Solubilization of rat liver vasopressin receptors as a complex with a guanine-nucleotide-binding protein and phosphoinositide-specific phospholipase C

Nambi AIYAR,* C. Frank BENNETT, Ponnal NAMBI, Wendy VALINSKI, Michael ANGIOLI, Michael MINNICH and Stanley T. CROOKE

Department of Molecular Pharmacology, Smith Kline and French Laboratories, Philadelphia, PA 19101, U.S.A.

Vasopressin V_1 receptors were solubilized from rat liver plasma membranes with the detergent lysophosphatidylcholine. [^3H]Arginine]vasopressin (AVP) binding to the solubilized preparations was specific and saturable, with a dissociation constant of 0.6 nM. Cross-linking of [^{125}I]vasopressin to the solubilized fraction, studied by SDS/polyacrylamide-gel-electrophoretic analysis, demonstrated the presence of a 65 kDa band which was specifically labelled with [^{125}I]vasopressin. Specific binding of [^3H]AVP to these solubilized receptors was decreased by guanine nucleotides, but not by adenosine 5'-[$\beta\gamma$ -imido]triphosphate. Addition of vasopressin increased specific binding of ^{35}S -labelled guanosine 5'-[γ -thio]triphosphate (GTP[^{35}S]) to the solubilized fractions, indicating co-solubilization of GTP-binding protein(s) [G-protein(s)] and vasopressin receptors. The solubilized fraction was insensitive to both cholera- and pertussis-toxin treatment. Immunoblotting of the solubilized fraction with antibodies specific for a phosphoinositide-specific phospholipase C (PI-PLC I) demonstrated the presence of a 60 kDa protein. Anti-PI-PLC I antiserum immunoprecipitated solubilized vasopressin-binding sites from rat liver (V_1), but not solubilized vasopressin-binding sites from hog kidney (V_2). Similar results were obtained with an anti-PI-PLC I IgG affinity column. The solubilized (V_1) receptors were enriched by ion-exchange and high-performance gel-filtration liquid chromatography. Vasopressin-binding activity was co-eluted with PI-PLC I and GTP[S]-binding activity on a DEAE-Sepharose column. The major vasopressin- and GTP[^{35}S]-binding activities were co-eluted with PI-PLC I activity at approx. 240 kDa suggesting that vasopressin receptors from rat liver membranes can be solubilized as a complex of receptor-coupler-effector by using the detergent lysophosphatidylcholine,

INTRODUCTION

A common mechanism for signal transduction utilized by many agonists comprises three major components: (1) a specific cell-surface receptor, (2) a GTP-binding protein and (3) an effector system [1,2]. Examples of different effector systems coupled to cell-surface receptors include phosphodiesterase, adenylate cyclase, phosphoinositide-specific phospholipase C (PI-PLC) and a second lipase, most probably phospholipase A_2 [1–5]. A single agonist may bind to multiple receptor subtypes, generating different second messengers. Vasopressin is an example of such an agonist. In renal collecting-duct epithelium, vasopressin binds to V_2 receptors, which are coupled to adenylate cyclase via an interaction with a stimulatory GTP-binding protein (G_s), resulting in an increase in water permeability [6]. Vascular smooth-muscle cells and rat liver exhibit vasopressin V_{1a} receptors, which stimulate phosphoinositide hydrolysis by activating a PI-PLC, resulting in smooth-muscle contraction and gluconeogenesis, respectively [7–9]. The vasopressin receptors involved in corticotropin release in adenohypophysis are classified as V_{1b} [10]. V_1 receptors in liver are also coupled in a negative manner to adenylate cyclase via

interactions with an inhibitory GTP-binding protein (G_i) [11]. In smooth-muscle cells V_1 receptors negatively modulate cyclic AMP and cyclic GMP accumulation, and activate PI-PLC without the involvement of G_i [12,13,8].

Although there is no evidence demonstrating a direct interaction between GTP-binding proteins and PI-PLC, it is generally accepted that a GTP-binding protein regulates PI-PLC in a manner analogous to adenylate cyclase. This is due to the observation that GTP and stable GTP analogues stimulate hydrolysis of polyphosphoinositides in permeabilized cells and cell membranes [14–18]. For the V_1 receptor, the identity of the GTP-binding protein which couples to PI-PLC is unknown [19–21]. However, in neutrophils and mast cells, chemotactic factors such as the peptide fMet-Leu-Phe and leukotriene B_4 stimulate PI-PLC through an interaction with a pertussis-toxin-sensitive GTP-binding protein [15,22].

Rat liver V_1 receptors have been solubilized from membranes apparently associated with a GTP-binding protein [19–21]. Association of the receptor with the GTP-binding protein required that the receptor be solubilized in the presence of agonist, or prebound

Abbreviations used: AVP, [8-arginine]vasopressin; PI-PLC, phosphoinositide-specific phospholipase C; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; G-protein, guanine-nucleotide-binding protein; PAGE, polyacrylamide-gel electrophoresis.

* To whom reprint requests should be addressed.

[19–21]. The GTP-binding protein solubilized with rat liver V_1 receptors was insensitive to pertussis-toxin treatment [19]. We have recently described the solubilization of unliganded V_1 receptors from rat liver membranes by using egg lysophosphatidylcholine [23]. Receptors solubilized with lysophosphatidylcholine exhibited saturation kinetics with [3 H]AVP when binding was performed in the presence of phospholipid vesicles [23]. The K_d of vasopressin binding to receptors solubilized in the absence of ligand with lysophosphatidylcholine (0.6 nM) compared very well with the K_d of vasopressin for membrane receptors (0.8 nM) [23] and was 10-fold lower than the K_d reported for agonist-bound V_1 receptors solubilized from rat liver membranes with the detergent CHAPS [21]. This clearly suggests that lysophosphatidylcholine-solubilized V_1 binding sites were in the high-affinity state even in the absence of agonist occupancy before solubilization.

PI-PLC has been purified from cytosolic fractions derived from several tissues, including rat liver, sheep seminal vesicle, bovine platelets, bovine brain and guinea-pig uterus [24–30]. Hofmann & Majerus [25] first reported that at least two immunologically distinct forms of PI-PLC exist. It is now known that there are at least five distinct isoenzymes of PI-PLC, based on immunological properties, molecular mass and substrate preference [25,27–30]. All five enzymes will hydrolyse PtdIns, PtdIns P and PtdIns P_2 under appropriate conditions. At concentrations of Ca^{2+} which mimic those in the resting cell (~ 100 nM), PtdIns P and PtdIns P_2 were preferred substrates [27–31]. It is currently unknown what role cytosolic PI-PLC plays in signal transduction. The substrates for PI-PLC are part of the cell membrane; therefore it can be presumed that, when the enzymes are catalytically active, they are membrane-associated. Evidence that cytosolic enzymes are involved in receptor-mediated phosphoinositide hydrolysis is provided by the finding that membrane-associated PI-PLC is immunologically identical with the cytosolic enzyme [29,32]. Further evidence was provided by the finding that cytosolic enzymes from platelets and brain are stimulated by stable GTP analogues, suggesting they are associated with a GTP-binding protein [33,34].

We have previously described the purification, characterization and molecular cloning of a PI-PLC from guinea-pig uterus [29,35], with properties similar to those of sheep seminal-vesicle PI-PLC I [25,31]. The purified enzyme, PI-PLC I, exhibited an apparent molecular mass of 60 kDa by SDS/PAGE. PI-PLC I hydrolysed PtdIns P and PtdIns P_2 at physiological concentrations of Ca^{2+} , was tightly associated with cell membranes, and was phosphorylated by protein kinase C [29], properties consistent with this enzyme being coupled to receptor-mediated phosphoinositide hydrolysis. In the present paper we report data to support further our conclusion that PI-PLC I is coupled to cell membrane receptors. Rat liver vasopressin receptors were solubilized as a complex containing V_1 receptors, a GTP-binding protein and PI-PLC I, thus identifying for the first time an apparent physical association between a PI-PLC and a cell-surface receptor.

MATERIALS AND METHODS

Materials

[8-Arginine]vasopressin (AVP) was from Bachem

(Torrance, CA, U.S.A.). [3 H]PtdIns P_2 (2.2 Ci/mmol), [3 H]AVP (64.3 Ci/mmol) and [125 I]AVP (2200 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). GTP, GTP[S], p[NH]ppA, L-lysophosphatidylcholine (from egg yolk, type 1) and polyethyleneimine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Asolectin was from Associate Concentrates (Woodside, NY, U.S.A.). GF/B filters were from Brandel (Gaithersburg, MD, U.S.A.). HSAB (*N*-hydroxysuccinimidyl 4-azido-benzoate) and BCA protein assay kit were from Pierce Chemical Co. (Rockford, IL, U.S.A.). DEAE-Sephacrose (Fast Flow) was from Pharmacia (Uppsala, Sweden). The TSK-4000 gel-filtration column was from Beckman. The antiserum against PI-PLC I was raised by using purified PI-PLC I from guinea-pig uterus as described previously [29].

Methods

Rat liver plasma membranes were prepared and solubilized with egg lysophosphatidylcholine as described previously [23]. Vasopressin-binding assays were done in triplicate by mixing solubilized preparation with phospholipid (asolectin) (7.5%, w/v) in 1:1 ratio and then incubating with [3 H]AVP in the absence (total) or presence (non-specific) of 5 μ M-AVP (unlabelled) for 60 min at 30 °C. After incubation the reaction was stopped by addition of 4 ml of ice-cold buffer A (10 mM-Na-Hepes, pH 7.4, 2 mM-MgCl $_2$ and 0.1% bovine serum albumin). The free ligand was separated from the bound ligand by filtration through GF/B glass-fibre filters (presoaked in 1% polyethyleneimine) [36]. The initial filtration was followed by two 5 ml washes with buffer A. The filtration was performed on a 24-sample cell harvester (Brandel). Non-specific binding was 20–30% of the total binding.

Chemical cross-linking of [125 I]AVP to the solubilized fractions

The solubilized fractions in phospholipid were incubated with 0.150 nM-[125 I]AVP in the absence and presence of 5 μ M unlabelled AVP for 1 h at 30 °C in buffer containing 30 mM-Na-Hepes, 5 mM-MgCl $_2$ and 0.1% albumin. At the end of incubation, [125 I]AVP-bound complex was separated from free [125 I]AVP by using Sephadex G-25 columns (Pharmacia PD-10), equilibrated with incubation buffer. The ligand–receptor complex was eluted in the initial 4.0 ml of buffer. To this fraction, freshly prepared HSAB in dimethyl sulphoxide (final concn. 2 mM) was added and incubated for 10 min at room temperature in the dark. The mixture was exposed to a model UVGL-25 mineral light lamp (UVP Inc.) at a distance of 6 cm for 3 min. The reaction product was then diluted with water and freeze-dried. The residue was resuspended in SDS-sample buffer and analysed by SDS/PAGE (10%-acrylamide gel).

The effects of GTP, GTP[S] and p[NH]ppA were assessed by measuring binding of [3 H]AVP to the solubilized fractions in the presence and absence of different concentrations of these nucleotides. GTP[35 S] binding was done as described by Northup *et al.* [37], with minor modifications. Assays were performed with 2 μ M-GTP[35 S] (4000 c.p.m./pmol) 10 mM-MgCl $_2$, 30 mM-Na-Hepes (pH 8.0) and 100 mM-NaCl, in a total volume of 50 μ l. After 30 min incubation at 30 °C, samples were diluted with 4 ml of incubation buffer and filtered on nitrocellulose filters. The filters were washed with

3 × 2 ml of buffer, dried and counted for radioactivity in a β -scintillation counter. Specific binding was 75–80 % of total binding.

Chromatographic separation of the lysophosphatidylcholine-solubilized rat liver plasma membranes

Lysophosphatidylcholine-solubilized rat liver membrane fraction was chromatographed on a DEAE-Sepharose column (3 cm × 9 cm). The column was equilibrated with buffer B, containing 20 mM-Na-Hepes (pH 7.4), 5 mM-MgCl₂ and 0.02 % egg lysophosphatidylcholine. The column was washed with equilibration buffer (30 ml) and then eluted with a 125 ml gradient of NaCl (0–1.0 M) in buffer B. Fractions (3 ml) were collected and assayed for [³H]AVP and GTP[³⁵S] binding and PI-PLC I enzyme activities. The major vasopressin-binding fractions (nos. 28–42) were pooled, concentrated and applied to a h.p.l.c. TSK gel 4000 PW column (0.8 cm × 25 cm). The column was equilibrated and eluted with 20 mM-Tris/HCl (pH 7.4), 5 mM-MgCl₂ and 0.005 % egg lysophosphatidylcholine, and 0.5 ml fractions were collected. Fractions were analysed for [³H]AVP and GTP[³⁵S] binding and PI-PLC I enzyme activities. The [³H]AVP binding was studied with 1 nM-[³H]AVP for 60 min at 30 °C in the absence or presence of 5 μ M unlabelled AVP. GTP[³⁵S] binding was studied with 1 μ M-GTP[³⁵S] in the absence or presence of 50 μ M unlabelled GTP[S] at 30 °C for 30 min.

PI-PLC enzyme activity was measured by the hydrolysis of [³H]PtdInsP₂. Samples (50 μ l) from each fraction were incubated with 20 μ M-[³H]PtdInsP₂ (sp. radioactivity 50000 d.p.m./nmol) in a reaction buffer containing 50 mM-BisTris (pH 7.0), 50 mM-KCl, 200 μ M-phosphatidylethanolamine, 0.3 % cholate, 100 μ M-CaCl₂, 0.01 % egg lysophosphatidylcholine and 2.5 mM-MgCl₂ in a final volume of 100 μ l, for 15 min at 37 °C. Reactions were terminated by addition of 250 μ l of chloroform/methanol/HCl (500:500:3, by vol.), followed by 75 μ l of 1 M-HCl/5 mM-EGTA. Samples of the aqueous phase were removed for determination of [³H]inositol 1,4,5-trisphosphate. The mass of PI-PLC I in each fraction was estimated by radioimmunoassays. Protein from each fraction was resolved on a SDS/10 % polyacrylamide gel. Adjacent lanes of the gel contained known amounts of purified guinea-pig uterus PI-PLC I (100 ng–1 μ g). Proteins were transferred to nitrocellulose paper, and probed with anti-PI-PLC I antibodies, followed by ¹²⁵I-labelled goat anti-rabbit IgG as previously described [29]. After autoradiography the quantity of PI-PLC I was estimated in each fraction by scanning densitometry (LKB, Uppsala, Sweden). The assays were linear between 100 ng and 1 μ g of protein.

Immunoaffinity absorption

IgG was purified from immune and preimmune serum by affinity chromatography on Protein A columns (Beckman, Palo Alto, CA, U.S.A.). The IgG was covalently coupled to CNBr-activated Sepharose-4B (Pharmacia). The final yield was 2.3 mg of protein/ml of gel. Solubilized rat liver membranes (1.6 mg of protein) were incubated with 2.0 ml of either preimmune-IgG-Sepharose or immune-IgG-Sepharose for 16 h at 4 °C. The column was washed with buffer B to remove unbound protein. Total [³H]AVP-binding activity in the flow-through fractions was measured as described above.

Protein measurement

This was done by the Pierce bicinchoninic acid (BCA) protein assay (Pierce Chemical Co.).

RESULTS

We have previously demonstrated that the rat liver vasopressin receptors were effectively solubilized with egg lysophosphatidylcholine [23]. [³H]AVP binding to solubilized rat liver membranes after mixing with phospholipids yielded a K_d of 0.6 ± 0.1 nM and total binding (B_{max}) of 1441 ± 52 fmol/mg of protein. The dissociation constant correlated well with that observed for membranes. Thus the solubilization of rat liver membranes with egg lysophosphatidylcholine preserved the high-affinity agonist-binding properties of V₁ receptors observed in membranes. The hetero-bifunctional cross-linking reagent HSAB was used to label vasopressin receptor covalently in the solubilized fraction. SDS/PAGE analysis followed by autoradiography revealed that the predominant vasopressin-binding component was a 65 kDa protein. Labelling of the 65 kDa band was inhibited (> 90 %) by unlabelled AVP and (> 60 %) by a V₁-receptor-specific antagonist,

Fig. 1. Cross-linking of ¹²⁵I-AVP to the solubilized fractions

Conditions are as described in the Materials and methods section. Lysophosphatidylcholine-solubilized rat liver membranes in the presence of phospholipid were incubated with ¹²⁵I-AVP with or without unlabelled ligands and then with 20 mM-HSAB. Lanes show solubilized fractions incubated (a) with ¹²⁵I-AVP alone, (b) with 5 μ M unlabelled AVP and (c) with 5 μ M V₁ antagonist.

Fig. 2. Effect of nucleotides on the specific binding of [^3H]AVP to solubilized liver membrane fractions in lipid micelles

Binding was performed in a reaction mixture containing 40 μg of lipid-micelle receptor protein, 1 nM- ^3H]AVP and indicated concentrations of GTP[S] (●), GTP (▲) or p[NH]ppA (■). The assay was performed at 30 °C for 60 min. The results are expressed as percentage inhibition of specific binding in the presence of nucleotides. The results shown are from one experiment conducted in triplicate which is representative of three similar experiments.

[d(CH₂)₅Tyr(Me)]AVP [38] (Fig. 1). No ^{125}I -labelled band was seen in the absence of cross-linking agent or the lipid-micelle fraction. Similar results have been reported for the covalent labelling of purified vasopressin V₁ receptors from rat liver with disuccinimidyl suberate [21]. The results strongly suggest that the 65 kDa band represents the ligand-binding site in the phospholipid fraction.

There is evidence that GTP and its analogues regulate vasopressin binding to liver membranes [39]. In the present study, GTP[S], a non-hydrolysable GTP analogue, decreased specific [^3H]AVP binding to the solubilized fraction in a dose-dependent manner (Fig. 2). GTP was also inhibitory, but less effective than GTP[S]. p[NH]ppA at concentrations between 1 μM and 1 mM had little effect. When binding was done with increasing concentrations of AVP, addition of GTP[S] to the solubilized fractions did not significantly alter the K_d of [^3H]AVP, but decreased B_{max} by 50% (results not shown). Ad-

Table 1. Effect of AVP on GTP[^{35}S] binding to rat liver membrane fractions

Binding was done for 30 min at 30 °C as described in the Materials and methods section in the presence or absence of 1 μM -AVP. The data presented are means $\pm$ S.E.M. of triplicate determinations from one experiment which is representative of two other experiments.

Fractions	Specific binding (pmol/mg)	
	No AVP	+ 1 μM -AVP
Solubilized	53.3 $\pm$ 2	81.9 $\pm$ 3
Solubilized in phospholipid	52.2 $\pm$ 4	108.3 $\pm$ 5

ditional evidence that a GTP-binding protein was coupled to vasopressin V₁ receptors was obtained when GTP[S] binding was performed in the absence and presence of vasopressin [40]. GTP[^{35}S] binding to the solubilized fraction was rapid, specific and saturable (results not shown). Addition of 1 μM -AVP potentiated GTP[S] binding to the solubilized fractions by 55% and 110% in the absence and presence of phospholipid respectively, suggesting that the solubilized vasopressin receptors are functionally coupled to G-protein(s) (Table 1). The absence of the ADP-ribosylated proteins induced by pertussis toxin or cholera toxin from the solubilized fraction containing V₁ receptors (results not shown) suggests that the G-protein involved is not G_i/G_o or G_s, and confirms the observation in membranes [19].

Previous studies have shown that vasopressin receptors are coupled to a PI-PLC through a cholera-toxin/pertussis-toxin-insensitive G-protein. To determine if vasopressin receptors were coupled to a PI-PLC in the solubilized fractions, it was first necessary to establish conditions for the enzyme assay which would permit PI-PLC to hydrolyse [^3H]PtdInsP₂ in the presence of the egg lysophosphatidylcholine used to solubilize the membranes. It was necessary to include a 10-fold molar excess of phosphatidylethanolamine and at least 0.3% cholate in the assays to detect PI-PLC enzyme activity in the solubilized fractions. Under these assay conditions rat liver membranes exhibited PI-PLC activity of 23.0 pmol/min per mg with 20 μM - ^3H]PtdInsP₂ as substrate, and the solubilized fractions exhibited a specific activity of 58.9 pmol/min per mg (Table 2). Therefore the conditions used to solubilize vasopressin receptors selectively [23] resulted in a 2.5-fold enrichment of PI-PLC enzyme activity. Both G-protein binding activity and PI-PLC in rat liver membranes and solubilized membrane fraction are in excess compared with vasopressin binding (Table 2). Numerous attempts to measure either a GTP[S]- or a vasopressin-stimulated PI-PLC activity under various assay conditions, including varying free Ca²⁺ concentrations from 65 nM to 100 μM , were unsuccessful. Thus the conditions required for detection of PI-PLC enzyme activity with exogenous substrate could dissociate PI-PLC from the receptor. Alternatively, PI-PLC may not be associated with the receptor under these solubilization conditions.

To address this question further, we utilized antibodies specific for a PI-PLC isoenzyme, PI-PLC I [29], to immunoprecipitate [^3H]AVP-binding activity from the solubilized liver membranes. To demonstrate that antibodies prepared against guinea-pig uterus PI-PLC I would cross-react with a similar enzyme in rat liver membranes, we performed immunoblot analysis of various rat liver subcellular fractions (Fig. 3). Anti-PI-PLC I antibodies recognized a 60 kDa protein in rat liver cytosol (Fig. 3, lane 2), rat liver membranes (Fig. 3, lane 3) and solubilized rat liver membranes (Fig. 3, lane 4). Consistent with the observations on enzyme activity, there was an enrichment of PI-PLC I in the solubilized fraction compared with membranes (Fig. 3, lanes 3 and 4). The PI-PLC I in rat liver co-migrated with purified guinea-pig uterus PI-PLC I (Fig. 3, lane 1). Preimmune immunoglobulins failed to react with proteins in any of the fractions (Fig. 3b). Anti-PI-PLC I antibodies were used to immunoprecipitate [^3H]AVP-binding activity in the solubilized fractions to demonstrate that [^3H]AVP-binding activity was solubilized in a complex with PI-

Table 2. [³H]AVP- and GTP[³⁵S]-binding and PI-PLC activities in rat liver membrane fractions

Assays were done as described in the Materials and methods section. The data presented are means $\pm$ S.E.M. of triplicate determinations from one experiment which is representative of two other experiments.

	[³ H]AVP binding		GTP[³⁵ S] binding		PLC activity		
	Specific (pmol/mg)	Total (pmol)	Specific (pmol/mg)	Total (pmol)	Specific (pmol/min per mg)	Total (pmol)	PI-PLC I (pmol/mg)
Rat liver membrane	0.38 $\pm$ 0.06	18.8	50.8 $\pm$ 2.3	2492	23.0 $\pm$ 1.6	1128.3	70
Solubilized fraction	1.35 $\pm$ 0.09	33.1	75.9 $\pm$ 4.8	1861	58.9 $\pm$ 13.6	1443.0	120
DEAE-Sepharose fraction pool	7.20 $\pm$ 0.12	29.1	306 $\pm$ 9	2209	529.4 $\pm$ 19	3811.7	150

PLC I. Addition of increasing concentrations of anti-PI-PLC I antibodies to the solubilized rat liver membranes, followed by protein A-Sepharose CL-4B, resulted in a maximal loss of 55 % of specific [³H]AVP-binding activity (Fig. 4), whereas preimmune IgG failed to remove [³H]AVP-binding activity from the supernatant (Fig. 4). The specificity of antibodies for receptors coupled to PI-PLC was demonstrated by immunoprecipitating V₂ receptors from pig kidney, which are coupled to adenylate cyclase. Even though PI-PLC I was detectable in pig kidney membrane and solubilized membranes (Fig. 3, lanes 6 and 7), antibodies prepared against PI-PLC I failed to remove [³H]AVP-binding activity from the solubilized membrane (results not shown).

To document further that V₁ receptors and PI-PLC I are complexed in the solubilized fraction, chromatographic fractionation experiments were performed. The material solubilized with egg lysophosphatidylcholine was applied to a DEAE-Sepharose column and eluted

with a linear NaCl (0–1.0 M) gradient. The eluted fractions were analysed for [³H]AVP binding, GTP[S]-binding and PI-PLC enzyme activity (Fig. 5a). Separation of the lysophosphatidylcholine-solubilized material on a DEAE-Sepharose column resulted in a 7-fold enrichment of [³H]AVP-binding activity, 4-fold enrichment of GTP[S]-binding activity, 10-fold enrichment of PI-PLC enzyme activity and 1.3-fold enrichment of PI-PLC I immunoreactivity (Table 2). One major peak of [³H]AVP-binding activity and PI-PLC activity was eluted from the DEAE-Sepharose column, whereas three broad peaks of GTP[S]-binding activity were resolved on the column. The major GTP[S]-binding activity co-eluted with [³H]AVP-binding and PI-PLC enzyme activity (Fig. 5a). Fractions containing [³H]AVP-binding activity were pooled, concentrated on an Amicon PM-30 membrane and separated by gel-filtration h.p.l.c. [³H]AVP-binding activity was eluted as a broad peak, of 350–120 kDa, with peak activity being co-eluted with PI-PLC I and one

Fig. 3. Identification of PI-PLC I in rat liver membrane fractions

Samples were separated by SDS/PAGE (10 % gel), electrophoretically transferred to nitrocellulose paper, and probed with either PI-PLC I antibodies (a) or preimmune antibodies (b), followed by ¹²⁵I-Protein A [28]. Lanes: (1) 0.25 μ g of purified guinea-pig uterus PI-PLC I, (2) 100 μ g of rat liver cytosolic proteins, (3) 100 μ g of rat liver membrane proteins, (4) 100 μ g of solubilized rat liver membranes, (5) 100 μ g of hog kidney cytosolic proteins, (6) 100 μ g of hog kidney membrane proteins, and (7) 100 μ g of solubilized hog kidney membranes. Autoradiographs were overexposed to demonstrate the presence of PI-PLC I in hog kidney fractions.

Fig. 4. Immunoprecipitation of AVP-binding activity by anti-PI-PLC I antibody

Lysophosphatidylcholine-solubilized rat liver membranes (220 µg of protein) were incubated with increasing amounts of anti-PI-PLC I antibody in 20 mM-Na-Hepes (pH 7.4)/1 mM-EGTA/5 mM-MgCl₂/250 mM-sucrose overnight at 4 °C. Immune complexes were precipitated with 300 ml of 50% (w/v) Protein A-Sepharose for 4 h at 4 °C. The immune complex bound to Protein A was pelleted by centrifuging at 10000 *g* for 5 min. AVP-binding activity in the supernatant was determined after mixing with phospholipid vesicles. Binding was done in triplicate with 1 nM-[³H]AVP for 60 min at 30 °C. Results are shown as specific [³H]AVP binding remaining in the supernatant after treatment with (A) buffer alone, (B) 200 µg of preimmune IgG, (C) 50 µg of anti-PI-PLC I IgG, and (D) 200 µg of anti-PI-PLC I IgG. The data shown are means ± S.E.M. of triplicate determinations from one representative experiment. Similar results were obtained in two other independent experiments.

of three GTP[S]-binding-activity peaks (Fig. 5*b*). PI-PLC enzyme activity migrated as one major peak of approx. 240 kDa, with a minor peak of activity present in the void volume (Fig. 5*b*). Immunoblots with anti-PI-PLC I antibody demonstrated a good correlation between PI-PLC enzyme activity and PI-PLC I immunoreactivity (results not shown). Rat liver cytosolic proteins were separated by gel-filtration h.p.l.c. under identical conditions and analysed for PI-PLC I immunoreactivity. Cytosolic PI-PLC I exhibited an apparent molecular mass of 60 kDa, which is in agreement with that reported for guinea-pig uterus cytosolic PI-PLC I [29]. These data suggest that the major [³H]AVP-binding activity peak eluted at 240 kDa may be a complex of vasopressin receptor, G-protein and PI-PLC I.

To rule out the possibility that [³H]AVP-binding sites were forming a non-specific precipitate after incubation with anti-PI-PLC I antibodies and Protein A-Sepharose, the fractions containing [³H]AVP-binding activity from the gel-filtration chromatography were pooled and then applied to either an anti-PI-PLC I IgG immunoaffinity column or to a preimmune-IgG affinity column. [³H]AVP-binding activity in the flow-through fractions was determined. The anti-PI-PLC I IgG affinity column retained 50–60% of the total [³H]AVP-binding activity, whereas the preimmune-IgG column retained less than 10% of the binding activity (Table 3). Attempts to elute [³H]AVP-binding activity from the antibody columns were not successful, probably owing to the harsh conditions required. These observations strongly suggest that solubilization of rat liver membranes with egg lysophosphatidylcholine results in a complex of receptor, G-protein and PI-PLC.

DISCUSSION

Studies from a number of laboratories have shown that vasopressin V₁ receptors in hepatocytes and smooth muscle are coupled to phospholipase C through a G-protein which is insensitive to both cholera toxin and pertussis toxin [8,9]. The key finding in the present paper is that, in the absence of agonist occupancy, a portion of the vasopressin V₁ receptors from rat liver membranes is solubilized as a complex containing the receptor, GTP-binding protein and PI-PLC I. The co-solubilization of a G-protein along with the vasopressin receptors is documented by (a) high-affinity agonist binding to the solubilized fractions, (b) inhibition of vasopressin binding by GTP and its stable analogues and (c) stimulation of GTP[³⁵S] binding by AVP. The co-solubilization of receptor with G-protein(s) is in agreement with those studies on the solubilization of dopamine D₁ [41] and adenosine A₁ [42] receptors, and in contrast with what has been observed with β- [43] and α₂- [44] adrenergic and dopamine D₂ [45] receptors, where high-affinity binding as well as the guanine-nucleotide sensitivity were lost once the receptors were solubilized. It is noteworthy that all the above receptors except vasopressin are coupled to adenylate cyclase, whereas V₁ receptors are coupled to PI-PLC.

The present study also suggests the presence of PI-PLC in the solubilized fraction as demonstrated by enzymic and immunoblot analyses. The enzyme PI-PLC is found in both cytosolic and membrane fractions. In rat liver, over 50% of total cellular PI-PLC was found to be membrane-associated. During chromatographic fractionation, the major vasopressin-binding activity was co-eluted with PI-PLC and G-protein(s).

Although we demonstrated that anti-PI-PLC I antibodies immunoprecipitated [³H]AVP-binding activity from solubilized rat liver membranes, we were unable to demonstrate an AVP- or guanine-nucleotide-dependent stimulation of PI-PLC enzyme activity in the solubilized membranes. One explanation for the lack of AVP stimulation of PI-PLC enzyme activity may be that PI-PLC was solubilized in a complex precoupled to the receptor and G-protein, and therefore further activation by agonist was not possible. Alternatively, the G-protein may exert a negative effect on PI-PLC enzyme activity which is relieved by coupling of agonist-occupied receptor to the GTP-binding protein, or by binding of GTP

Fig. 5. (a) Ion-exchange chromatography and (b) gel filtration of solubilized rat liver membranes

(a) Lysophosphatidylcholine-solubilized rat liver membranes were chromatographed on a DEAE-Sepharose column (Fast Flow; Pharmacia) as described in the Materials and methods section. Results show the A_{280} (—), specific [³H]AVP binding (○), GTP[³⁵S]γS (Δ) and PI-PLC activity (□). Arrows indicate the retention volumes of standard proteins of known molecular mass (kDa). (b) Fractions 28–42 from the DEAE-Sepharose column (Fig. 5a) were pooled, concentrated by Amicon ultrafiltration and subjected to gel filtration on TSK 4000 PW as described in the Materials and methods section. Symbols as in panel (a).

analogues to the G-protein [46]. The use of cholate in the enzyme assays may also relieve the negative effects of the GTP-binding protein, resulting in full activation of the enzyme. Cholate may also dissociate PI-PLC from the G-protein so that agonist-dependent activation of the enzyme was no longer possible. In addition, conditions which were optimal for measurement of PI-PLC activity

(cholate) were not suitable for measuring vasopressin binding. Finally, we have calculated that there is at least 100-fold greater quantity of PI-PLC I in rat liver membranes as compared with V_1 receptors. Therefore it is unlikely that all membrane-associated PI-PLC I, as well as G-protein, is in a complex with V_1 receptors. The immunoprecipitation experiments suggest that only 50–60 % of the V_1 receptors are complexed with PI-PLC I, which is supported by the h.p.l.c. gel-filtration profile (Fig. 5b). Therefore it may not be possible to detect a 5 % increase in PI-PLC activity under the current assay conditions.

There are at least five distinct PI-PLC isoenzymes in mammalian tissues [25,27–29]. These enzymes exhibit different molecular masses on SDS/PAGE, differ in antigenic properties, and more recently have been demonstrated to have different amino acid sequences [25,27–29,35,47–50]. It is at present unknown what function each of the different PI-PLC isoenzymes performs in cells. It is tempting to speculate that different PI-PLC isoenzymes are functionally coupled to distinct classes of cell-surface receptor (e.g. PI-PLC I to vasopressin V_1 receptors, PI-PLC II to another type of receptor etc.). The tissue-distribution data for the different PI-PLC isoenzymes support such a conclusion [25,28,51].

In summary, we have presented data which for the first time describes the solubilization of a cell-surface receptor

Table 3. [³H]AVP binding to TSK-4000 fractions preincubated with immunoaffinity resin

Fractions from the TSK-4000 column, enriched in [³H]AVP-binding activity, were pooled and incubated with either a preimmune-IgG-Sepharose column or an anti-PI-PLC IgG-Sepharose column as described in the Materials and methods section. [³H]AVP binding in the flow-through fractions was measured as previously described. The data presented are means ± S.E.M. of triplicate determinations.

	Binding (pmol)	
	Expt. 1	Expt. 2
Total activity used	1.42 ± 0.8	0.82 ± 0.20
Supernatant from preimmune-IgG-Sepharose	1.24 ± 0.12	0.72 ± 0.15
Supernatant from anti-PI-PLC IgG-Sepharose	0.72 ± 0.10	0.40 ± 0.15

(V_i) as a complex with the coupler (G-protein) and effector (PI-PLC).

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REFERENCES

1. Spiegel, A. M. (1987) *Mol. Cell. Endocrinol.* **49**, 1–16
2. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
3. Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. W. & Wilson, D. B. (1986) *Science* **234**, 1519–1526
4. Okajima, F. & Ui, M. (1984) *J. Biol. Chem.* **259**, 13863–13871
5. Carman-Krzan, M. (1986) *Prog. Med. Chem.* **23**, 42–89
6. Jard, S. (1983) *Curr. Top. Membr. Transp.* **18**, 255–285
7. Michell, R. H., Kirk, C. J. & Billah, M. M. (1979) *Biochem. Soc. Trans.* **7**, 861–865
8. Aiyar, V. N., Nambi, P., Stassen, F. L. & Crooke, S. T. (1986) *Life Sci.* **39**, 37–45
9. Exton, J. H. (1987) *Kidney Int.* **32**, Suppl. **23**, S-68–S-76
10. Guillon, G., Kirk, C. J. & Balestre, M. N. (1986) *Biochem. J.* **240**, 189–196
11. Morgan, N. G., Shipp, C. C. & Exton, J. H. (1983) *FEBS Lett.* **163**, 277–281
12. Nambi, P., Whitman, M., Stassen, F. L. & Crooke, S. T. (1986) *J. Pharmacol. Exp. Ther.* **237**, 143–146
13. Nambi, P., Whitman, M., Gessner, G., Aiyar, N. & Crooke, S. T. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8492–8495
14. Litosch, I., Wallis, C. & Fain, J. N. (1985) *J. Biol. Chem.* **260**, 5464–5471
15. Smith, C. D., Cox, C. C. & Snyderman, R. (1986) *Science* **232**, 97–100
16. Cockcroft, S. & Gomperts, B. D. (1985) *Nature (London)* **314**, 534–536
17. Wallace, M. A. & Fain, J. N. (1985) *J. Biol. Chem.* **260**, 9527–9530
18. Uhing, R. H., Jiang, H., Prpic, V. & Exton, J. H. (1985) *FEBS Lett.* **188**, 317–320
19. Fitzgerald, T. J., Uhing, R. J. & Exton, J. H. (1986) *J. Biol. Chem.* **261**, 16871–16877
20. Bojanic, D. & Fain, J. N. (1986) *Biochem. J.* **240**, 361–365
21. Fishman, J. B., Dickey, B. F. & Fine, R. E. (1987) *J. Biol. Chem.* **262**, 14049–14055
22. Anderson, T., Schlegel, W., Monod, A., Krause, K.-H., Stendahl, O. & Lew, D. P. (1986) *Biochem. J.* **240**, 333–340
23. Aiyar, N., Nambi, P., Stassen, F. L. & Crooke, S. T. (1987) *Mol. Pharmacol.* **32**, 34–36
24. Takenawa, T. & Nagai, Y. (1981) *J. Biol. Chem.* **256**, 6769–6775
25. Hofmann, S. L. & Majerus, P. W. (1982) *J. Biol. Chem.* **257**, 6461–6469
26. Hakata, H., Kambayashi, J. & Kosaki, G. (1982) *J. Biochem. (Tokyo)* **92**, 929–935
27. Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G. & Rhee, S. G. (1987) *J. Biol. Chem.* **262**, 12511–12518
28. Ryu, S. H., Suh, P. G., Cho, K. S., Lee, K. Y. & Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6649–6653
29. Bennett, C. F. & Crooke, S. T. (1987) *J. Biol. Chem.* **262**, 13789–14797
30. Rebecchi, M. J. & Rosen, O. M. (1987) *J. Biol. Chem.* **262**, 12526–12532
31. Wilson, D. B., Bross, T. E., Hofmann, S. L. & Majerus, P. W. (1984) *J. Biol. Chem.* **259**, 11718–11724
32. Lee, K.-Y., Ryu, S. H., Suh, P.-G., Choi, W. C. & Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5540–5544
33. Baldassare, J. J. & Fisher, G. J. (1986) *J. Biol. Chem.* **261**, 11942–11944
34. Deckmyn, H., Tu, S. M. & Majerus, P. W. (1986) *J. Biol. Chem.* **261**, 16553–16558
35. Bennett, C. F., Balcarek, J., Varrichio, A. & Crooke, S. T. (1988) *Nature (London)* **334**, 268–270
36. Aiyar, N., Valinski, W., Nambi, P., Minnich, M., Stassen, F. L. & Crooke, S. T. (1989) *Arch. Biochem. Biophys.* **268**, 698–706
37. Northup, J. K., Smigel, M. D. & Gilman, A. G. (1982) *J. Biol. Chem.* **257**, 11416–11423
38. Kruszynski, M., Lammek, B., Manning, M., Seto, J., Halder, J. & Sawyer, W. H. (1980) *J. Med. Chem.* **23**, 364–368
39. Cantau, B., Keppens, S., DeWulff, H. & Jard, S. (1980) *J. Receptor Res.* **1**, 137–168
40. Senogles, S. E., Benovic, J. L., Amlaiky, N., Unson, C., Milligan, G., Vinitsky, R., Spiegel, A. M. & Caron, M. G. (1987) *J. Biol. Chem.* **262**, 4860–4867
41. Niznik, H. B., Otsuka, N. Y., Dumbrille-Ross, A., Grigoriadis, D., Tirpak, A. & Seeman, P. (1986) *J. Biol. Chem.* **261**, 8397–8406
42. Stiles, G. L. (1985) *J. Biol. Chem.* **260**, 6728–6732
43. Limbird, L. E. & Lefkowitz, R. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 228–232
44. Smith, S. K. & Limbird, L. E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4026–4030
45. Kilpatrick, B. F. & Caron, M. G. (1983) *J. Biol. Chem.* **258**, 13528–13534
46. Fain, J. N., Wallace, M. A. & Wojcikiewicz, R. J. H. (1988) *FASEB J.* **2**, 2569–2574
47. Stahl, M. L., Ferenz, C. R., Kelleher, K. L., Kriz, R. W. & Knopf, J. L. (1988) *Nature (London)* **332**, 269–272
48. Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. & Rhee, S. G. (1988) *Cell* **54**, 161–169
49. Katan, M., Kriz, R. W., Totty, N., Philip, R., Meldrum, E., Aldape, R. A., Knopf, J. L. & Parker, P. J. (1988) *Cell* **54**, 171–177
50. Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. & Rhee, S. G. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5419–5423
51. Suh, P.-G., Ryu, S. H., Choi, W. C., Lee, K.-Y. & Rhee, S. G. (1988) *J. Biol. Chem.* **263**, 14497–14504

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