Guanine nucleotide regulation of [³H]vasopressin binding to liver plasma membranes and solubilized receptors

Evidence for the involvement of a guanine nucleotide regulatory protein

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A guanine nucleotide regulatory protein may be involved in vasopressin-receptor-mediated polyphosphoinositide breakdown in rat liver. Therefore we examined the effects of the non-hydrolysable guanine nucleotide guanosine 5'-[β -imido]triphosphate (p[NH]ppG) on [³H]vasopressin ([³H]AVP) binding to hepatic plasma membranes and detergent extracts. [³H]AVP bound to a single set of high-affinity binding sites in membranes. Addition of p[NH]ppG decreased the affinity of receptor binding without altering the maximal binding capacity. The rate of dissociation of [³H]AVP from membrane-bound receptors was also enhanced by p[NH]ppG. Solubilization of [³H]AVP-prelabelled membranes with dodecyl β -D-maltoside resulted in a [³H]AVP-receptor complex that was unstable in solution. Incubation of these extracts for 5 min at 30 °C resulted in a 40% loss of bound [³H]AVP, whereas in the presence of p[NH]ppG there was a 54% loss. However, when membranes were prelabelled with [³H]AVP and p[NH]ppG and then solubilized, the resulting hormone-receptor complex was still temperature-labile but insensitive to the further addition of p[NH]ppG. The molecular size of soluble vasopressin receptors was estimated by gel filtration. The [³H]AVP-receptor complex was eluted as a single peak with an apparent molecular size of 258 kDa. However, no peak was detected when solubilized extract was made from membranes prelabelled with [³H]AVP and p[NH]ppG, suggesting that this receptor complex had dissociated during chromatography. It is possible therefore that the high- M_r complex contains the hormone, its receptor and a guanine nucleotide binding protein.

INTRODUCTION

There is increasing evidence to suggest that a novel guanine nucleotide binding protein is involved in the transduction of hormone-mediated responses involving polyphosphoinositide turnover (see reviews by Joseph, 1985; Litosch & Fain, 1986). The first suggestions for this came from radioligand-binding studies, which showed that guanine nucleotides decreased agonist, but not antagonist, affinities in several tissues and receptor systems involving the phosphoinositide breakdown pathway (for examples see Cantau *et al.*, 1980; Goodhardt *et al.*, 1982; Evans *et al.*, 1985). One of these, the hepatic vasopressin receptor, has been extensively characterized both biochemically (Hems *et al.*, 1976; Michell *et al.*, 1979; Tolbert *et al.*, 1980; Lynch *et al.*, 1985) and pharmacologically (Michell *et al.*, 1979; Cantau *et al.*, 1980). In contrast with the kidney V_2 receptor, the liver V_1 receptor is not coupled to adenylate cyclase, but mediates its glycogenolytic effects via a cyclic AMP-independent mechanism involving the elevation of intracellular Ca^{2+} concentration secondary to the breakdown of phosphoinositides (Michell *et al.*, 1981).

The increase in cytosolic Ca^{2+} as a result of hormone action is believed to be regulated by a product of inositol lipid hydrolysis, *myo*-inositol 1,4,5-trisphosphate, which is formed as a result of phospholipase C action on membrane-bound phosphatidylinositol 4,5-bisphosphate (Berridge, 1984; Williamson *et al.*, 1985; Fain, 1986). However, the events occurring between receptor occupa-

tion by agonist and phospholipase C activation are at present unclear. By analogy to the well-characterized adenylate cyclase system, it is possible that a guanine nucleotide binding protein may act as a coupling protein between receptor and catalytic component. In order to investigate this possibility, we examined the effects of p[NH]ppG on membrane-bound and detergent-solubilized hepatic vasopressin receptors and on the apparent molecular size of these receptors as measured by gel filtration.

MATERIALS AND METHODS

Materials

[8-L-arginine][*Phe*-3,4,5-³H]Vasopressin ([³H]AVP, 53 Ci/ mmol) was obtained from NEN. Ultrogel AcA34 was from LKB, Bio-Gel P30 from Bio-Rad, dodecyl β -D-maltoside from Calbiochem, p[NH]ppG from Boehringer Mannheim, [arginine]vasopressin and bacitracin were from Sigma, and bovine serum albumin was from Armour. M_r markers (indicated in the text) were from either Sigma or Bio-Rad. All other reagents were of analytical grade.

Preparation of liver membranes

Hepatic membranes were prepared from Sprague-Dawley rats (150–200 g) by a modification of the method of Pilkis *et al.* (1974). Partially purified plasma membranes were obtained at the 48.2%/42.5%-sucrose interface, followed by washing in 1 mM-KHCO₃, pH 7.2,

Abbreviations used: p[NH]ppG, guanosine 5'-[β -imido]triphosphate; AVP, [arginine]vasopressin; G_s and G_i , the guanine nucleotide regulatory proteins that couple receptors to stimulation and inhibition of adenylate cyclase respectively.

by centrifuging at 40000 *g* for 15 min. The pellet was resuspended in 1 mM-KHCO₃, pH 7.2, and stored in batches at -80 °C for up to 1 month without significant loss of [³H]AVP-binding activity.

Binding assays

Membranes were thawed and incubated in a final volume of 250 μ l in assay buffer (50 mM-Tris/HCl, pH 7.4, and 5 mM-MgCl₂) containing bacitracin and bovine serum albumin (each 1 mg/ml) and the indicated concentrations of [³H]AVP. To determine non-specific binding, separate incubations were performed in the presence of 5 μ M unlabelled vasopressin. After incubation for 20 min at 30 °C (the time necessary for equilibration of binding), the incubations were terminated by the addition of 3.5 ml of ice-cold assay buffer, followed by vacuum filtration over Whatman GF/B glass-fibre filters. These were then washed with 3 $\times$ 5 ml of assay buffer, and radioactivity associated with the filters was counted by liquid-scintillation spectroscopy. For dissociation experiments, after equilibration of the membranes with ligand, an excess of unlabelled vasopressin (5 μ M final concn.) was added to the tubes to prevent reassociation of label, and incubations were continued for the indicated times.

For experiments involving the prelabelling of receptors before solubilization, membranes (2–3 mg of protein) were incubated as above, except that the final volume was 2 ml. After the incubation period, the tubes were rapidly cooled and all subsequent steps were performed at 0–4 °C. To separate bound from free ligand, the tubes were centrifuged in a Microfuge at 12000 *g* for 10 min, followed by two further washes in assay buffer.

Solubilization of vasopressin receptors

The pellet containing prelabelled [³H]AVP–receptor complex was resuspended in 0.55 ml of solubilizing buffer, consisting of 100 mM-Hepes (adjusted to pH 7.4 with NaOH), 50 mM-NaCl, 5 mM-MgCl₂ and 0.3% dodecyl β -D-maltoside (see Boer *et al.*, 1983). The membranes were gently mixed for 30 min at 0 °C, followed by centrifugation at 110000 *g* for 1 h at 0 °C. The clear yellow supernatant (approx. 2 mg of protein/ml) was used directly in all subsequent steps. By this procedure, approx. 50% of the hepatic vasopressin receptors were solubilized.

Measurement of soluble hormone–receptor complex

[³H]AVP bound to soluble receptors was separated from free dissociated ligand on Bio-Gel P30 columns (0.5 cm $\times$ 9 cm). Radioactivity eluted in the void volume (0.5–1.5 ml) represented binding to macromolecular complexes.

For estimation of molecular size, the solubilized material (0.5 ml) was applied to a 1.6 cm $\times$ 50 cm column containing Ultrogel AcA34 which had been pre-equilibrated with buffer consisting of 50 mM-Tris/HCl, pH 7.4, 50 mM-NaCl, 5 mM-MgCl₂ and 0.1% dodecyl β -D-maltoside. The flow rate was 10 ml/h, and 1 ml fractions were collected. Radioactivity was counted by liquid-scintillation spectroscopy, and background radioactivity was subtracted from the results. The gel-filtration column was calibrated with soluble marker proteins of known *M_r* as follows; thyroglobulin (669000), apoferritin (443000), β -amylase (200000), alcohol dehydrogenase (150000), bovine serum albumin (66000), carbonic anhydrase (29000) and myoglobin (17000).

Fig. 1. p[NH]ppG modulation of [³H]AVP receptor affinity in liver membranes

(a) Saturation of specific [³H]AVP binding. Incubations were performed in the absence (■) or presence (●) of 100 μ M-p[NH]ppG as described in the Materials and methods section. (b) Scatchard plot of the same data, from which *K_d* and *B_{max}* values were derived. The results shown are from representative experiments performed in duplicate, which were repeated three times.

Protein

Protein was measured by the method of Bradford (1976) with bovine γ -globulin as standard.

Expression of results

In all cases except where indicated, the results are expressed as specific binding, which is defined as the difference between total and non-specific binding.

RESULTS

Incubation of liver membranes with increasing concentrations of [³H]AVP revealed that binding was saturable and of high affinity (Fig. 1a). Scatchard plots (Fig. 1b) were linear, indicating binding to a single set of non-co-operative binding sites, with a dissociation constant (*K_d*) of 2.93 ± 0.74 nM (mean $\pm$ S.E.M., *n* = 4) and maximal binding (*B_{max}*) of 625 ± 137 fmol/mg of protein. In agreement with previous results (Cantau *et al.*, 1980), addition of the non-hydrolysable guanine nucleotide p[NH]ppG to the incubation resulted in a decrease in the affinity of vasopressin receptors for [³H]AVP without a change in *B_{max}*; mean values were

Fig. 2. Guanine nucleotide effects on the time course of dissociation of specific [3 H]AVP binding

Membranes were pre-equilibrated with 10 nM-[3 H]AVP, after which an excess of unlabelled vasopressin (5 μ M final concn.) was added (zero time) in the absence (■) or presence (●) of 100 μ M-p[NH]ppG and incubations were continued for the indicated times. Inset shows semi-logarithmic plot of the same data. The results shown are expressed as means $\pm$ S.E.M. for three experiments performed in duplicate.

K_d 12.2 ± 1.2 nM and B_{max} 613 ± 31 fmol/mg of protein ($n = 4$). To investigate the guanine nucleotide effect further, the dissociation of [3 H]AVP was examined (Fig. 2). In control membranes, [3 H]AVP binding slowly dissociated, but if p[NH]ppG was included in the assay, the degree of dissociation was enhanced. Analysis of kinetic replots (Fig. 2, inset) indicated that dissociation was of first order, with half-lives of 64 min and 38 min for control and p[NH]ppG-incubated membranes respectively.

The effect of p[NH]ppG on the dissociation of ligand from soluble receptors was also examined. Despite the relative stability of the [3 H]AVP-receptor complex in membranes at 30 $^{\circ}$ C, once the membranes were solubilized, the complex was unstable. Indeed, even during soluble-receptor preparation (30 min solubilization, 1 h centrifugation), approx 30% of receptor-bound [3 H]AVP dissociated and was eluted with the total volume from the Bio-Gel columns.

When soluble receptors were prepared from [3 H]AVP-preincubated membranes, incubation of the soluble extract for 5 min at 30 $^{\circ}$ C resulted in a 40% decrease in the amount of receptor-bound [3 H]AVP as compared with the same extract kept on ice (Table 1). In the presence of p[NH]ppG the [3 H]AVP-receptor complex became even more unstable, resulting in an enhanced degree of dissociation (54%), in agreement with the results obtained in membranes. It should be noted that reassociation of ligand with soluble receptors should not be a factor, owing to the low free vasopressin concentration and because [3 H]AVP binding to detergent extracts is minimal. In separate experiments, membranes were prelabelled with [3 H]AVP in the presence of p[NH]ppG, followed by solubilization. The p[NH]ppG-pretreated soluble extract was then incubated in the same fashion as control extract. In contrast with control extracts, [3 H]AVP binding was still temperature-sensitive,

Table 1. Effect of p[NH]ppG on the binding of [3 H]AVP to soluble vasopressin receptors

(A) Solubilized receptors were prepared after preincubation of membranes with 10 nM-[3 H]AVP. Samples were then taken and incubated at 30 $^{\circ}$ C for 5 min in the absence or presence of 100 μ M-p[NH]ppG. Receptor-bound [3 H]AVP was separated from free ligand on Bio-Gel P30 columns. (B) Incubations were performed as above, except that membranes were preincubated with 20 nM-[3 H]AVP and 100 μ M-p[NH]ppG. Separate incubations were performed in the presence of 5 μ M unlabelled vasopressin to determine non-specific binding, which was subtracted from the results. The results are expressed as the percentage of remaining bound radioactivity relative to incubations kept on ice and are means $\pm$ S.E.M. of three experiments: * significant effect of p[NH]ppG ($P < 0.05$; paired t test).

	A	B
No addition	60 ± 2	58 ± 7
p[NH]ppG	$46 \pm 3^*$	54 ± 9

but was insensitive to the further addition of nucleotide (Table 1).

The molecular size of the control and p[NH]ppG-preincubated hormone-receptor complex was examined by AcA34 gel filtration. [3 H]AVP-prelabelled solubilized receptor was eluted from the column as a single broad peak (Fig. 3a), which was quite distinct from non-specific binding. In addition to the receptor-bound peak, a second peak was observed near the total volume, corresponding to free [3 H]AVP, which was eluted primarily in fractions 107–113. This peak accounted for approx. 90% of total eluted radioactivity, indicating that most of the [3 H]AVP dissociated from specific and non-specific sites during the time taken to run the column (approx. 11 h). The molecular size of the soluble [3 H]AVP-receptor complex as analysed from the calibration curve in Fig. 4 was 258000 ± 7000 Da ($n = 3$). In marked contrast with controls, the elution profile of soluble vasopressin receptors prepared from membranes that had been preincubated with [3 H]AVP and p[NH]ppG closely followed that obtained for non-specific binding, no detectable peak being observed (Fig. 3b).

DISCUSSION

Several reports have suggested the involvement of a guanine nucleotide binding protein in hormone-mediated polyphosphoinositide metabolism. Vasopressin has been shown to stimulate a low- K_m GTPase in hepatocyte membranes (Fain *et al.*, 1985). Moreover, guanine nucleotide effects have been clearly demonstrated in permeabilized cells (Gomperts, 1983; Haslam & Davidson, 1984), cell-free systems (Litosch *et al.*, 1985; Litosch & Fain, 1985; Wallace & Fain, 1985; Litosch *et al.*, 1986; Uhing *et al.*, 1986) and receptor-binding assays (Cantau *et al.*, 1980; Goodhardt *et al.*, 1982; Evans *et al.*, 1985). In agreement with Cantau *et al.* (1980), who used GTP, p[NH]ppG decreased the affinity of vasopressin for its receptor, as reflected by an increase in its rate of dissociation. Similar effects have been observed with agonist binding to β -adrenoceptors, whose coupling to a

Fig. 3. AcA34 gel filtration of soluble vasopressin receptors

(a) Soluble receptors were prepared as described in the Materials and methods section after prelabelling membranes with 10 nM [3 H]AVP in the absence (■) or presence (●) of 5 μ M unlabelled vasopressin (representing non-specific binding). Free [3 H]AVP, which had dissociated from specific and non-specific binding sites, was eluted from the column between fractions 107 and 113. (b) Gel filtration of soluble receptors after preincubation of membranes with 20 nM [3 H]AVP and 100 μ M p[NH]ppG. No receptor-bound peak is detectable, although the amount of free [3 H]AVP eluted was normally greater as compared with non-specific binding, indicating that it had dissociated from bound sites during the column run. The results presented are representative examples which were repeated twice. V_0 = void volume of column.

guanine nucleotide regulatory protein is well characterized (Lefkowitz & Williams, 1977; Limbird *et al.*, 1980). These observations suggest that the vasopressin receptor may be coupled to a guanine nucleotide regulatory protein in a similar manner.

A popular theory for receptor coupling to G proteins has been the ternary-complex model proposed by DeLean *et al.* (1980). According to this, occupation of a receptor by an agonist leads to the formation of a high-affinity complex consisting of hormone (H), receptor (R) and guanine nucleotide regulatory protein (G). Dissociation of the complex occurs on addition of GTP, to produce a GTP-liganded G protein and low-affinity HR complex. Testing of this hypothesis with the vasopressin receptor has been simplified somewhat by the observation that vasopressin binds to a single set of high-affinity binding sites in the

Fig. 4. Estimation of the molecular size of soluble [3 H]AVP-receptor complex

The AcA34 column was calibrated with the indicated marker proteins, and the molecular size of soluble vasopressin receptors assessed from data in Fig. 3(a) and two replicate experiments. In these three experiments the receptor-bound peaks were found at fractions 57, 57 and 58, which corresponds to a molecular size (mean $\pm$ S.E.M.) of 258000 ± 7000 Da.

absence of p[NH]ppG. This is in marked contrast with the more commonly studied adrenergic receptors, which exhibit mixed populations of high- and low-affinity agonist binding sites in the absence of guanine nucleotides. Possibly the vasopressin-receptor complex exists entirely as a high-affinity HRG complex, which is completely dissociated to the lower-affinity HR complex in the presence of p[NH]ppG. With this in mind, the effect of p[NH]ppG on the two proposed receptor states was investigated. As expected, p[NH]ppG only enhanced [3 H]AVP release from its receptor if soluble extracts were prepared from membranes not pretreated with the nucleotide. This supports the hypothesis that the [3 H]AVP-receptor complex contains a guanine nucleotide binding protein as well as the receptor. The differences in the two proposed complexes should also be apparent as differences in the molecular size of the vasopressin receptor, as has been shown for adenylate cyclase-coupled β -adrenoceptors (Limbird & Lefkowitz, 1978; Limbird *et al.*, 1980) and renal vasopressin receptors (Guillon *et al.*, 1984). However, it must be emphasized that, in contrast with the adenylate cyclase system, little is known about the coupling of receptors to phospholipase C. It is prudent therefore to consider the possibility of additional components in this system besides the HRG complex which may influence the apparent size of the [3 H]AVP-receptor complex. Gel filtration of soluble vasopressin receptors indicated a M_r far in excess of the 68000 reported in studies using photoaffinity-labelling techniques (Boer & Fahrenholz, 1985). No accurate estimate can be made of the actual size of this complex, because unknown amounts of the detergent are bound to the membrane protein (see Tanford & Reynolds, 1976). However, further support for at least a G-protein-associated complex is given in that guanine nucleotide pretreatment of membranes resulted in a soluble complex whose M_r could not be estimated, owing to its instability. Attempts to measure the size of unoccupied vasopressin receptors (presumably not associated with

G) have in our hands been unsuccessful, since, on solubilization, binding of the hormone is minimal (D. Bojanic, unpublished work). These studies therefore await the development of a radiolabelled vasopressin antagonist which, by analogy with the β -adrenoceptor system, should not induce the coupling of receptor to G and hence would enable the molecular size of R to be measured.

The identity of the guanine nucleotide regulatory protein remains to be determined. Suggestions for the involvement of G_i or a closely related protein have come from studies which demonstrated that pertussis toxin inhibited agonist-induced activation of phosphoinositide breakdown in adipocytes (Moreno *et al.*, 1983), human polymorphonuclear leucocytes (Smith *et al.*, 1985; Verghese *et al.*, 1985), mast cells (Nakamura & Ui, 1985) and rabbit neutrophils (Shefcyk *et al.*, 1985). These effects may be atypical, however, since receptor-mediated events associated with phosphoinositide breakdown are insensitive to pertussis toxin in several cell types (Evans *et al.*, 1985; Martin *et al.*, 1985; Masters *et al.*, 1985; Houslay *et al.*, 1986; Uhing *et al.*, 1986). Clearly, further clarification of this issue is necessary. Although our results cannot identify the subtype of G protein involved, the evidence presented here suggests that the phosphoinositide-linked G protein is coupled to receptor in a manner similar to G_s and G_i . Further studies are needed to determine the relationship of this regulatory protein with phospholipase C activation.

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