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# A New Linear $V_{1A}$ Vasopressin Antagonist and Its Use in Characterizing Receptor/G Protein Interactions

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## SUMMARY

We characterized a new iodinated, high affinity, linear $V_{1a}$ vasopressin antagonist, phenylacetyl-D-Tyr(Et)Phe-Gln-Asn-Lys-Pro-Arg-Tyr-$NH_2$. The antagonist bound specifically to the $V_{1a}$ vasopressin receptor in crude rat liver membranes with an apparent $K_d$ value of 0.168 nM. This affinity is ~1 order of magnitude greater than that of the natural agonist, vasopressin. The inhibitory activity of the antagonist can be demonstrated by its inability to elicit activation and uncoupling of G proteins from the receptor. Thus, after occupancy of receptor sites in rat liver membranes with labeled antagonist and detergent solubilization, the labeled receptor (~60 kDa) was eluted as a stable 400-kDa complex on size-exclusion chromatography. In contrast, when the receptor sites were occupied by the agonist [$^3$H]vasopressin, the receptor eluted as a 60-kDa peak. Coincubation of membranes with iodinated antagonist and an excess of unlabeled vasopressin caused both reduced antagonist binding and a complete shift from the 400-kDa to the 60-kDa peak. The addition of vasopressin to unliganded 400-kDa fractions resulted in a 75% increase in [$^{35}$S]guanosine-5′-*O*-(3-thio)triphosphate binding activity, indicating that the 400-kDa fraction contains complexes between the $V_{1a}$ receptor and G proteins. The vasopressin-elicited increase was inhibited by antagonist. Using specific antibodies and immunoadsorption to protein A/Sepharose columns, we found that G protein isotypes $G_{\alpha q/11}$, $G_{\alpha i3}$, and $G_{\alpha s}$, and effector enzymes PLC-$\beta$1, PLC-$\gamma$2 and PLA-2 were associated with the antagonist-labeled receptor in the 400-kDa fraction. Because the 400-kDa complex was found in the absence of ligand, the $V_{1a}$ receptor and the appropriate G proteins and effector enzymes are likely preassociated with each other and do not aggregate after antagonist addition. The association of $V_{1a}$ receptor with the different specific G proteins and effector enzymes is consistent with the multiple actions of vasopressin on liver cells. Antibodies directed against a portion of the carboxyl-terminal domain of the $V_{1a}$ receptor interacted with 60-kDa antagonist-occupied receptor but not with receptor in the 400-kDa complex. These results suggest that the carboxyl-terminal region of the receptor is sterically hindered when coupled to G proteins. The iodinated linear vasopressin antagonist therefore allows stable receptor/G protein complexes and can be an important tool (along with the antisera) for use in the study of factors that control $V_{1a}$ receptor/G protein coupling.

AVP receptors are members of the superfamily of G protein-coupled receptors. Distinct yet similar vasopressin receptor subtypes, which are expressed by separate genes (1), are found in the liver ($V_{1a}$), anterior pituitary [$V_{1b}$ ($V_3$)], and kidney ($V_2$). $V_1$ receptors are associated with polyphosphoinositide metabolism; $V_2$ receptor activity is mediated by cAMP. $V_{1a}$ receptors in liver membranes have been solubilized and characterized (2–6). Incubation of liver membranes with GTP$\gamma$S reduces the affinity of the receptor for ligand, presumably by dissociating a receptor/G protein complex (5). A nearly homogeneous preparation of rat liver $V_{1a}$ receptor has an apparent molecular weight of 58,000, which is in agreement with affinity cross-linking results with crude membrane preparations (6).

A number of selective AVP agonists and antagonists have served as powerful tools to elucidate the physiological and pathophysiological roles of AVP (7, 8). Manning *et al*. (8) showed that acyclic analogues of AVP have potent antagonistic properties, and they synthesized eight variants with a Tyr-$NH_2$ residue at the carboxyl terminus for radioiodination (9). One has been characterized in detail (10), whereas another, LVPA, is the subject of the current study. This peptide is of interest because it has an 8-Lys that can be derivatized for photoaffinity labeling or labeling with biotin or used with bifunctional cross-linking agents. One purpose of the current

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**ABBREVIATIONS:** AVP, arginine vasopressin; GTP$\gamma$S, guanosine-5′-*O*-(3-thio)triphosphate; LVPA, phenylacetyl-D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-Tyr-$NH_2$; PLC, phospholipase C; PLA, phospholipase A; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]2-hydroxy-1-propanesulfonate.

study was to characterize binding and functional properties of this peptide. We noted that unlike tritiated agonist, the iodinated antagonist allows $V_{1a}$ receptor to remain complexed to G proteins and effector enzymes during solubilization. As such, the iodinated antagonist takes on even greater usefulness by allowing the identification of the G proteins and to discriminate between coupled and uncoupled forms of the $V_{1a}$ receptor.

## Experimental Procedures

**Materials.** [*tyrosyl*-2,6-$^3$H]AVP (17.4 Ci/mmol) and Na$^{125}$I were purchased from Amersham (Arlington Heights, IL). CHAPSO was purchased from Pierce Chemical (Rockford, IL). Rabbit polyclonal antibodies to mouse $G_{\alpha q/11}$ (residues 341–359 of $G_{\alpha q}$ and $G_{\alpha 11}$), mouse $G_{\alpha q}$ (residues 13–29), rat $G_{\alpha s/olf}$ (residues 377–394 of $G_{\alpha s}$, differing from $G_{\alpha olf}$ by a single residue), rat $G_{\alpha i3}$ (residues 345–354), rat PLC-$\beta$1 (residues 1204–1216), human PLC-$\gamma$2 (residues 1213–1232), human PLA-2 (residues 1–216), and corresponding peptides used for the antibodies preparation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum to murine $G_{\alpha q/14}$ was directed against the peptide $H_2$N-CQLNREFNLV-COOH, corresponding to residues 350–359 of the carboxyl terminus of $G_{\alpha 14}$ (11). LVPA (9) was a gift from Dr. Maurice Manning (Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH). The other AVP analogues were purchased from Peninsula Laboratories (Belmont, CA). [$^{35}$S]GTP$\gamma$S (1156 Ci/mmol, was purchased from DuPont-New England Nuclear Research Products (Boston, MA). Mastoparan was purchased from Sigma Chemical (St. Louis, MO).

**Iodination of linear AVP antagonist.** LVPA was monoiodinated to a specific activity of ~2000 Ci/mmol, and the labeled peptide was purified by high performance liquid chromatography, as described previously for the preparation of iodinated oxytocin antagonist (12).

**Binding studies.** Binding parameters were determined by incubating liver membranes (50 $\mu$g) with increasing concentrations of $^{125}$I-LVPA (8–1100 pM) for 16 hr at 4° in a final volume of 150 $\mu$l. Assay buffer was composed of 50 mM Tris-maleate, 10 mM $MnCl_2$, 0.1% (w/v) gelatin, 1% (w/v) bovine serum albumin (Fraction V), and 0.05% (w/v) bacitracin, pH 7.5. Nonspecific binding was determined by the addition of 0.1 $\mu$MAVP for each $^{125}$I-LVPA concentration point. Bound and free ligands were separated by filtration through microporous glass-fiber filters (Whatman GF/C) presoaked in 0.3% (v/v) polyethyleneimide in water, with the use of a Titertek Cell Harvester 530 (Flow Laboratories, Irvine, Scotland). For competition experiments, 50-$\mu$g samples of liver membrane proteins were incubated with 0.43 pM $^{125}$I-LVPA (~20,000 cpm) and increasing concentrations of peptides. We determined membrane protein concentrations by using Micro BCA Protein Assay Reagent (Pierce Chemical) and bovine serum albumin as standard.

**Solubilization of occupied receptor.** Livers from adult female Sprague-Dawley rats were homogenized in 10 volumes of buffer composed of 10 mM Tris, 1.5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.4, at 4°.[3] The homogenate was centrifuged 20,000 × *g* for 10 min, and the supernatant was recentrifuged at 165,000 × *g* for 30 min at 4°. The microsomal pellet was resuspended in 50 mM Tris-maleate, pH 7.6, containing 10 mM $MnCl_2$ and stored at −70° at concentrations ranging from 7 to 18 mg of membrane protein/ml. Aliquots were thawed, repelleted at 165,000 × *g* for 30 min, adjusted to a concentration of 9 mg of membrane protein in 2 ml of binding buffer (20 mM HEPES, 10 mM $MnCl_2$, pH 7.6), and resuspended by sonication. Labeled agonist or antagonist (~1 × 10$^6$ cpm) was incubated with the membranes for 1 hr at 22°. Unbound ligand was removed by centrifuging the membranes at 165,000 × *g* for 30 min at 4°. The resulting pellets were homogenized in 0.9 ml of solubilization solution composed of 7.5 mM CHAPSO, 20 mM HEPES, 10 mM $NaN_3$, 2 mM EDTA, and 20 mM ATP, pH 7.6, and incubated on ice for 30 min with constant stirring. The extract was centrifuged at 165,000 × *g* for 30 min. The supernatant was adjusted to a final concentration of 5.0 mM CHAPSO by the addition of 20 mM HEPES, and a mix of proteolysis inhibitors to a final concentration of 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g of leupeptin/ml, and 10 $\mu$g of apoprotinin/ml was added. The samples were either used immediately for chromatography or stored at −70° for several days until used.

**Size-exclusion chromatography.** Columns for chromatography were equilibrated with DOC buffer (5 mM deoxycholate, 20 mM HEPES, and 2 mM EDTA, pH 7.4). Two types of columns were used: Sepharose 6L-CB (Pharmacia, Piscataway, NJ) (1.5 × 100 cm) and Superose 12 HR 10/30 (Pharmacia). Solubilized extracts (0.5 ml) were applied to the columns, and 2- and 0.5-ml fractions, respectively, were collected. Radioactivity in each fraction was measured using a gamma counter, and in some instances specific fractions were pooled and used for further studies. Molecular size markers were purchased from Pharmacia.

**Measurement of [$^{35}$S]GTP$\gamma$S binding.** Aliquots of the 400-kDa peak eluted from Superose 12 HR 10/30 columns were adjusted to 3.5 $\mu$g of protein in 50 $\mu$l and assayed for [$^{35}$S]GTP$\gamma$S binding by a modification of the method of Hepler *et al.* (13). The final sample (100 $\mu$l) contained 50 mM HEPES, pH 7.2, 3 mM EGTA, 20 mM $MgCl_2$, 50 $\mu$M GDP, 100 mM NaCl, 0.1% deoxycholic acid, 50 $\mu$M GTP$\gamma$S, and 3 nM [$^{35}$S]GTP$\gamma$S. Assays were performed in the presence or absence of 0.1 $\mu$M AVP or 1 $\mu$M unlabeled LVPA. Mastoparan (50 $\mu$M) was used as positive control. The samples were incubated for 20 min at 30°. Bound and free [$^{35}$S]GTP$\gamma$S was separated by gel permeation through 1-ml Sephadex G-50 spin-columns (equilibrated with 20 mM HEPES, pH 7.2, and 5 mM deoxycholate). Radioactivity in the void volume fraction was quantified by liquid scintillation counting.

**Production of polyclonal antisera to the rat $V_{1a}$ receptor.** Antisera were produced by Immuno-Dynamics (La Jolla, CA). The synthetic peptide $H_2$N-CHSMAQKFAKDDSDS-COOH, which is a 15-mer peptide extending to 10 residues from the carboxyl-terminal tail of the rat $V_{1a}$ receptor (1), was coupled to keyhole limpet hemocyanin using the heterobifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester. Two rabbits were hyperimmunized with the conjugate. The IgG fraction was purified from antisera using protein A/Sepharose CL-4B (Sigma) columns, as described previously (14), or antibodies were affinity purified by adsorption to peptide/bovine serum albumin complex coupled to Carbolink Gel according to the manufacturer's instructions (Pierce Chemical). The hapten was coupled to bovine serum albumin in the same manner as for hemocyanin.

**Immunoadsorption of $V_{1a}$ receptor complexes.** Aliquots (400 $\mu$l) of $^{125}$I-LVPA-labeled fractions obtained after size selection on Superose 12 HR 10/30 column were incubated with anti-$G_\alpha$ and anti-phospholipase polyclonal antibodies at 4° for 4 hr. To verify antibody specificity, antibodies were preincubated for 90 min with the corresponding haptens (amounts as described in figure legends) before addition of the 400-kDa fraction. The concentration of each antibody was 10 $\mu$g in 100 $\mu$l of phosphate-buffered saline, containing 0.2% gelatin and 1% bovine serum albumin. After incubation, the samples were loaded onto protein A/Sepharose CL-4B columns. Each column was composed of 150 $\mu$l of gel in a 1-ml syringe, which was preequilibrated with 10% bovine serum albumin at room temperature in DOC buffer and then rinsed with 2 ml of DOC buffer. After application of the samples, the columns were rinsed five times with 1 ml of DOC buffer, and adsorbed and unadsorbed radioactivity was measured using a gamma counter.

**Immunoaffinity column chromatography.** A 2.5-ml aliquot of solubilized rat liver membrane proteins labeled with $^{125}$I-LVPA was applied to a 10-ml immunoaffinity column, which consisted of 20 mg of rabbit polyclonal $V_{1a}$ receptor antibodies (protein A purified from antiserum) immobilized to Carbolink Gel according to the manufacturer's instructions (Pierce Chemical). After incubation overnight at

[3] Care of the animals was in accord with institutional guidelines.

4°, the gel was rinsed with DOC buffer to wash out unbound material, and proteins adsorbed to the column were eluted with 50 mM triethanolamine containing 5 mM DOC, pH 11.5. The columns were regenerated by rinsing with phosphate-buffered saline until pH was restored to 7.4, and the columns could be used several more times.

**Immunoblotting.** Fractions from columns were concentrated with a Centricon-10 concentrator (Amicon, Beverly, MA). Samples were subjected to SDS-polyacrylamide gel electrophoresis (10% or 12.5% polyacrylamide). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon P, Amersham) using a semidry blotter (Buchler Instruments). Nonspecific interactions were blocked with a 5% dry milk solution in phosphate-buffered saline/Tween 20 overnight at 4°. The membranes were incubated with antibody for 1 hr at room temperature, followed by a 1-hr incubation with horseradish peroxidase-labeled anti-rabbit Ig antibody from donkey. The ECL system (Amersham) was used for detection.

## Results

Scatchard analyses showed that LVPA apparently bound to a single class of noninteracting sites in crude rat liver membranes, with an apparent $K_d$ value of 0.168 ± 0.004 nM and $B_{max}$ value of 32.7 ± 4.2 fmol/mg of protein (values are mean ± standard error; five determinations) (Fig. 1, *top*). The ligand specificity of the receptor sites was measured by the ability of AVP and several analogues to compete for binding with $^{125}$I-LVPA. The regressions were parallel, indicating a common set of binding sites (Fig. 1, *bottom*). The rank orders of affinities for the peptides were des-Gly-[phenylacetyl$^1$,D-Tyr(*O*-ethyl)$^2$,Lys$^6$,Arg$^8$]vasopressin > [(β-mercapto-β,β-cyclopentamethylene propionic acid)$^1$,Tyr(-methyl)$^2$,Arg$^8$]vasopressin > [Arg$^8$]vasopressin > oxytocin > [Thr$^4$,Gly$^7$]oxytocin > pressinoic acid. The first two peptides, which are cyclic AVP antagonists (15), had lower relative $K_i$ values than AVP. Oxytocin and the selective oxytocin agonist [Thr$^4$,Gly$^7$]oxytocin had greater relative $K_i$ values than AVP. Pressinoic acid, which contains the six-residue ring of AVP but lacks the three-residue tail, bound with low affinity.

Comparison of the size of solubilized receptor binding to [$^3$H]AVP versus iodinated antagonist by gel-exclusion chromatography on columns of Sepharose 6B CL showed that the complexed agonist eluted as a peak ~60 kDa, whereas the complexed antagonist eluted as a 400-kDa peak (Fig. 2A). The 60-kDa form apparently has a reduced affinity for the ligand because unbound [$^3$H]AVP was eluted even though all of the ligand was bound before solubilization. The presence of the 400-kDa peak was largely absent in the lactating rat mammary gland, which has a preponderance of oxytocin receptors (16; data not shown). Incubation of liver mem-

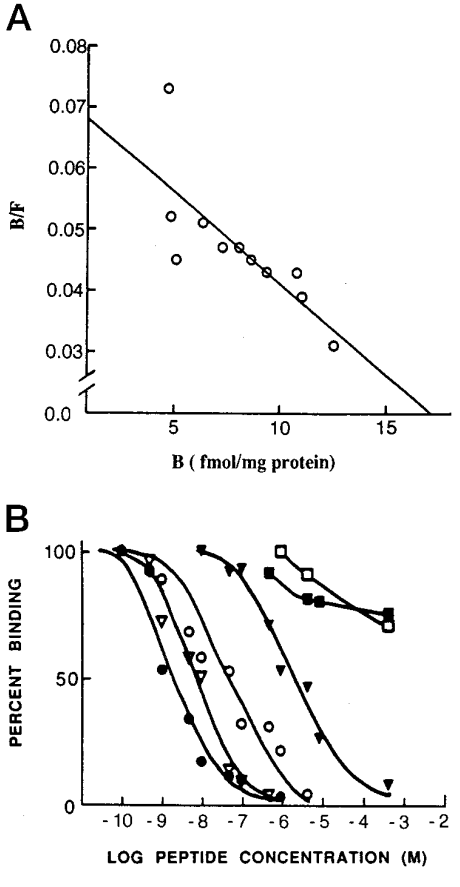


**Fig. 1.** Binding of $^{125}$I-LVPA by rat liver membranes. *Top*, Scatchard analysis. Data are from one of five replicate experiments. *Bottom*, ligand specificity. Inhibition of $^{125}$I-LVPA binding by rat liver membranes with increasing concentrations of des-Gly-[phenylacetyl$^1$,D-Tyr(O-ethyl)$^2$,Lys$^6$,Arg$^8$]vasopressin (●), [1-(β-mercapto-β,β-cyclopentamethylene propionic acid-Tyr(O-methyl)$^2$-Arg$^8$-vasopressin) (▽), Arg$^8$-vasopressin (○), oxytocin (▼), [Thr$^4$,Gly$^7$]oxytocin (■), and pressinoic acid (□). Results are expressed in percentage of maximal specific binding and are from one of four representative experiments.

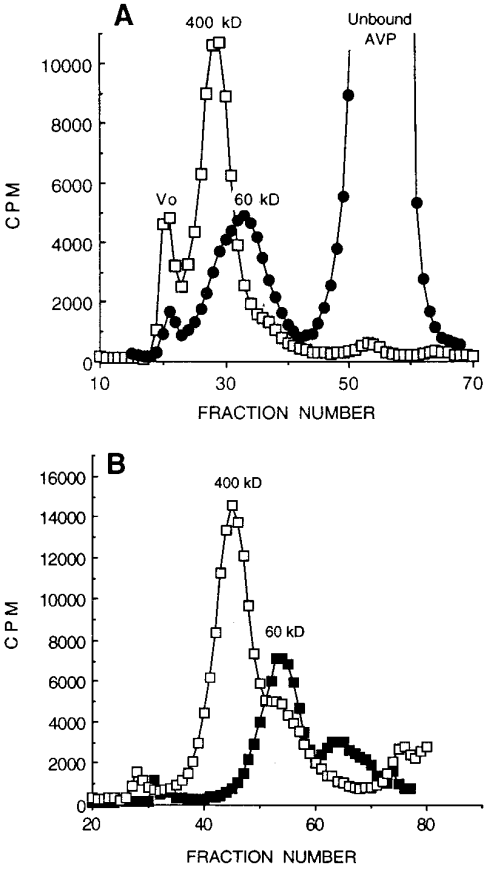


**Fig. 2.** Size separation of $V_{1a}$ vasopressin receptor binding sites in solubilized liver membranes. A, Comparison of the binding of [$^3$H]AVP and $^{125}$I-LVPA to solubilized receptor, as shown by gel filtration on a column of Sepharose 6B-CL. Two-milliliter fractions were taken. □, $^{125}$I-LVPA. ●, [$^3$H]AVP. B, Addition of 100 nM AVP and $^{125}$I-LVPA (■) before solubilization resulted in the complete loss of the labeled 400-kDa binding complex and the appearance of $^{125}$I-LVPA in the 60-kDa fraction. Fractions of 1.25 ml were taken for greater resolution.

branes with iodinated antagonist (~0.2 nM) and 100 nM AVP resulted in reduced binding and a complete shift from the 400-kDa peak to the 60-kDa peak (Fig. 2B). These findings indicate that AVP destabilizes the 400-kDa complex while allowing reduced binding of $^{125}$I-LVPA to the 60-kDa receptor. The $V_{1a}$ receptor has a higher affinity for $^{125}$I-LVPA than AVP itself (Fig. 1B).

To determine whether the antagonist causes the receptor to associate with other proteins or whether the antagonist binds to existing high-molecular-weight receptor complexes, we carried out size selection of the solubilized receptor in the absence of antagonist. Fractions containing the receptor were identified by immunoblotting using antisera directed against residues near the carboxyl terminus of the rat $V_{1a}$ receptor. The specificity of affinity purified antibodies for the rat $V_{1a}$ receptor was shown with immunoblot analysis. The antibodies reacted with a ~60-kDa band from rat liver membranes (Fig. 3), corresponding in size to the purified receptor (5, 6). The specific receptor band was absent from comparable amounts of membranes from rat kidney, which contain a preponderance of vasopressin $V_2$ receptors. No cross-reactivity was seen with human $V_{1a}$ receptors in human amnion/chorion, as would be expected from the different amino acid sequence (Fig. 3). Receptor immunoreactivity was found in the 400-kDa fraction regardless of whether the binding site was occupied by antagonist, suggesting that the 400-kDa receptor/protein complex is preformed (Fig. 4B). In agreement with the radioactivity profile of labeled antagonist (Fig. 4A), little receptor was present in the 60-kDa form. Tentative identification of receptor-associated proteins in the 400-kDa fraction was made by immunoblot analyses, using antisera to several G protein $\alpha$ subunits. Significant quantities of $G_{\alpha q}$ (Fig. 4C) and $G_{\alpha i}$ (Fig. 4D) classes were found in the 400-kDa and smaller fractions. $G_{\alpha s}$ was also present in the 400-kDa peak but absent in the fraction corresponding to free $G_s$ (Fig. 4E).

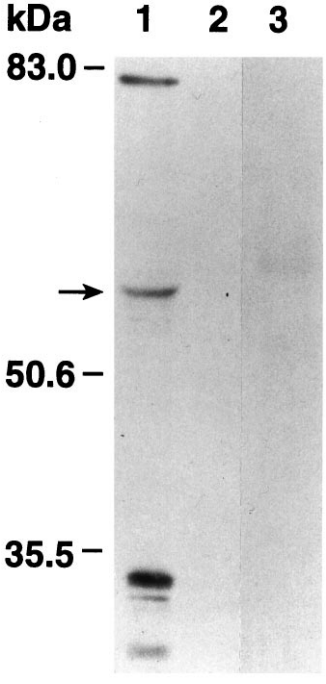


**Fig. 3.** Specificity of antibodies to the carboxyl-terminal region of the rat $V_{1a}$ receptor, as determined by immunoblotting. Cell membranes proteins (50 $\mu$g/lane) from rat liver (*lane 1*), rat kidney (*lane 2*), and human amnion/chorion (*lane 3*) were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon, and immunoblotted with affinity purified antibodies against the $V_{1a}$ receptor. A 60-kDa band, corresponding to the mass of the receptor, was seen only in rat liver. Several additional bands (~82 kDa, 32 kDa, and lower molecular mass) were found in rat liver membranes.

A direct demonstration of the association of $V_{1a}$ vasopressin receptors with G proteins in the unliganded 400-kDa peak was shown by AVP-induced [$^{35}$S]GTP$\gamma$S binding. The addition of AVP to the 400-kDa fraction caused a 75% increase in [$^{35}$S]GTP$\gamma$S binding ($p < 0.05$), which was inhibited by the LVPA (Fig. 5). The antagonist itself was ineffective in stimulating [$^{35}$S]GTP$\gamma$S binding. Vasopressin-activated binding was ~60% that induced by mastoparan, a peptide toxin from wasp venom that mimics receptors by activating $G_i$ and $G_o$ (17). In support of the lack of effect of LVPA on [$^{35}$S]GTP$\gamma$S binding, the addition of GTP$\gamma$S (0.1 mM) or GTP (20 mM) to $^{125}$I-LVPA-bound rat liver membranes before solubilization had no effect on the amount of 400-kDa complex formed (data not shown).

The identities of G proteins in the $^{125}$I-labeled receptor complex were determined by isolation of the complex by size selection to eliminate unassociated G proteins, followed by the addition of specific G protein $\alpha$ subunit antibodies and immunoadsorption to protein A/Sepharose CL-4B. After extensive rinsing of each protein A column, the amount of G protein-coupled labeled receptor was measured. The addition of anti-$G_{\alpha q/11}$ or $G_{\alpha i3}$ antibodies each resulted in adsorption of >20% of the labeled complex to protein A compared with 3% adsorption by preimmune, protein A-purified IgG (Fig. 6, *top*). In contrast, there was little or no effect of anti-$G_{\alpha 14}$ and antibodies directed against the amino-terminal domain of $G_{\alpha q}$.

Antibodies directed against $G_{\alpha s/olf}$ also bound to labeled complex, but the amount was consistently less than that seen with anti-$G_{\alpha q/11}$ and $G_{\alpha i3}$. To determine whether the notable binding of $G_{\alpha q/11}$ and $G_{\alpha i3}$ was specific, the two were incubated separately with the peptide used for immunization. The haptens competed with labeled binding sites in a ligand-specific manner; each peptide was competitive only with its corresponding antibody (Fig. 6, *bottom*).

Antagonist-labeled complexes were also examined for the presence of possible effector enzymes by the use of antibodies to PLC-$\beta$1, PLC-$\gamma$2, and PLA-2. Approximately 11% of the complex was adsorbed by either anti-PLC-$\beta$1 or -PLA-2 antibodies, whereas ~7% was adsorbed with anti-PLC-$\gamma$2 (Table 1). The specific binding of the anti-PLC-$\beta$1 and anti-PLC-$\gamma$2 antibodies was significantly reduced by preincubation with 10 $\mu$g of specific hapten (Table 1). Because of the relatively large size of the PLA-2 peptide used for immunization (216 residues), 10 $\mu$g of peptide was apparently too small of a molar concentration to compete with PLA-2 for antibody binding sites.

When crude LVPA-liganded complex from solubilized membranes was adsorbed to immobilized antireceptor antibodies, approximately half of the radioactivity was bound to the column (Fig. 7, *top*). In contrast, only ~6% of the total radioactivity was retained on preimmune IgG columns (data not shown). The same relative proportion of bound and unbound labeled complex was found over a range in protein concentrations loaded onto the immunoaffinity column, indicating that the unadsorbed fraction was not due to column

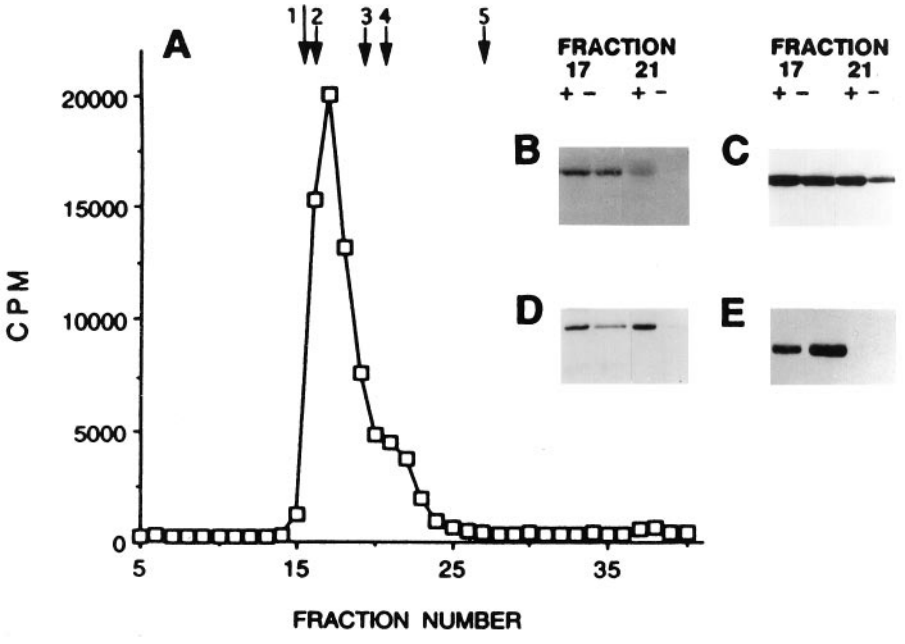


**Fig. 4.** Presence of receptor and G proteins in the 400-kDa fraction before and after $^{125}$I-LVP binding. Immunoblot analyses of Superose 12 fractions 17 and 21 (400- and 60-kDa peaks, respectively) (A), using antisera to (B) the $V_{1a}$ receptor, (C) $G_{\alpha q/11}$, (D) $G_{\alpha i3}$, or (E) $G_{\alpha s}$. Liver membranes were solubilized either before (−) or after (+) occupancy by $^{125}$I-LVPA. Estimated molecular masses of the immunoreactive protein bands in were (B) 59–60 kDa, (C) 42–43 kDa, (D) 41–42 kDa, and (E) 45 kDa, respectively. The gel filtration standards used were (*1*) thyroglobulin (670 kDa), (*2*) apoferritin (443 kDa), (*3*) β-amylase (200 kDa), (*4*) bovine serum albumin (66 kDa), and (*5*) carbonic anhydrase (29 kDa).

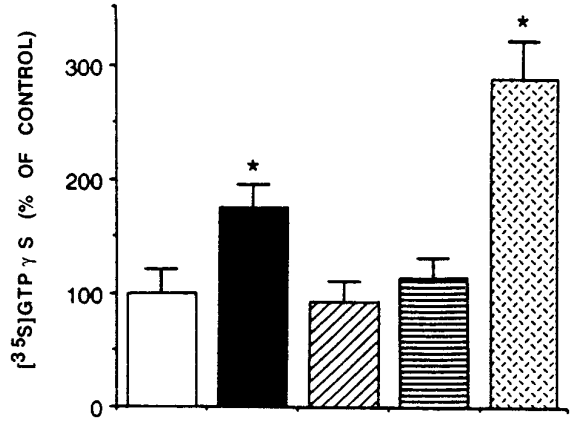


**Fig. 5.** Vasopressin stimulation of [$^{35}$S]GTPγS binding in the 400-kDa peak. Liver membranes were solubilized in the absence of ligand and fractionated on Superose 12 HB 10/30 columns (as shown in Fig. 4A). Fractions corresponding to the 400-kDa peak were incubated with [$^{35}$S]GTPγS and either buffer (□), 0.1 μM AVP (■), 1 μM LVPA (▨), 0.1 μM AVP plus 1 μM LVPA (▤), or 50 μM mastoparan ( ). Binding in the presence of buffer alone (control) was 294 ± 54 fmol/μg of protein (mean ± standard error). Values are mean ± standard error of triplicate experiments. *, Binding was significantly different ($p < 0.05$, Student's *t* test) from the control.

overloading (data not shown). Size-exclusion analysis showed that the unadsorbed fraction was ~400 kDa and not free ligand that had dissociated from the receptor (data not shown). Incubation overnight of the solubilized extract with anti-receptor IgG apparently results in a greater dissociation of free receptor from the 400-kDa complex than seen in the Superose 12 HB 10/30 studies (which were done without any prior incubation), accounting for only 50% of the receptor appearing in the 400-kDa form.

Immunoblotting of both unbound and eluted fractions from the immunoaffinity column, using antisera directed against the receptor, indicated that about equal amounts of the receptor in the crude extract were in both fractions (Fig. 7, *bottom*). Immunoblots of the unadsorbed and eluted fractions with antisera directed against $G_{\alpha q/11}$ and $G_{\alpha i3}$ indicated that G protein was present only in the unadsorbed fractions (Fig. 7, *bottom*). In view of the findings shown in Fig. 4, the results indicate that the interaction of receptor with G protein prevented the receptor from being immunoadsorbed to the anti-$V_{1a}$ receptor affinity column. Only free receptor was bound to the column.

## Discussion

Phaa-D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-$NH_2$ has been shown to be a highly potent and selective linear $V_1$ octapeptide antagonist (15). To increase the usefulness of this analogue, Manning *et al.* (9) added a carboxyl-terminal Tyr-$NH_2$ as a potential radioiodination site. The noniodinated analogue was a strong $V_1$ antagonist ($p_2$ = 8.64) and very weak $V_2$ agonist (~0.001 unit/mg) (9). The current report is the first characterization of the iodinated peptide. The apparent $K_d$ value of 0.168 nM for iodinated antagonist-liver membrane interactions is approximately one third greater than the value (0.06 nM) observed for the analogue containing an arginine at position 6 instead of a lysine (10) and ~1 order of magnitude less than an analogue containing valine in position 4 instead of glutamine and having a Tyr-$NH_2$ carboxyl-terminal residue at position 8 (18). Aside from the obvious advantages of having a highly selective and high affinity antagonist that can be radioiodinated for use with small amounts of tissue or for autoradiographic studies, the lysine residue at position 6 allows the formation of derivatives for photoaffinity and fluorescent labeling and for biotinylation. An additional important application for the antagonist is delineated in the current study: the ability to maintain stable $V_{1a}$ receptor/G protein complexes for identifying the G proteins and for use with conformational probes. Our findings, using both the antagonist and receptor antibodies, suggest that receptor/G protein-effector enzyme complexes are preformed and relatively stable in the absence of agonist. In addition to the $V_{1a}$ receptor, other G protein-coupled receptors seem to exist in preformed complexes with G proteins;

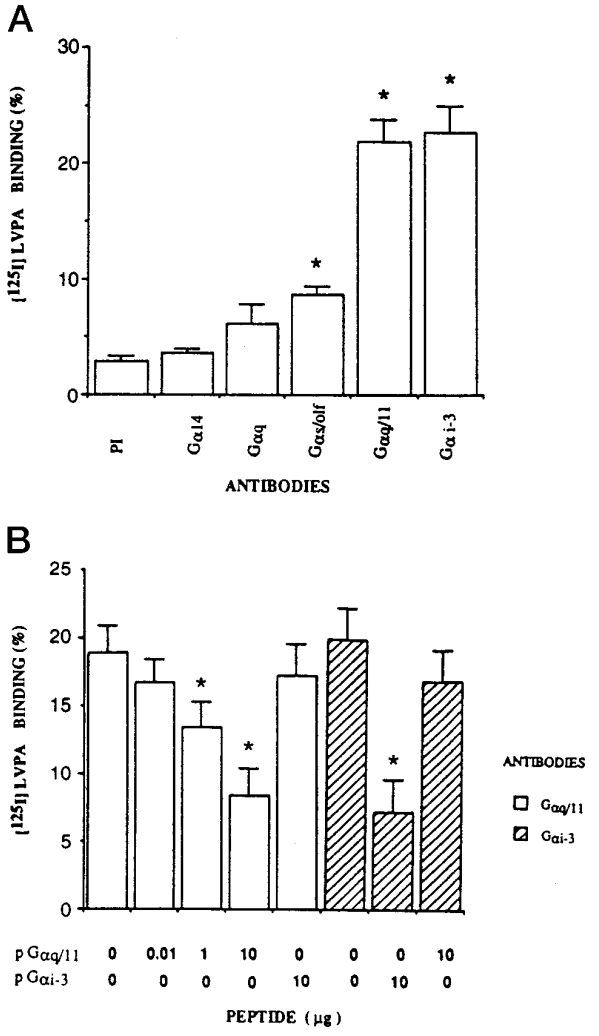


**Fig. 6.** G proteins associated with $V_{1a}$ vasopressin receptors in the 400-kDa complex. *Top*, immunoadsorption of the $^{125}$I-labeled receptor complex (400 kDa) with antibodies to G protein $\alpha$ subunits. $^{125}$I-LVPA-labeled complex was incubated with 10 $\mu$g of the IgG fraction of each of the antisera indicated, and the antibody-bound complex was adsorbed to a protein A/Sepharose column. Values are mean ± standard error of four or more experiments. *, $p < 0.05$ compared with preimmune serum antibodies. *Bottom*, binding specificity of anti $G_{\alpha q/11}$ and $G_{\alpha i3}$ antibodies. $^{125}$I-LVPA-labeled complex was incubated with 10 $\mu$g of either anti-$G_{\alpha q/11}$ or -$G_{\alpha i3}$ antibodies and peptide hapten $pG_{\alpha q/11}$ and $pG_{\alpha i3}$. The values, from which nonspecific binding (*PI*) was subtracted, are mean ± standard error of three or more determinations. *, $p < 0.05$ compared with samples with no competing peptide.

these include $A_1$ adenosine (19), C5a (20), opioid (21), and $\alpha_2$-adrenergic (22) receptors.

The identification of $G_{q/11}$ coupling to the $V_{1a}$ receptor is consistent with the results of Wange *et al.* (23), who found increased photoaffinity labeling of $G_{q/11}$ with [$^{32}$P]$\gamma$-azidoanilido GTP after treatment of rat liver membranes with AVP. Activation of $G_{q/11}$ likely accounts for AVP stimulation of increases in cytosolic levels of free $Ca^{2+}$ (24) and phosphoinositide release (25) in the liver. The association of $G_{\alpha i3}$ with the $V_{1a}$ receptor was an unexpected finding but in retrospect is consistent with several earlier observations: AVP inhibited both glucagon- and forskolin-induced hepatic cAMP accumulation, presumably by a direct effect on adenylyl cyclase activity (26). Rat hepatocytes treated with pertussis toxin,

TABLE 1

**Immunoadsorption of 400-kDa receptor complex with antibodies to phospholipases**

Isolated [$^{125}$I]LVPA-labeled receptor complex was immunoprecipitated with polyclonal antibodies against PLC-$\beta$1, PLA-2, or PLC-$\gamma$2 in the presence or absence of specific peptide used for antibody preparation, as described in Materials and Methods. Preimmune antibodies, used as control, precipitated 2.32 ± 0.29% of [$^{125}$I]LVPA bound to protein A Sepharose. Values are mean ± standard error of three or more experiments.

| Antibody to enzyme | Peptide | [$^{125}$I]LVPA bound to protein A Sepharose |
|---|---|---|
| | *μg* | *% of input* |
| PLC-$\beta$1 | 0 | 11.13 ± 1.33 |
| PLC-$\beta$1 | 10 | 3.64 ± 0.34[a] |
| PLA-2 | 0 | 10.66 ± 0.81 |
| PLA-2 | 10 | 11.56 ± 0.14 |
| PLC-$\gamma$2 | 0 | 6.81 ± 0.50 |
| PLC-$\gamma$2 | 10 | 3.71 ± 0.26[a] |

[a] Binding was significantly different ($p < 0.05$, Student's *t* test) in comparison with sample without peptide.

which inhibits $G_i$ but not $G_{q/11}$, lost both AVP- and GTP$\gamma$S-stimulated $Ca^{2+}$ influx, under conditions in which AVP-stimulated release of $Ca^{2+}$ from intracellular stores was unaffected (27). Inhibition was also obtained with anti-$G_{\alpha i1-2}$ antibody and $G_{\alpha i2}$ peptide but not with a $G_{\alpha i3}$ peptide (27). Additional studies have indicated that $G_{i2}$ is involved in AVP and store-activated $Ca^{2+}$ inflow in hepatocytes (28). Because the anti-$G_{\alpha i3}$ antibodies used in our study cross-react with $G_{\alpha i2}$, it is not possible to determine the relative contribution of each to the 400-kDa complex. AVP stimulation of arachidonic acid release by rat aortic smooth muscle cells is also mediated by a pertussis toxin-sensitive G protein, and the effect is independent of phosphoinositide hydrolysis (28). $G_{i3}$ has also been shown to regulate PLC-mediated intracellular $Ca^{2+}$ release and PLA-2-mediated arachidonic acid release in Chinese hamster ovary cells (29). These collective observations strongly suggest that pertussis toxin-sensitive G proteins, such as $G_i$, are important mediators of AVP action.

The association of $G_s$ with a smaller fraction of $V_{1a}$ receptors is a new observation and might be indicative of a mechanism by which AVP opens voltage-gated membrane $Ca^{2+}$ channels (30). Differences in the amounts of labeled receptor complex immunoadsorbed by the different G protein antibodies could be an indication of the relative proportion of $V_{1a}$ receptor associated with each $\alpha$ subunit subtype, but we cannot draw any definitive conclusions without knowing the affinities of each of the antisera for their respective epitopes.

Polyclonal antibodies to $G_{\alpha q/11}$, $G_{\alpha i3}$, $G_{\alpha s/olf}$ and $G_{\alpha 14}$ were directed against peptides corresponding to the carboxyl termini of their respective $G_\alpha$ subunits. $G_{\alpha 14}$ IgG was ineffective in immunoadsorbing any of the $V_{1a}$ receptor complex, even though the antiserum should bind $G_{\alpha q/11}$ because of overlap in amino acid sequence between the two peptides. Because we used polyclonal antisera, there might be a preponderance of antibodies directed against $G_{\alpha 14}$ rather than $G_{\alpha q}$ in the particular preparation of antiserum used, accounting for its lack of effect. Antibody directed against the amino-terminal region of $G_{\alpha q}$ was not effective, perhaps because of inaccessibility of the amino-terminal region in either the native or complexed conformation. The amino terminus of $G_{\alpha q}$ has been shown to be important for its interactions with PLC-$\beta$1 and m1 muscarinic cholinergic receptor (31).

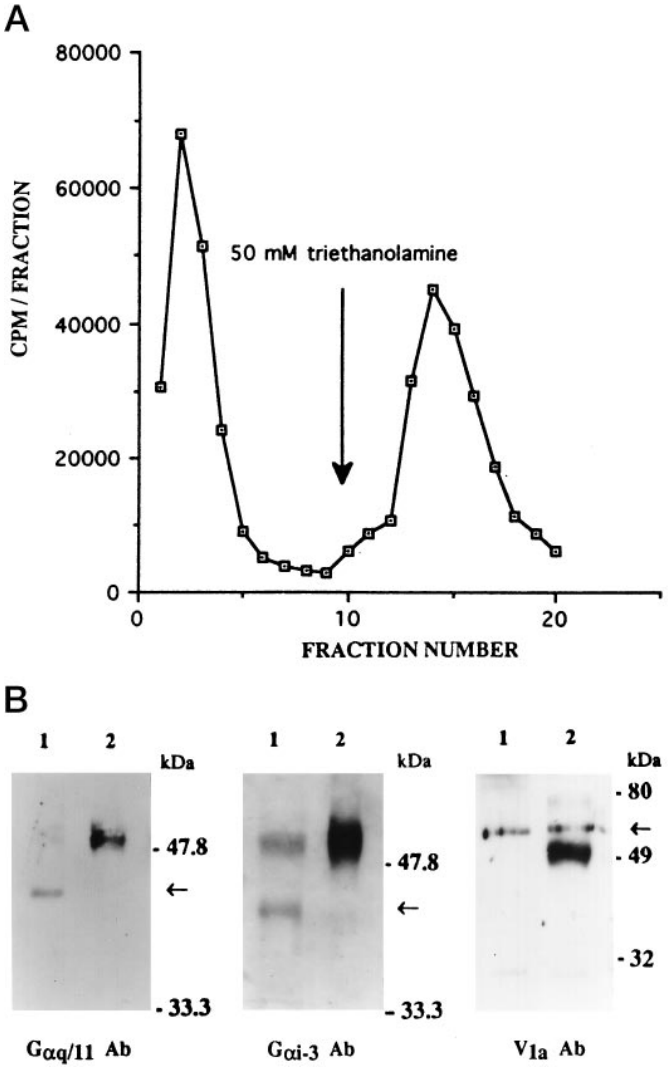


**Fig. 7.** The $V_{1a}$ vasopressin receptor is not bound to an immunoaffinity column when the receptor is associated with $G_{q/11}$ and $G_{i3}$. *Top*, immunoadsorption of prefractionated, solubilized $^{125}$I-LVPA-labeled receptor complex to immobilized IgG fraction of antisera directed against the carboxyl-terminal tail of the rat vasopressin $V_{1a}$ receptor. After application of the sample and rinsing of unbound material, elution was performed with 50 mM triethanolamine. *Bottom*, the unadsorbed (*lanes 1*) and eluted fractions (*lanes 2*) were separately pooled and subjected to immunoblot analysis, using antibodies to $G_{\alpha q/11}$, $G_{\alpha i3}$, and $V_{1a}$ receptor (*left*, *middle*, and *right*, respectively). *Arrows*, relevant bands. Sloughing of IgG from the affinity columns results in the appearance of a major 50-kDa IgG heavy-chain band in the eluted fractions.

We also found that the $V_{1a}$ receptors are associated with PLC and PLA-2. PLC has previously been shown to be associated with solubilized rat liver $V_{1a}$ receptors (32). The principal fraction of effector enzymes associated with the $V_{1a}$ receptor was PLC-$\beta$1. A smaller, but significant fraction of receptors was bound to PLC-$\gamma$2, suggesting that AVP stimulation of inositol phosphate production results from activation of more than one isoform of PLC. Activation of PLC-$\gamma$2 occurs by tyrosine phosphorylation, which involves interactions between PLC-$\gamma$ and receptor tyrosine kinases (33). The mechanism of interaction between $V_{1a}$ receptor and PLC-$\gamma$2 is not presently understood, but activation of PLC-$\gamma$2 might be a pathway involved in the mitogenic activity of AVP (34–35).

We found that when receptor is part of the 400-kDa complex, it does not react with antibodies directed against the region near the carboxyl-terminal end. The antibody, however, binds to uncomplexed $V_{1a}$ receptor and to SDS-denatured receptor. A possible explanation for these findings is that interaction of the receptor with G protein involves the carboxyl-terminal tail, which is hindered from interaction with receptor antibodies when complexed to G proteins. The carboxyl termini of several G protein-coupled receptors, along with specific cytoplasmic loops, have been implicated in G protein interactions (36, 37). Splice variants of the carboxyl-terminal tail of the prostaglandin $EP_3$ receptor subtypes specify the specific isotype of G protein that is coupled (i.e., $G_s$, $G_i$, or $G_s/G_i/G_q$) (38). Schneider *et al*. (40) found that a core region of the PTH receptor composed of the three intracellular loops can interact promiscuously with different G proteins and that the carboxyl terminus of the full-length receptor directs the specific interaction with $G_s$. In addition to contributing to G protein isotype specificity, the carboxyl-terminal domain can be essential for activity. Irie *et al*. (50) found that $EP_3$ receptors truncated at the carboxyl terminus retained the ability to physically associate with $G_{i2}$ but that there was no forskolin-induced inhibition cAMP accumulation or GTPase stimulation.

We are not certain whether changes in the accessibility of the antibody to the region near the carboxyl-terminal domain of the $V_{1a}$ receptor reflect overall conformational changes in the molecule on interaction with other proteins in the complex or more local conformational changes due to interactions with G or other proteins. Because there are both potential palmitoylation and phosphorylation sites in the carboxyl-terminal domain of the $V_{1a}$ receptor (1), inaccessibility of the antibody may be a reflection of covalent modification that are requisite for interaction with other proteins. The antibody, in conjunction with $^{125}$I-LVPA, should prove useful in future investigations to clarify whether covalent modifications of the vasopressin $V_{1a}$ receptor are involved in G protein coupling.

In summary, the inability of the antagonist to stimulate [$^{35}$S]GTP$\gamma$S binding is indicative of the inability of the antagonist-occupied receptor to assume a conformation that activates G proteins. As a consequence, the preformed receptor/G protein/effector enzyme complex is maintained in the presence of antagonist. Thus, $^{125}$I-LVPA is an important tool, along with conformational probes, to characterize previously unrecognized G protein and effector enzymes that interact with the $V_{1a}$ receptor. Furthermore, the high specific activity of the labeled antagonist, compared with tritiated compounds, and the high affinity for $V_{1a}$ receptors allow the use of relatively small amounts of membrane material for these studies.

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