V1aR presumably by activating PKC. Phosphorylation of unoccupied V1aR stimulated by PMA did not result in a loss of binding sites from the cell.
The consequences of treating the cells overnight with 10 nM AVP receptor without changing the basal level of phosphorylation were also investigated. As illustrated in Fig. 5, incorporation of radioactivity into the V1aR phosphorylation (data not shown). The consequences of treating the cells overnight with 10 µM PMA to down-regulate PKC activity are shown in Fig. 6. After the PMA treatment, AVP-stimulated phosphorylation of the receptor was 40 ± 4% of the increase over the basal level detected in cells not treated with PMA, but it remained transient. The fact that inactivation of PKC by staurosporine or by overnight treatment with PMA did not prevent rapid loss of phosphate from the V1aR suggested that phosphorylation of the sites modified by PKC did not play a role in the lability of the phosphate groups incorporated into the receptor protein possibly by GRKs.

To confirm the role of GRKs in staurosporine-resistant phosphorylation, the dominant negative GRK2 mutant K220M was co-transfected with the V1aR into HEK 293 cells (18). AVP-stimulated receptor phosphorylation was assessed in the presence and absence of staurosporine to inhibit PKC activity. Although only a modest 26 ± 9% (n = 6) reduction was detected in all the experiments performed (data not shown), these experiments confirmed the involvement of GRKs in V1aR phosphorylation. These experiments were the only ones carried out in transiently transfected cells; all other determinations were done with stably transfected cell clones.

Desensitization of the V1a Vasopressin Receptor—As mentioned above, treatment of the cells with 100 nM AVP promoted the rapid loss of more than 70% of the binding sites from the plasma membrane. Thus, a significant impairment in the ability of the hormone to stimulate the production of inositol phosphates (IPs) was expected due to the reduction in receptor number. As illustrated in Fig. 7A, examination of the time course of AVP-stimulated IP accumulation revealed that the rate of production of inositol phosphates remained constant even 40 min after the addition of AVP. The possible interference of 20 mM LiCl with receptor sequestration was examined, but none was detected (data not shown). The rate of phosphoinositide accumulation was examined at shorter times searching for evidence of rapid desensitization. As shown in the inset panel in Fig. 7A, the rate of accumulation of IPs was higher in the first 30 s and quickly decayed to a lower rate of sustained activity. The sustained activity observed could be due to the following: 1) the receptors present on the cell surface, or 2) to sustained activity of the internalized receptor-G-protein complex. To distinguish between these possibilities, the effect of the V1aR antagonist TyrPhaar in the accumulation of IPs was tested (19). Four minutes after addition of 100 nM AVP, the hormone was removed and replaced by 1 µM TyrPhaar, and the effect of the antagonist on PLC activity was measured at different times. As illustrated in Fig. 7B, addition of the antagonist rapidly stopped the accumulation of IPs indicating that it was mediated by receptors present on the cell surface. Overnight treatment of transfected cells with PMA to down-regulate PKC activity failed to alter the fast and slow rates of accumulation of inositol phosphates, suggesting that there was no

**FIG. 3.** Sequestration of V1aR. Cells were treated with 100 nM AVP at 37 °C for the times indicated, followed by removal of the hormone as described under “Experimental Procedures.” The numbers of binding sites left on the plasma membrane were measured with [3H]AVP. The values reported are mean ± S.E. of three independent experiments and are expressed as percentage of the total number of sites measured on the plasma membrane of untreated cells.

**FIG. 4.** PMA stimulated V1aR phosphorylation. A, [32P]Orthophosphate-labeled cells expressing the epitope-tagged V1aR were treated with 100 nM PMA for the time indicated on the abscissa. Receptor immunoprecipitation and SDS-PAGE were performed as described under “Experimental Procedures.” Phosphorylation is expressed as percentage of the phosphate incorporation promoted by 1 min of AVP treatment. Data are reported as the mean ± S.E. of five independent experiments. B, after pretreating the cells with 100 nM PMA for the indicated time, the binding sites present on the plasma membrane were quantified using [3H]AVP as described under “Experimental Procedures.”
involvement of PKC in this mild desensitization (data not shown).

The speed of recycling of the internalized receptor to the cell surface was also examined. After removal of AVP, fast recycling of receptor to the cell surface was detected, a process that had been described for the V1aR in smooth muscle vascular cells (8). As shown in Fig. 8, the receptor internalized during a 10-min AVP treatment recycled rapidly to the cell surface following removal of AVP with an isotonic acid wash and return of the cells to normal growth conditions. At the last time point analyzed, the number of binding sites on the cell surface had increased to values very similar to those present in the non-treated cells indicating that only a small percent of the receptors had been degraded after internalization.

DISCUSSION

Occupancy of the V1aR by agonist promoted phosphorylation of the receptor protein. At variance from what had been observed with most receptors of this family, the phosphorylation reached maximum values very quickly and was transient in the continuous presence of AVP. This was an unexpected finding since in the same HEK 293 cell line the V2 vasopressin receptor was found to reach maximal phosphorylation at a slower rate and to maintain the phosphate incorporated into the protein for as long as 30 min in the presence of AVP. Transient phosphorylation in the continuous presence of ligand has been reported

Fig. 5. Effect of staurosporine on V1aR phosphorylation. [32P]Orthophosphate-labeled cells expressing the epitope-tagged V1aR were treated 1 min with 1 μM staurosporine, followed by activation of the receptor with AVP for the times indicated on the abscissa. Staurosporine was present until the cells were lysed. The values reported are mean ± S.E. of three independent experiments expressed as percentage of the phosphate incorporated stimulating the receptor for 30 s with AVP.

Fig. 6. Effect of PKC down-regulation on V1aR phosphorylation. Cells expressing the epitope-tagged V1aR were used for this experiment. After overnight treatment with 10 μM PMA to down-regulate PKC, treated and control cells were labeled with [32P]orthophosphate and exposed to 100 nM AVP for the times indicated. The receptor was isolated by immunoprecipitation as described under “Experimental Procedures.” A representative experiment is shown. Extracts of cells not exposed to AVP are identified as C (control).

Fig. 7. AVP-stimulated accumulation of inositol phosphates. Cells were labeled with myo-[3H]inositol, and the IPs produced after addition of 100 nM AVP in presence of 10 mM LiCl were extracted and measured as described under “Experimental Procedures.” A, sustained AVP stimulation of PLC. The inset panel illustrates the time course of accumulation in the first 2 min. The values reported are mean ± S.E. of three independent experiments. B, effect of a V1aR antagonist on sustained AVP stimulation of PLC. Inositol phosphate accumulation was measured in the presence of 100 nM AVP as in A. After 4 min the medium was replaced with a solution containing 1 μM (TyrPhaa) or 100 nM AVP .

Fig. 8. V1aR recycling after sequestration. Sequestration of the V1a was stimulated by treating the cells for 10 min with 100 nM AVP. The hormone was then removed by acid wash, and the cells were returned to 37 °C in growth medium for the indicated times to allow the return of receptors to the plasma membrane. The number of binding sites was measured with 30 nM [3H]AVP in the presence and absence of 5 μM unlabeled AVP to determine total and nonspecific binding, respectively.
for three other receptors as follows: the α1β-adrenergic and the bradykinin B2 receptors expressed in fibroblasts (20, 21), and the cholecystokinin (CCK) receptor native to acinar cells (22). Phosphorylation of the α1β-adrenergic and the bradykinin receptor peaked about 5 min after addition of ligand and started showing a reduction in the phosphate content 10 min later. Phosphorylation of the CCK receptor in acinar cells reached maximum values 1–2 min after addition of the ligand and returned to basal levels after 20 min. However, when the CCK receptor was expressed in Chinese hamster ovary cells, it remained phosphorylated at its maximum level in the continuous presence of CCK (23). Compared with these proteins the V1aR was the fastest in reaching maximum phosphorylation.

The data indicated that protein kinase C and other kinases, most likely GRKs, were involved in phosphorylating the V1aR. The possibility that phosphorylation of the V1aR by PKC caused the fast de-phosphorylation observed was not supported by comparison with other G-protein-coupled receptors. For example the somatostatin (24), angiotensin AT1 A (25), and thromboxane A2 (26) receptors do not lose their phosphate groups in the continuous presence of the ligand, although PKC contributes to their ligand-promoted phosphorylation. This assumption is consistent with the finding that neither down-regulation of PKC by PMA nor inhibition of its activity by staurosporine had a significant effect on the kinetics of phosphorylation and dephosphorylation of the V1aR. The longer lived phosphorylated forms of the V2 vasopressin and other receptors expressed in HEK 293 cells indicated that transient phosphorylation is not dependent on the host cells, rather it is an intrinsic property of V1aR protein. Expression of the dominant negative GRK2 reduced ligand-promoted V1aR phosphorylation in the presence of staurosporine by 26% strengthening the assumption that the protein is a substrate for GRK. This supposition is in accordance to the reported ability of GRKs to phosphorylate other receptors of this subfamily. The identity of the GRK(s) that phosphorylate this receptor remains unresolved since the six known variants of the enzyme have been detected in HEK 293 cells. To examine whether the constitutive V1aR of hepatocytes and smooth muscle vascular cells exhibits transient phosphorylation, it will be necessary to develop antibodies with good affinity for the receptor protein.

In addition to phosphorylation, AVP promoted internalization of the V1aR. Although the experiments presented established a strong temporal correlation between phosphorylation and internalization of the V1aR, our laboratory has reported the ligand-promoted internalization of the non-phosphorylated V2 vasopressin receptor (4). This finding suggests that GRK phosphorylation might not be necessary for the internalization of the ligand occupied receptor, but to prove this point would require identification of the sites phosphorylated by GRK(s) in the V1aR followed by the characterization of the behavior of this mutant V1aR in internalization experiments.

Removal of the hormone revealed prompt recycling of the receptor to the cell surface. The recycled receptor accumulated relatively fast on the plasma membrane, and the number of sites reached values similar to those present at the beginning of the experiment suggesting that recycling occurred without significant degradation of V1aR molecules. The return of the de-phosphorylated receptor protein to the cell surface would be in agreement with the hypothesis of Krueger et al. (3) that defines internalization as a necessary step for the receptor to be de-phosphorylated and thus regain its activity, i.e. to reverse ligand-promoted inactivation.

Similar to phosphorylation, PLC activation occurred very rapidly after hormone addition, followed immediately by a decrease in the rate of accumulation of inositol phosphates. The attenuation of PLC activity was not as pronounced as expected from the significant loss of receptors from the cell surface. Fig. 7 illustrates that the rate of IP accumulation was sustained for more than 30 min in the presence of hormone, while after 5 min more than 75% of the receptor remained inside the cell. Desensitization of the V1aR happened within the first 2 min after addition of hormone as illustrated in the inset panel of Fig. 7, and there was no evidence of progressive loss of PLC activity during prolonged incubations at variance with what has been described for in vitro systems that are desensitized until the activity of the effectors practically ceases (27). The sustained level of PLC activity suggested that the apparent desensitization of the rate of IP accumulation detected initially was due to the fast sequestration of the phosphorylated receptor resulting in a decrease in the number of proteins mediating PLC stimulation. The ability of a V1aR antagonist to inhibit the sustained accumulation of IPs that followed indicated that the activity of PLC was maintained by receptors present on the cell surface, whose number was constantly replenished by recycling of the protein.

Consistent with the hypothesis that a conformational change induced by ligand binding is required to trigger receptor internalization (4), PMA-induced phosphorylation of unoccupied receptor was not sufficient to provoke a reduction of cell surface receptors. These data are compatible with the report that a truncated V1aR missing the putative PKC sites on the carboxyl terminus still undergoes ligand-induced desensitization, probably caused by ligand-induced disappearance of receptor from the cell surface. No information on V1aR phosphorylation was provided (28).

Some of the phosphate incorporated in response to PMA was removed during the 1st h, implying that dephosphorylation could take place without the receptor being transferred to the endosomal compartment. The phosphate incorporated by PKC into the ligand-free receptor was cleared at a much slower rate than the phosphate incorporated into the ligand-bound receptor (Figs. 2, 4, and 6). Two alternative hypotheses are suggested by the observations: that AVP-promoted internalization of the V1aR translocates the receptor to endosomal compartments that are enriched in phosphatases responsible for the fast dephosphorylation, or that the phosphorylated occupied receptor acquires a conformation that makes it a better substrate for the phosphatases regardless of its subcellular localization. The experiments presented could not distinguish between these two possibilities; they only established a strong temporal correlation between the disappearance of the incorporated phosphate and the fast internalization of the receptor.

A noticeable amount of phosphate was present in the resting receptor. The changed migration in acrylamide gels caused by the incorporation of additional phosphate promoted by receptor activation suggests that there are qualitative as well as quantitative differences among the phosphorylation sites. This observation is reminiscent to what has been described for the β2-adrenergic receptor for which multiple cAMP-dependent protein kinase and GRK phosphorylation sites have been demonstrated to play different roles in desensitization and sequestration (2). No functional consequences of PKC-catalyzed phosphorylation were detected for the V1aR in the experiments described.

In conclusion, activation of the V1aR by AVP promotes phosphorylation of the receptor by GRK and PKC. PKC phosphorylation by itself does not promote V1aR sequestration or alter its desensitization. AVP-promoted phosphorylation is fast and quickly reversible in the continuous presence of ligand, a characteristic that seems intrinsic to the receptor protein. Phosphorylation and sequestration of the ligand occupied receptor are concurrent and seemed to participate in desensitization.
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