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Evidence for Two Molecular Forms of Solubilized Vasopressin Receptors in Rat Kidney Membranes

Regulation by Guanyl Nucleotides

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SUMMARY

As previously reported, it is possible to solubilize vasopressin-receptor complexes formed in rat kidney membranes by the use of Triton X-100. Ultracentrifugation on sucrose gradients and elution through molecular sieving columns of these soluble extracts revealed the existence of two molecular forms of vasopressin-receptor complexes (molecular weight = 200,000 and 100,000, respectively). These two molecular forms of vasopressin-receptor complexes can be partially purified and exhibit different properties: (a) The light form is more sensitive to thermal dissociation than is the heavy form. (b) The presence of guanyl nucleotide affects the dissociation rate of only the heavy form of the vasopressin-receptor complex. (c) The light form seems to be convertible to the heavy form by increasing the duration of incubation between the membranes and the tritiated hormone. (d) Guanyl nucleotides affect the distribution of the two molecular forms of the receptor (decrease of the relative amount of the heavy form). These data provide evidence for interaction between vasopressin-receptor complexes (light form) and another protein component, which may be a GTP-binding protein.

INTRODUCTION

As is the case with other hormones of neurotransmitters, vasopressin is known to interact with specific cell surface receptors located on kidney plasma membranes and to activate membrane-bound adenylate cyclase enzyme (1–3). Guanyl nucleotides play an important role in the coupling between receptor and adenylate cyclase. They decrease receptor affinity for the hormone and increase the sensitivity of adenylate cyclase activation by hormone (see refs. 4 and 5 for review).

At least three distinct components are implicated in the hormonal activation of adenylate cyclase: (a) the receptor, containing a specific binding site for hormone or neurotransmitter; (b) The adenylate cyclase enzyme, transforming ATP into cyclic AMP; and (c) GTP-binding protein(s), being responsible for mediating the effects of GTP and hormones on adenylate cyclase catalytic activity (see ref. 6 for review).

Several authors have already demonstrated the existence of interactions between two components of the system during hormonal activation of adenylate cyclase. Citri and Schramm (7) have shown that the first step in enzyme activation consists of interactions between the receptor and the GTP-binding protein. Solubilization experiments have also demonstrated the existence of such complexes (8, 9). Alternatively, we and others have demonstrated that adenylate cyclase activation by guanyl nucleotide results from the formation of a complex between the catalytic component of the enzyme and a GTP-binding protein of 40,000 M_r (10, 11). Such results raised the problem of the number of GTP binding proteins involved in hormonal adenylate cyclase activation. Recent work by Northup *et al.* (12, 13) indicates that the purified GTP-binding protein of rabbit liver is composed of two distinct molecules of 45,000 and 35,000 daltons. The association of these two subunits represents the so-called G/F protein of 70,000 M_r. Similar results were obtained on other systems such as human and turkey erythrocytes (14, 15). However, these studies did not discern whether the GTP-binding protein(s) associated with the hormonal receptor is(are) the molecule(s) directly associated with the adenylate cyclase catalytic unit. Data of Stadel *et al.* (16) do resolve this for the adenylate cyclase coupled with the *beta*-adrenergic receptor, although molecular weight was not determined for these GTP-binding proteins.

The aim of the work presented here was to study the molecular properties of solubilized vasopressin-receptor

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complexes. It suggests, as was demonstrated for the *beta*-adrenergic receptor, that interactions between vasopressin-receptor and a GTP-binding protein exist.

MATERIALS AND METHODS

Products. Tyr^2,Lys^8-[^{3}H]vasopressin (8.5 Ci/mmol) was prepared and purified by affinity chromatography using a neurophysin-Sepharose column as previously described (17). Radiochemical purity and biological activity of the labeled hormone were determined as reported earlier (18). β-Galactosidase from *Escherichia coli*, Gpp(NH)p,[1] catalase from beef liver, and alcohol dehydrogenase from yeast were purchased from Boehringer Mannheim. Bio-Gel P-30 was from Bio-Rad Laboratories (Richmond, Calif.); cytochrome *c* from horse heart was from Sigma Chemical Company (St. Louis, Mo.). Triton X-100 was obtained from Koch Light Laboratories; deuterated water (99.8% pure) was from CEA (Saclay France); Ultrogel ACA 34 was purchased from LKB Instruments (Rockville, Md.). AVP and LVP were from Ferring. Cyclo dV-DAVP was provided by Dr. Manning (Medical College of Ohio, Toledo).

Membrane preparation. The method used to prepare rat renal medullary membrane has already been described (18). Animals used were Wistar rats weighing 150–250 g. The inner medulla of the kidney was dissected and homogeneized in a Potter-Elvehjem homogenizer at 4° in a buffered isotonic solution: 5 mM Tris-HCl (pH 7.4)/3 mM $MgCl_2$/1 mM EDTA/250 mM sucrose. The pellet was collected from the homogenate by centrifugation (15 min at 600 × *g*) and washed three times in a hypotonic solution: 5 mM Tris-HCl (ph 7.4)/3 mM $MgCl_2$/1 mM EDTA. The final pellet was used immediately.

Binding of [^{3}H]vasopressin to intact membranes. Rat renal membranes (3–5 mg of protein per milliliter) were generally incubated for 15 min at 30° in a medium containing 15 nM [^{3}H]vasopressin, 100 mM Tris-HCl (pH 7.4), 0.75 mM $MgCl_2$, and 0.25 mM EDTA. Under these conditions the fractional saturation of kidney receptors was found to be close to 80% (18). The amount of [^{3}H]vasopressin specifically bound to kidney membranes was determined on an aliquot of the membrane suspension collected at the end of the incubation period. Bound hormone was separated from free hormone using a Millipore filtration technique previously described (18). All determinations were corrected for nonspecific binding (residual radioactivity was measured in the presence of 5 μM unlabeled vasopressin). At the end of incubation in the presence of [^{3}H]vasopressin, membrane samples were rapidly cooled in an ice bath. All further manipulations were performed at 4° to minimize hormone-receptor dissociation. Free [^{3}H]vasopressin present in the cooled sample was eliminated by centrifuging (5 min at 20,000 × *g*) and washing membranes three times in a buffer containing 10 mM $MgCl_2$ and 100 mM Tris-HCl, pH 8 (pH measured at room temperature).

Solubilization of hormone-receptor complexes. The procedures leading to the solubilization of the vasopressin-receptor complexes formed on intact membranes have been described (19). The kidney membranes previously incubated with tritiated vasopressin and washed to eliminate free hormone were incubated at 0° for 45 min in the presence of 100 mM Tris-HCl (pH 8)/10 mM $MgCl_2$/5 mM EDTA/200 mM NaCl/0.3% (v/v) Triton X-100. The final protein concentration in the extracts was 2.4–4.0 mg/ml. After incubation in the presence of detergent, a nonsedimentable fraction of solubilized membrane was separated at 0° by a rapid (5 min) centrifugation at 20,000 × *g*. This rapid centrifugation did not correspond to the usual criterion for solubilization (100,000 × *g* for 60 min). However, data presented in this paper suggest that our procedure is nontheless valid, since the solubilized material found in the supernatant is included in Ultrogel ACA-34 column and not pelleted in the sucrose gradient (see Fig. 1). Most (80%–90%) of the radioactivity bound to membrane was recovered in the supernatant used for ultracentrifugation and gel filtration experiments. Control experiments (19) have shown that (a) the radioactivity found in the supernatant corresponds to free [^{3}H]LVP and to [^{3}H]LVP associated with macromolecular components having a molecular weight of at least 40,000. (b) The presence of free [^{3}H]LVP indicates that, during solubilization, partial hormone-receptor dissociation occurred. The radioactivity associated with macromolecular components represents the soluble form of the native hormone-receptor complexes formed on intact membranes, since some properties of the membranous hormone-receptor complexes were also observed in the soluble preparations (sensitivity to guanyl nucleotides when dissociation was performed at 30°). (c) The dissociation rate of the radioactivity associated with solubilized macromolecular components is compatible with ultracentrifugation and filtration experiments ($t_{1/2}$ dissociation time at 4° = 7 hr).

Separation of soluble receptor bound from radioligand was accomplished by filtration through a Bio-Gel P-30 column (0.5 × 10 cm). Details of the separation procedure have already been described (19).

Sucrose gradient ultracentrifugation of soluble extracts. Linear gradients (4.0 ml) were prepared by using an Isco gradient formed from 3% and 10% sucrose solutions in H_2O or ^2H_2O (98% of ^2H_2O final concentration). These solutions were buffered with 100 mM Tris-HCl (pH 8)/10 mM $MgCl_2$/1 mM EDTA/0.1% (v/v) Triton X-100. Samples (300 μl) of solubilized extracts were layered on top of the gradient together with cytochrome *c* and alcohol dehydrogenase. Centrifugation was carried out in a Spinco L2-65B centrifuge model at 40,000 rpm at 4° (SW 60 rotor) for 14 hr. After centrifugation, 200-μl fractions were collected from the gradient using an Isco gradient fractionator. The amount of vasopressin-receptor complexes present in each fraction was measured by filtration through Bio-Gel P-30 columns. Calibration curves for the determination of apparent sedimentation coefficents were constructed according to the method of Martin and Ames (20).

Gel filtration of soluble extracts. An Ultrogel ACA-34 column (1.6 × 40 cm) was equilibrated with an elution buffer identical with that used for preparing the gradient solutions, with the exception of the sucrose concentration, which was 75 mM. A portion (1.2 ml) of solubilized material was layered on top of the column together with the following markers: β-galactosidase, catalase, cytochrome *c*, alcohol dehydrogenase, $K_3Fe(SCN)_6$, and phage T_4. Fractions of 1 ml were collected at a flow rat of 10 ml/hr. The amount of solubilized hormone-receptor present in each column fraction was determined after filtration through Bio-Gel P-30 columns. The elution volumes of the hormone-receptor complex and markers were expressed in terms of K_{av} according to the method of Laurent and Killander (21): $K_{av} = (V - V_o)/(V_f - V_o)$, where V_o, V_f, and V represent the elution volumes of phage T_4, of $K_3Fe(SCN)_6$, and of the fraction considered, respectively. Assay conditions and values for the hydrodynamic parameters of markers used to calibrate gradients and columns were previously specified (11).

Protein determination and radioactivity measurements. Protein content was estimated by the method of Lowry *et al.* (22), using bovine serum albumin as standard. Radioactivity measurements were performed by liquid scintillation spectrophotometry.

RESULTS

Hydrodynamic parameters of solubilized vasopressin-receptor complexes. The hydrodynamic parameters of solubilized hormone-receptor complexes were derived from gel filtration and ultracentrifugation experiments. Figure 1 illustrates the result of typical experiments. Mean values obtained for all determinations performed are illustrated in Table 1. Ultrogel column elution profiles of radioactivity bound to macromolecular components of solubilized kidney membrane (Fig. 1A) show three peaks. The first component (15%) was recovered in the void volume of the column and probably represents [^{3}H]vasopressin bound to partially solubilized material,

[1] The abbreviations used are: Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; LVP, lysine vasopressin; AVP, arginine vasopressin; cyclo dV-DAVP (1-(β-mercapto-β-cyclopentamethylene propionic acid), 4-valine,8-D-arginine vasopressin.

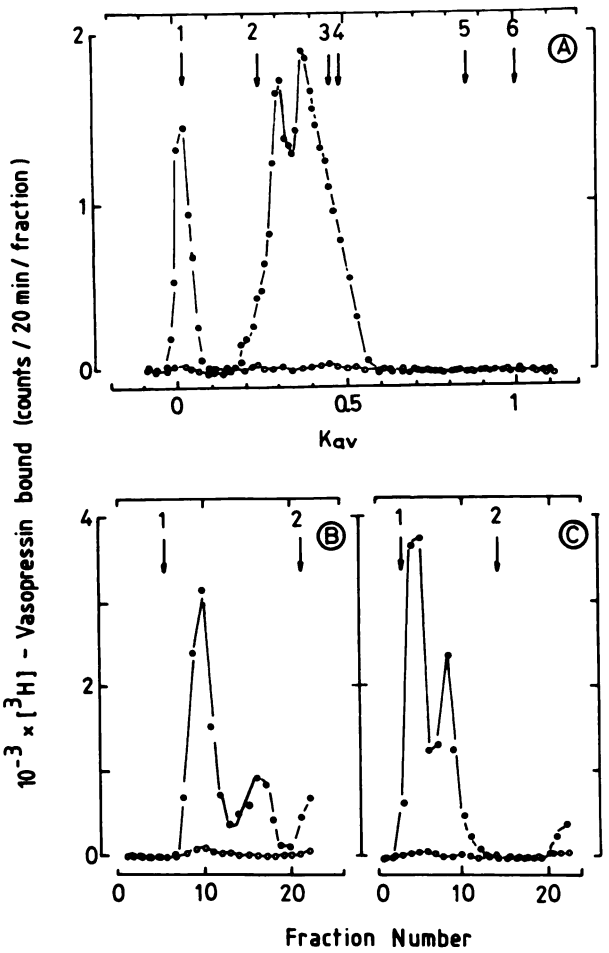

FIG. 1. *Gel filtration and ultracentrifugation profiles of solubilized [³H]vasopressin-receptor complexes*

Rat kidney membranes (4.0 mg of protein per milliliter) were incubated for 15 min at 30° in a medium containing 100 mM Tris-HCl (pH 8) 10 mM $MgCl_2$, and 15 nM [³H]LVP (●) or in the same medium plus 3 μM unlabeled vasopressin (○). Membranes were then solubilized at 0° in the presence of Triton X-100 (for details see Materials and Methods). The supernatant containing the solubilized vasopressin-receptor complexes was layered together with enzyme markers on top of either the chromatographic column (A) or H_2O (B) or ^2H_2O (C) 3–10% linear sucrose gradient. Gel filtration was performed at 4° for 7 hr as described under Materials and Methods. In each column fraction, marker activities and radioactivity bound to macromolecular components were determined and plotted as a function of the corresponding K_{av} values (see Materials and Methods). *Arrows* indicate the elution peaks of the calibration markers used: *1* = phage T_4; *2* = β-galactosidase; *3* = catalase; *4* = alcohol dehydrogenase; *5* = cytochrome *c*; *6* = $K_3Fe(SCN)_6$. Over-all recovery of bound radioactivity from the column was 70–80%. Gradients were centrifuged and fractionated as described under Materials and Methods. The marker activities and the radioactivity bound to macromolecular components were determined. *Arrows* indicate the elution peaks of the calibration markers used (*1* = cytochrome *c*; *2* = alcohol dehydrogenase).

which was not eliminated during the 20,000 × *g* centrifugation procedure used to obtain a detergent-solubilized preparation (see Materials and Methods). The remainng bound radioactivity was eluted in two symmetrical peaks (Stokes' radii = 5.6 and 6.5 nm). When a similar experiment was performed on material solubilized from membranes incubated with [³H]vasopressin and an excess of unlabeled hormone, all three peaks disappeared. The radioactivity detected under such circumstances corresponded to nonspecific [³H]vasopressin binding and was not significantly different from the background of the scintillation liquid spectrophotometer used.

Figure 1B and C represents the sedimentation profiles of solubilized vasopressin-receptor complexes after ultracentrifugation through H_2O or ^2H_2O linear sucrose gradients. As previously observed (Fig. 1A), two symmetrical peaks of bound radioactivity were detected. Moreover, the radioactivity found in the bottom fractions of the gradients probably corresponds to the partially solubilized hormone-receptor complexes. The apparent sedimentation constant of the two forms of the hormone-receptor complexes measured in H_2O gradients were 3.4 S and 6.1 S, respectively (see Table 1). The correponding values measured in ^2H_2O gradients were used to calculate the partial specific volume of the solubilized proteins studied. The parameters of these two forms of solubilized hormone-receptor complexes were significantly different (0.777 and 0.744 $ml \cdot g^{-1}$, respectively for the heavy and the light forms) (Table 1). Estimates of the physicochemical parameters of the two solubilized forms of receptors are given in Table 1. The functional coefficient (f/f_0) of 1.73 suggests that the solubilized vasopressin-receptor complexes are asymmetrical in solution.

Pharmacological identification of the two forms of solubilized vasopressin receptor. As represented in Fig. 2, when 50% of specific [³H]vasopressin binding to the membrane preparation was reduced by the addition of unlabeled peptide (AVP or cyclo dV-DAVP), the soluble radioactivity found in the regions where the two forms of hormone-receptor complexes migrated was also decreased by a factor of 2. Similar results were obtained when other vasopressin analogues were used (agonist or antagonist peptides of the antidiuretic response of vasopressin on kidney).

Influence of detergent/protein ratio on the amount of the two forms of solubilized vasopressin-receptor. Modifications of the detergent to protein ratio during solubilization procedures did not alter the apparent sedimentation constant nor the relative amounts of the two forms of solubilized vasopressin-receptor complexes (Table 2). The apparent solubilization yield of hormone-receptor complexes was slightly lowered by decreasing the protein concentration of the extract. More precise analysis of this phenomenon shows that an increase in the rate of vasopressin-receptor complex dissociation during solubilization accounts for such an apparent decrease in successfully solubilized receptors rather than a modification of the ability of Triton X-100 to solubilize membranous proteins (data not shown). Taken together, the above data indicate that the heavy form of the hormone-receptor complex is not a consequence of partial solubilization of the light form.

Guanyl nucleotide effects on solubilized vasopressin-receptor complexes. A partial separation of the two forms of the solubilized hormone-receptor complexes was performed by ultracentrifugation (see Fig. 2). These partially purified extracts exhibited different kinetic properties. As shown in Fig. 3, the light form of the hormone-

TABLE 1

Effect of membrane concentration on solubilization of vasopressin-receptor complexes

Rat kidney membranes differing in their protein concentrations were incubated for 30 min at 30°. Membranes were then washed, solubilized, and centrifuged as described under Materials and Methods. The radioactivity present in the supernatant and associated with the macromolecular component was determined on one aliquot of each extract by filtration on a Bio-Gel P-30 column (see Materials and Methods). The apparent solubilization yield of the vasopressin-receptor complexes was calculated as the ratio of radioactivity associated with macromolecular components of the supernatant and the radioactivity of the detergent-treated membrane extracts. For these calculations, corrections for nonspecific binding were realized. The remaining supernatant fractions were layered on top of 3–10% linear sucrose gradients together with calibration markers. The centrifugation and the gradient fractionation were carried out as described under Materials and Methods. Radioactivity associated with the macromolecular component was determined on each fraction by filtration on a Bio-Gel P-30 column. This elution pattern of the calibration markers used was determined to calculate the apparent sedimentation constant of each peak of radioactivity. The relative amount of each peak of radioactivity was deduced from these curves by measuring the area under each peak.

Protein concentration of the extract	Vasopressin-receptor complexes			
	Solubilization yield	Apparent sedimentation constant (Svedberg units)		Relative amount of the heavy form
		Light form	Heavy form	
mg/ml	%			%
2.0	33 + 3 (4)	3.2 + 0.1 (4)	6.0 + 0.1 (4)	55 + 1 (4)
3.1	45 + 2 (3)	3.2 + 0.2 (3)	6.1 + 0.1 (3)	53 + 3 (3)
4.5	44 + 4 (5)	3.3 + 0.1 (5)	6.2 + 0.1 (5)	54 + 2 (5)

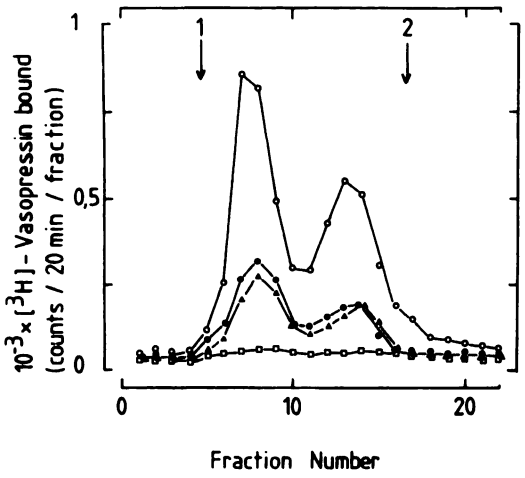

FIG. 2. *Binding specificities of the two soluble vasopressin receptors*

Rat kidney membranes (3.0 mg of protein per milliliter) were incubated for 30 min at 30° in a medium containing 100 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 15 mM [^{3}H]LVP, and various other unlabeled peptides (○, none; ▲, AVP, 4 mM; ●, cyclo dV-DAVP, 18 mM; □, LVP, 10 μM). The AVP and cyclo dV-DAVP concentrations were adjusted to reduce the specific binding of [^{3}H]vasopressin on rat kidney membranes by a factor of 2. The concentration of unlabeled LVP is that used for nonspecific experiments. Vasopressin-receptor complexes formed on intact membranes were then solubilized and subjected to ultracentrifugation through sucrose gradient as described in legend to Fig. 1. Radioactivity bound to macromolecular components was determined for each gradient fraction. *Arrows* indicate the elution peak of the calibration markers used (*1* = cytochrome *c*; *2* = alcohol dehydrogenase).

receptor complex is more sensitive to dissociation at 30° than is the heavy form. Moreover, addition of guanyl nucleotide to the incubation medium greatly increased the rate of dissociation of the heavy form of the hormone-receptor complex. Such results were not observed with the light form of the hormone-receptor complex. As seen in Fig. 4 and Table 3, addition of guanyl triphosphonucleotides produced a decrease in vasopressin-specific binding to membrane and in the relative amount of the heavy form of the hormone receptor complex. This reduction was dependent on the concentration of Gpp(NH)p present in the incubation medium and was almost complete at 0.1 mM. Adenyl triphosphonucleotide seems to be less active, since addition of 0.1 mM ATP in the incubation medium produces only a 40% decrease in the relative amount of the heavy form of the receptor. The nucleoside triphosphates did not produce any modification of the apparent sedimentation constant of the two solubilized forms of vasopressin receptors (see Table 3). As the solubilization yield of vasopressin-receptor was not statistically modified upon triphosphonucleotide treatment, it is possible to determine the absolute quantities of each form of membranous vasopressin receptors. These calculations show that (a) the amount of the light form of the receptor remained unchanged even after addition of triphosphonucleotide (this would imply that triphosphonucleotide affects the binding of vasopressin only on the heavy form of the receptor). (b) The free receptors, probably liberated after the action of triphosphonucleotides on the heavy form of vasopressin-receptor complexes, could not bind tritiated vasopressin again. However, additional experiments were necessary to prove this hypothesis.

Modification of the relative amount of the heavy form of the vasopressin receptor. As depicted in Table 3, partition of the two forms of solubilized vasopressin-receptor complexes depends on the duration of incubation of kidney membranes in the presence of [^{3}H]vasopressin. Their apparent sedimentation constants remain unchanged regardless of the incubation period tested (Table 3). However, the relative amount of the heavy form increases gradually with the time of incubation (68% increase during 55 min of incubation). This phenomenon cannot be accounted for by (a) any [^{3}H]vasopressin degradation, since the high-pressure liquid chromatographic profile of the tritiated hormone remained unchanged even after a 60-min incubation at 30° in the presence of

TABLE 2

Molecular parameters of solubilized vasopressin-receptor complexes

All values are the means ± standard error of the mean for the number of determinations indicated in parentheses. Stokes radii were determined according to the method of Laurent and Killander (21). Standard sedimentation constants, partial specific volume, frictional ratio, and apparent molecular weight were calculated as previously described (11). The % of detergent bound to the solubilized hormone-receptor complexes was determined according to Smigel and Fleischer (23). As described by Tanford and Reynolds (24), these calculations give only estimates of the parameters studied, since these proteins are membranous.

Parameters	Value for	
	Light receptor	Heavy receptor
Stokes radius, Rs (Å)	56 ± 1 (8)	65 ± 1 (6)
Apparent sedimentation coefficient, S_{app} (Svedberg units)	3.4 ± 0.1 (29)	6.1 ± 0.2 (27)
Standard sedimentation constant, S_{20_w} (Svedberg units)	3.6	6.2
Partial specific volume, V (ml/g)	0.777 ± 0.01 (6)	0.744 ± 0.01 (30)
Frictional ratio, f/f_0	1.74	1.73
Apparent molecular weight, M_r	100,000	200,000
Detergent bound to protein (% of wt.)	21	17
Molecular weight of protein moiety	80,000	160,000

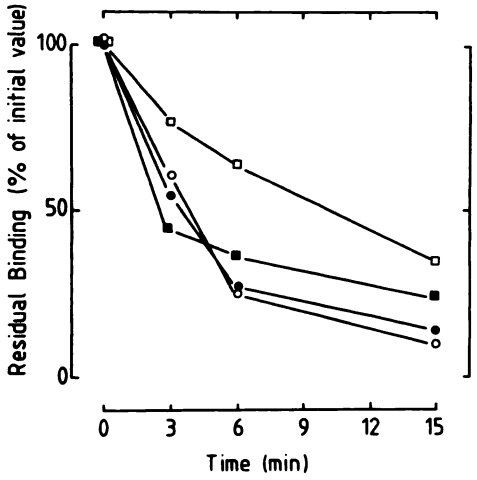

FIG. 3. *Kinetic properties of partially purified solubilized vasopressin-receptor complexes*

Rat kidney vasopressin-receptor complexes were solubilized as described in legend to Fig. 1. The soluble extracts were layered on top of an H_2O 3–10% linear sucrose gradient. After the ultracentrifugation, gradients were collected (see Materials and Methods). The fractions in which each type of hormone-receptor complexes migrated were gathered separately (□, ■, heavy form of the receptor; ○, ●, light form of the receptor). These partially purified extracts were incubated at 30° in the presence (●, ■) or in the absence(○, □) of 50 μM Gpp(NH)p. The radioactivity remaining bound to the macromolecular component was determined after various incubation periods by Bio-Gel P-30 filtration (see Materials and Methods). Results are expressed as percentage of the radioactivity present in the unwarmed, corresponding extracts and are the means of two experiments.

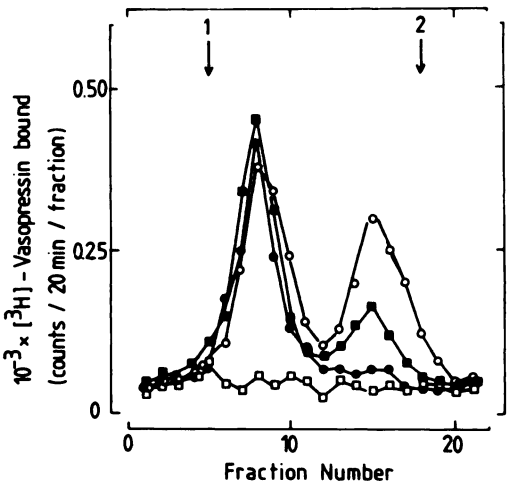

FIG. 4. *Effect of triphosphonucleotides on the relative amount of the solubilized vasopressin-receptor complexes*

Rat kidney membranes (3.5 mg of protein per milliliter) were incubated for 30 min at 30° with 15 nM [^{3}H]vasopressin under different conditions [○, without effector; □, with 1 μM unlabeled vasopressin; ●, with 0.1 mM Gpp(NH)p; ■, with 0.1 mM ATP plus creatine kinase (1 mg/ml) and creatine phosphate (1.5 mg)]. Vasopressin-receptor complexes formed on intact membranes were then solubilized and subjected to ultracentrifugation through sucrose gradients as described in legend to Fig. 1. Radioactivity bound to macromolecular components was determined for each gradient fraction. *Arrows* indicate the elution peak of the calibration markers used (*1* = cytochrome *c*; *2* = alcohol dehydrogenase).

kidney membranes (results not shown); (b) a modification of the apparent solubilization yield of vasopressin-receptor complexes (Table 3).

DISCUSSION

The results presented above demonstrate the existence of two different species of solubilized vasopressin-receptor complexes differing in their molecular and kinetic parameters. As demonstrated earlier (19), these soluble forms of bound radioactivity cannot originate from non-specific rebinding of free [^{3}H]vasopressin to detergent micelles or to macromolecular components of solubilized membranes. Moreover, soluble forms of bound radioactivity represent the vasopressin-receptor complexes formed on intact membranes, since, as indicated in Fig. 2, the decrease in [^{3}H]vasopressin binding to intact membranes due to the addition of unlabeled vasopressin analogues leads to an identical decrease in the labeling of the two forms of solubilized vasopressin receptor. Partial or incomplete solubilization of a single class of vasopressin receptor, leading to the existence of different molecular species of solubilized hormone-receptor complexes,

TABLE 3

Influence of time and guanyl nucleotides on the relative amount of the two forms of solubilized vasopressin-receptor complexes

Rat kidney membranes (4.0 mg of protein per milliliter) were incubated at 30° with 15 nM tritiated vasopressin in the presence or absence of triphosphonucleotides during various periods. The specific binding of [^{3}H]vasopressin to membranes was measured on an aliquot of each sample by Millipore filtration (see Materials and Methods). The remaining membranes were washed, solubilized, and centrifuged as described under Materials and Methods. The supernatant containing the radioactivity was layered on top of sucrose gradients. Ultracentrifugation and gradient fractionation were carried out as described under Materials and Methods. Determinations of the apparent solubilization yield, of the apparent sedimentation constant, and the relative amount of the two forms of vasopressin-receptor complexes were calculated as indicated in legend to Table 2. All results are the means ± standard error of the mean of the number of determination indicated in parentheses.

Incubation condition		Vasopressin specific binding	Vasopressin-receptor complexes			Relative amount of heavy form of the receptor
Time of incubation at 30°	Triphosphonucleotide added		Apparent solubilization yield	Apparent sedimentation constant (Svedberg units)		
				Light form	Heavy form	
min		*pmole/mg protein*	%			%
5	—	0.21 + 0.01 (3)	41 + 3 (3)	3.3 + 0.2 (5)	6.1 + 0.2 (5)	30 + 4 (5)
15	—	0.26 + 0.01 (3)	45 + 4 (6)	3.3 + 0.1 (6)	6.2 + 0.2 (6)	41 + 2 (6)
30	—	0.23 + 0.02 (3)	46 + 2 (9)	3.2 + 0.1 (9)	6.0 + 0.1 (9)	56 + 2 (9)
60	—	0.23 + 0.04 (3)	45 + 4 (5)	3.3 + 0.1 (5)	6.1 + 0.1 (5)	57 + 4 (5)
30	Gpp(NH)p, 50 μM	0.15 + 0.02 (3)	35 + 3 (3)	3.4 + 0.1 (4)	6.1 + 0.1 (4)	30 + 3 (4)
30	Gpp(NH)p, 100 μM	0.14 + 0.02 (3)	37 + 3 (3)	3.3 + 0.1 (4)	6.0 + 0.1 (4)	6 + 2 (4)
30	ATP, 100 μM	0.19 + 0.02 (3)	40 + 4 (3)	3.3 + 0.1 (5)	6.0 + 0.1 (5)	33 + 3 (5)

may also be excluded since (a) the elution profiles of the different peaks of soluble forms of bound radioactivity are symmetrical (Fig. 1) and (b) the apparent sedimentation constant and the relative amount of each form of solubilized vasopressin-receptor are unchanged even after modification of the detergent to protein ratio (Table 1).

The determination of the molecular parameters of the two solubilized vasopressin-receptor complexes raises the question of the nature of these two forms. On the basis of their molecular weight, the heavy form of the receptor may represent a dimer of the light form (molecular weight = 200,000 and 100,000 for the heavy and light forms, respectively) (see Table 1). However, it is unlikely that the association of two forms of receptor complexes insensitive to guanyl nucleotide can produce a complex that is sensitive to guanyl nucleotides. In comparison with the *beta*-adrenergic system, where similar results were obtained (16), the heavy form of the vasopressin receptor probably represents a complex between the light form of the receptor and another membranous component (probably a GTP-binding protein). This hypothesis remains to be verified, since we have not yet identified or purified the GTP-binding proteins in our extract. Despite the error associated with the determination of the hydrodynamic parameters of solubilized membrane proteins (24), our study allows an estimate of the molecular weight of the protein found associated with the vasopressin receptor (difference between the molecular weights of the two vasopressin-receptor complexes). The value we obtained (90,000) is in accord with those determined directly for the guanyl nucleotide-binding protein (called G/F) known to interact with the *beta*-adrenergic receptor (15).

Earlier studies on the same system have demonstrated the existence of a GTP binding protein of 40,000 M_r associated with the adenylate cyclase moiety after activation by guanyl nucleotides (10).[2] These results would signify that two distinct GTP-binding proteins may be engaged in the molecular mechanisms leading to the activation of rat kidney adenylate cyclase by vasopressin. One of them (90,000 M_r) would be capable of interaction with the vasopressin-receptor complex; the other (40,000 M_r), of interaction with the catalytic unit of adenylate cyclase. On the basis of our results, such a hypothesis cannot be excluded. Nevertheless, as a great number of analogies exists between hormonal and neurotransmitter activation of adenylate cyclase, it seems reasonable to consider that the GTP-binding protein of 40,000 M_r liberated during the hormonal activation processes would represent a subunit of the 90,000 M_r molecule, in line with recent work on the *beta*-adrenergic receptor coupled to adenylate cyclase. Indeed, studies by Northup *et al.* (12, 13) have revealed that this GTP-binding protein is composed of two distinct subunits (45,000 and 35,000 M_r) and can be partially dissociated by the addition of guanyl nucleotide. Furthermore, Stadel and co-workers (16) have demonstrated the interactions between a GTP-binding protein, the *beta*-adrenergic receptor, and the adenylate cyclase moiety of the same system. Moreover, addition of guanyl nucleotides decreased the interactions between receptor and GTP-binding protein and increased those between the GTP-binding protein and adenyl cyclase (8, 25, 26).

According to this hypothesis, the scheme of hormonal activation of adenylate cyclase may be clarified. The formation of a ternary complex between the hormone, its specific receptor, and a GTP-binding protein of 90,000 M_r represents the first step in this mechanism. The interaction of GTP with this ternary complex leads, then, to the liberation of the 40,000 M_r GTP-binding protein, which then interacts with the catalytic moiety of the adenylate cyclase and activates the enzyme. The forma-

[2] G. Guillon, D. Butlen, and R. Rajerison, unpublished results.

tion of a ternary complex between hormones (or neurotransmitter), their specific receptors, and a GTP-binding protein appears to represent a crucial step in the activation of adenylate cyclase in a number of hormonal and neurotransmitter systems (9, 10, 27–29).

As shown in Table 3, the light form of the receptor seems to be interconvertible with the heavy form by increasing the duration of the incubation between [^{3}H] LVP and membranes, since the relative amount of each form of solubilized receptor varies without any significant modification of the specific binding of vasopressin to membranes. The rate of vasopressin receptor interconversion is relatively slow as compared with the rate of vasopressin activation of adenylate cyclase (on this system, maximal adenylate cyclase activation by 15 nM vasopressin was reached after an incubation of 15 min at 30°). Under these conditions, only 35% of the receptor is in the heavy form (see Table 3). These data reveal an important problem, since receptor interconversion appears necessary for adenylate cyclase activation. Different hypotheses may be proposed: (a) the rate of vasopressin-receptor interconversion may be more rapid in intact cells than in partially purified plasma membranes; (b) maximal adenylate cyclase activation by hormone may be obtained with partial vasopressin receptor interconversion if the vasopressin receptor molecules are in excess of the molecules of adenylate cyclase.

In conclusion, the findings reported here provide strong evidence for the existence of a ternary complex between hormone, vasopressin-receptor, and a GTP-binding protein. Such results would signify that the molecular mechanisms implicated in hormonal or neurotransmitter activation of adenylate cyclase are similar regardless of the effector or biological system studied.

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