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Biochemistry

Molecular complexes involved in the regulation of adenylate cyclase

(cytoskeleton/N-protein/guanyl nucleotides/β-adrenergic receptor)

NAJI E. SAHYOUN, HARRY LEVINE III, JONATHAN DAVIS, GEORGE M. HEBDON, AND PEDRO CUATRECASAS

Department of Molecular Biology, The Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709

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ABSTRACT **Selective extraction of the adenylate cyclase regulatory protein (N-protein) from pigeon erythrocyte plasma membranes provided evidence for its cytoskeletal association. Cholate, but not Triton X-100 or digitonin, was effective in solubilizing the ADP-ribosylated N-protein. The labeled protein complex or components thereof that were associated with the Triton-insoluble cytoskeleton (shells) could be partly released by 0.1 mM EDTA; 1 M KCl in the presence of Triton X-100 achieved complete solubilization. 5′-Guanylyl imidodiphosphate (p[NH]ppG) and NaF, activators of adenylate cyclase, promoted the release of the regulatory protein from the cytoskeleton but $MnCl_2$, an "uncoupler" of the adenylate cyclase system, had the opposite effect. The solubilized, labeled N-protein was able to bind specificially to rat erythrocyte inside-out vesicles in the presence of divalent cations. A proteolytic product of inside-out vesicles inhibited the binding of the N-protein to fresh vesicles. Three molecular species which contained the M_r 45,000 polypeptide component of the N-protein were identified by gel permeation chromatography and by sucrose density gradient velocity sedimentation. p[NH]ppG appeared to convert the two larger molecular complexes to a smaller molecular entity. Such a molecular dissociation might be relevant to the effects of guanyl nucleotides on the activity of adenylate cyclase and on the affinity of hormone receptors.**

Activation of the adenylate cyclase system by hormones or GTP analogs appears to involve changes in the molecular associations among the receptor, the regulatory nucleotide-binding protein (N-protein), and the catalytic component. Gel permeation chromatography (1), sucrose density gradient sedimentation (2, 3), and target-irradiation analysis (4) have provided evidence for these molecular changes. Furthermore, selective extraction (5) and reconstitution (6) experiments suggested that constituents of the adenylate cyclase complex were bound to the cytoskeleton in a manner that depended on the state of activation of the enzyme (6). This report focuses on several molecular interactions of the N-protein and on their regulation by agents that activate adenylate cyclase. We have also attempted to solubilize, separate, and characterize the putative molecular complexes that result from these interactions. This approach may help to elucidate further the mechanism of activation of the enzyme as well as the mechanism of the regulation of the number and the affinity of hormone receptors that act on adenylate cyclase.

Our results indicate that the N-protein binds to the inner aspect of the plasma membrane, partly through specific protein-protein interactions. 5′-Guanyl imidodiphosphate p[NH]ppG and NaF appear to release the N-protein from the cytoskeleton, whereas $MnCl_2$, which "uncouples" the regulatory component from the catalytic component (7), seems to increase the association between the N-protein and the cytoskeleton. Three different molecular complexes which contain the ADP-ribosylated M_r 45,000 polypeptide component of the N-protein have been isolated. p[NH]ppG induces the formation of a small molecular species which contains the M_r 45,000 polypeptide. This dissociative reaction may be required for activation of the N-protein. The results are discussed in the context of a molecular model which depicts features of the mechanism of activation of adenylate cyclase by p[NH]ppG.

MATERIALS AND METHODS

Cholera toxin was purchased from Schwarz/Mann. Digitonin was supplied by Fisher. [α-^{32}P]ATP (40–60 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear. [α-^{32}P]NAD was synthesized from [α-^{32}P]ATP as described (8). Erythrocytes were obtained from male Sprague–Dawley rats (125–150 g) and from White Carneau pigeons. Rat reticulocytes were produced by the phenylhydrazine injection method (1). Pigeon erythrocytes were lysed by freeze-thawing, and the plasma membrane fraction was separated as reported (9). Rat reticulocyte and erythrocyte ghosts were made by hypotonic lysis (10) and they were resealed in the presence of $MgCl_2$ (11). Inside-out vesicles were prepared by incubation of the erythrocyte ghosts in 0.1 mM sodium phosphate buffer (pH 7.6) at 37°C (12). The N-protein of erythrocyte ghosts and plasma membranes was labeled by ADP-ribosylating the M_r 45,000 polypeptide in the presence of cholera toxin and 60 μM [α-^{32}P]NAD (5–7 Ci/mmol) (9); 65–80% of the total membrane protein ADP-ribosylation was accounted for by the specific labeling of the M_r 45,000 polypeptide.

Sucrose density gradients (5–20%) were prepared in cellulose nitrate tubes. The gradients contained 25 mM Tris·HCl (pH 7.6), 0.1% Triton X-100 (all detergent concentrations are expressed as wt/vol), and 5 mM $MgCl_2$. Samples (0.2 ml) were loaded and the gradients were centrifuged in a Beckman SW 50.1 rotor at 45,000 rpm for 14–16 hr at 4°C, and 0.2-ml fractions were collected. Gel permeation chromatography was performed by applying 0.5-ml samples to 26-ml Ultrogel AcA-34 columns and eluting with 50 mM Tris·HCl, pH 7.6/0.1% Triton X-100/5 mM $MgCl_2$; 0.8-ml fractions were collected. The buffers used in all experiments contained 30 μg of phenylmethylsulfonyl fluoride per ml.

The labeled M_r 45,000 polypeptide contained in the fractions from the sucrose density gradients and from the AcA-34 columns was separated from other membrane proteins by Na-DodSO$_4$/polyacrylamide gel electrophoresis; the gels were then subjected to autoradiography with the assistance of a fluorescent screen (5). The Coomassie blue-stained gels and the autoradiograms were analyzed quantitatively by standardized

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Abbreviation: p[NH]ppG, 5′-guanyl imidodiphosphate.

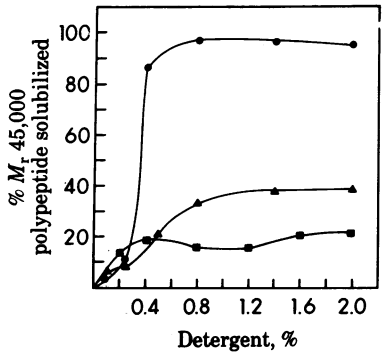

FIG. 1. Solubilization of the M_r 45,000 polypeptide with different detergents. ADP-ribosylated pigeon erythrocyte plasma membranes (3.7 mg of protein per ml) were extracted with 4 vol of 50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$ containing the indicated concentration of detergent. After 15 min at 0°C, the detergent extracts were centrifuged at 160,000 × g_{max} and the ADP-ribosylated polypeptide was assayed in the various supernatants. The M_r 45,000 polypeptide that was not solubilized could be recovered quantitatively in the detergent shells. The amount of labeled M_r 45,000 polypeptide in the detergent supernatants and detergent pellets was assessed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by autoradiography. ●, Cholate; ▲, Triton X-100; ■, digitonin. The results are expressed as percentage of the total labeled M_r 45,000 polypeptide.

densitometric scanning (5). The same technique was used to quantitate the amount of labeled M_r 45,000 polypeptide in other experiments. Protein concentrations were determined by the Coomassie Blue R-250 method (13).

RESULTS

Solubilization of the M_r 45,000 Cholera Toxin Substrate. The association of the N-protein with the plasma membrane was studied by selective detergent extraction. Different classes of detergents solubilized different quantities of the M_r 45,000 polypeptide from pigeon erythrocyte plasma membranes (Fig. 1). At the highest concentration of detergent, the amounts of the labeled polypeptide released were 23% with digitonin, 38% with Triton X-100, and 100% with cholate. Lubrol PX and Nonidet P-40 were similar to Triton X-100. Band 3 displayed a different trend of solubilization: 5% was released by cholate and 55% was solubilized by Triton X-100 or digitonin. The solubilization characteristics of adenylate cyclase, the M_r 45,000 polypeptide and other membrane proteins are compared elsewhere (5).

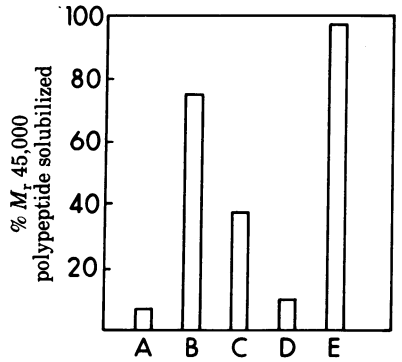

FIG. 2. Solubilization of the M_r 45,000 polypeptide associated with the detergent shells. ADP-ribosylated pigeon erythrocyte plasma membranes (4.2 mg of protein per ml) were extracted with 20 vol of 2.0% Triton X-100/5 mM $MgCl_2$ for 30 min at 0°C. The resulting detergent shells were resuspended in buffer (0.6 mg of protein per ml) and then were extracted for 30 min as follows: A, 1.0% Triton X-100 at 0°C; B, 1.0% Triton X-100 at 37°C; C, 0.1 mM EDTA, pH 7.6, at 37°C; D, 1 M KI at 0°C; and E, 1 M KCl/0.5% Triton X-100 at 0°C. All extraction media (except C) contained 50 mM Tris·HCl (pH 7.6). The 160,000 × g_{max} supernatants were assayed for the labeled M_r 45,000 polypeptide.

The subpopulation of the M_r 45,000 polypeptide that remained associated with the detergent shells after maximal extraction with Triton X-100 at 0°C could be solubilized further (Fig. 2). Treatment of the Triton shells with 1 M KCl in the presence of detergent at 0°C or with 1% Triton X-100 alone at 37°C achieved almost complete solubilization of the labeled polypeptide, whereas approximately one-third was solubilized by incubation with 0.1 mM EDTA (pH 7.6) at 37°C. A similar pattern of solubilization was observed when rat erythrocyte or reticulocyte ghosts were studied.

Binding of the Solubilized M_r 45,000 Polypeptide to Erythrocyte Inside-Out Vesicles. A binding assay was developed to investigate directly the putative interaction of the N-protein with other plasma membrane components. The detergent-solubilized protein containing the labeled M_r 45,000 polypeptide could bind to rat erythrocyte inside-out vesicles (Table 1), provided that the final concentration of detergent was <0.15%. The binding required divalent cations and was decreased significantly in the presence of a 4-fold excess of unlabeled crude M_r 45,000 polypeptide. Resealed erythrocyte ghosts failed to

Table 1. Binding of labeled M_r 45,000 polypeptide to rat erythrocyte membrane preparations

Membrane preparation	Addition	Binding, fmol/mg membrane protein
Resealed ghosts	10 mM $MgCl_2$	20
Inside-out vesicles	10 mM $MgCl_2$	230
Inside-out vesicles	10 mM $MnCl_2$	330
Inside-out vesicles	5 mM EDTA	45
Inside-out vesicles	5 mM EDTA + 0.1 M NaCl or 0.1 M KCl	60
Trypsinized inside-out vesicles	10 mM $MgCl_2$	80
Inside-out vesicles	10 mM $MgCl_2$ supernatant of trypsinized vesicles	125
Inside-out vesicles	10 mM $MgCl_2$ + unlabeled crude M_r 45,000 polypeptide extract	95
Inside-out vesicles	10 mM $MgCl_2$ + heat-denatured unlabeled M_r 45,000 polypeptide extract	180

The reaction mixture of the binding assay contained 80–170 μg of rat erythrocyte membrane protein and 120 fmol of labeled M_r 45,000 polypeptide in 200 μl of 50 mM Tris·HCl, pH 7.6/0.08% Triton X-100. The labeled M_r 45,000 polypeptide was solubilized from pigeon erythrocyte plasma membranes with 0.5% Triton X-100 at 0°C. The reaction mixture was incubated at 23°C for 30 min, and then chilled and centrifuged at 36,000 × g_{max} over 15% sucrose in a Microfuge tube (14). The resulting pellets were subsequently assayed for the amount of bound M_r 45,000 polypeptide. Treatment of the inside-out vesicles with trypsin was performed by exposing the vesicles (2.8 mg of protein per ml) to trypsin at 10 μg/ml for 15 min at 0°C; the proteolysis was stopped by adding soybean trypsin inhibitor. Trypsin that had been preincubated with soybean trypsin inhibitor was used in a control incubation. The soluble (160,000 × g_{max} supernatant) products of the trypsin treatment were used to block the binding of the M_r 45,000 polypeptide. Under the conditions of the assay, the binding was complete after 20 min at 23°C. The binding was also linear with respect to the amount of rat erythrocyte membrane vesicles used (80–170 μg) and with respect to the amount of labeled M_r 45,000 polypeptide (50–150 fmol). The concentration of the unlabeled M_r 45,000 polypeptide used for displacement (last two lines) was 4 times greater than the concentration of the labeled polypeptide.

Table 2. Effect of cations and activators on detergent solubilization of the labeled M_r 45,000 polypeptide

Agent added	Polypeptide solubilized, % of control
50 mM Tris·HCl at pH 7.6 (control)	100
0.1 M NaCl	103
0.1 M KCl	107
5 mM $MgCl_2$	97
20 mM $MgCl_2$	85
50 mM $MgCl_2$	55
5 mM $MnCl_2$	70
20 mM $MnCl_2$	8
50 mM $CaCl_2$	38
12 mM NaF	155
100 μM p[NH]ppG	165

ADP-ribosylated pigeon erythrocyte plasma membranes were suspended in 50 mM Tris·HCl (pH 7.6) at 3.5 mg of protein per ml. The membranes were exposed to divalent cations in the presence of a reducing agent (7) at 23°C for 15 min. Other membrane samples were pretreated with 100 μM p[NH]ppG or 12 mM NaF for 15 min at 37°C in the presence of 5 mM $MgCl_2$. Then the various membrane samples were chilled and extracted with 3 vol of buffer containing 2.0% Triton X-100 and the corresponding concentration of the agent with which the membranes had been pretreated. The samples were centrifuged at 160,000 × g_{max} for 10 min in a chilled Beckman Airfuge, and the supernatants were assayed for the labeled M_r 45,000 polypeptide.

display any significant binding. Mild treatment of the inside-out vesicles with trypsin abolished more than half of the binding. Concomitantly, trypsin digestion generated a factor from the inside-out vesicles which appeared to block appreciably the binding of the ADP-ribosylated M_r 45,000 polypeptide to fresh, untreated vesicles. This factor was different from the M_r 45,000 polypeptide itself because similar trypsin treatment of ADP-ribosylated inside-out vesicles failed to release the labeled polypeptide.

Assessment of the true number of binding sites on the inside-out vesicles was not feasible because of the presence of endogenous M_r 45,000 polypeptide. The exogenously added, labeled polypeptide might exchange with the endogenous pool or the number of binding sites might exceed the amount of the endogenous M_r 45,000 polypeptide. Furthermore, the labeled polypeptide could bind directly to the inside-out vesicles or the binding might require another component of the N-protein of which the M_r 45,000 polypeptide was a subunit (15).

Regulation of the Plasma Membrane Associations of the M_r 45,000 Polypeptide. The previous experiments provided evidence for the interaction of the N-protein with other (possibly cytoskeletal) protein(s) on the inner aspect of the plasma membrane. Therefore, it was of interest to study the effects of modulators of adenylate cyclase activity on this interaction. Relatively high concentrations of divalent cations decreased the solubilization of the M_r 45,000 polypeptide from membranes by Triton X-100 (Table 2). $MnCl_2$ was more effective than $MgCl_2$ or $CaCl_2$. Moreover, $MnCl_2$, but not $MgCl_2$ or $CaCl_2$, diminished by 50% the maximal amount of band 3 solubilized by Triton X-100. These effects were reversed by washing away the divalent cations prior to detergent extraction. p[NH]ppG and NaF promoted the release of the M_r 45,000 polypeptide into the detergent supernatant. The effect of GTP was similar to that of p[NH]ppG, but ATP, GDP and GMP did not alter the pattern of solubilization. Similarly, 10 μM *l*-isoproterenol and 10 μM *l*-propranolol were without effect. The maximal response to p[NH]ppG occurred after 3 min at 37°C and it was readily reversed by washing away the nucleotide prior to detergent treatment.

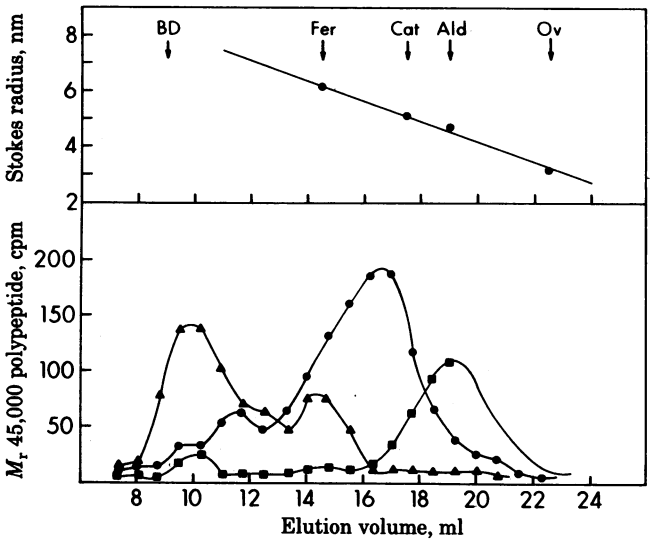

FIG. 3. Ultrogel AcA-34 fractionation of macromolecules containing the labeled M_r 45,000 polypeptide. ADP-ribosylated pigeon erythrocyte plasma membranes (2.9 mg of protein/ml) were extracted with 3 vol of 2.0% Triton X-100/50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$ at 0°C for 30 min. The 300,000 × g_{max} supernatant was saved and the pellet was extracted in 3 vol of 1.0% Triton X-100/50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$ for 30 min at 37°C and the 300,000 × g_{max} supernatant was obtained. The 0.1 mM EDTA extract was prepared by washing 1 ml of the membrane suspension three times with 10 ml of chilled 0.1 mM EDTA (pH 7.6) followed by incubation in 3 ml of 0.1 mM EDTA (pH 7.6) at 37°C for 30 min and the 300,000 × g_{max} supernatant was separated; 0.5-ml samples of the various supernatants were applied to 26-ml AcA-34 columns and 0.8-ml fractions were collected to be analyzed for their content of labeled M_r 45,000 polypeptide. ●, Triton X-100 at 0°C; ▲, Triton X-100 at 37°C; ■, 0.1 mM EDTA. BD, blue dextran 2000; Fer, ferritin; Cat, catalase; Ald, aldolase; Ov, ovalbumin.

Association of the M_r 45,000 Polypeptide with Different Molecular Complexes. We separated different molecular species containing the labeled M_r 45,000 polypeptide. Both gel permeation chromatography (Fig. 3) and sucrose density gradient velocity sedimentation (Fig. 4) revealed three such mo-

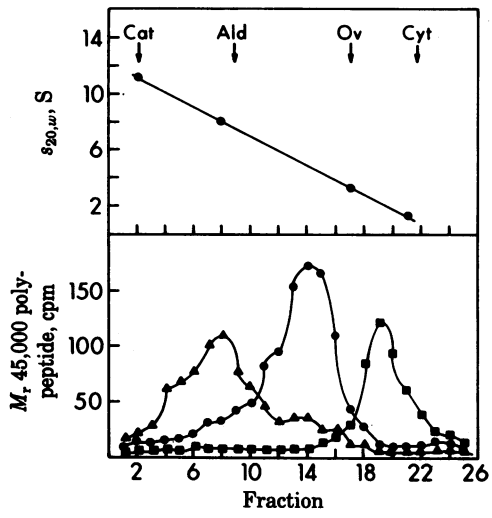

FIG. 4. Sucrose density gradient velocity sedimentation of macromolecules containing the labeled M_r 45,000 polypeptide. The supernatants were prepared as in Fig. 3. The bottom of the gradient is to the left. ●, Triton X-100 at 0°C; ▲, Triton X-100 at 37°C; ■, 0.1 mM EDTA at pH 7.6. Cat, catalase; Ald, aldolase; Ov, ovalbumin; Cyt, cytochrome *c*.

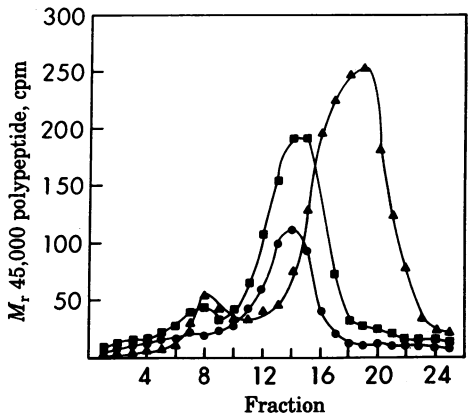

FIG. 5. Effect of p[NH]ppG (▲) and NaF (■) on the sedimentation rate of the labeled M_r 45,000 polypeptide in sucrose density gradients. ADP-ribosylated pigeon erythrocyte plasma membranes (3.5 mg of protein per ml) were pretreated with 100 μM p[NH]ppG or 12 mM NaF and were extracted with Triton X-100 as in Table 1. Sedimentation and sample analysis were performed as in Fig. 4. ●, Control.

lecular complexes. The Stokes radii and the sedimentation coefficients were a function of the method of solubilization. Extraction of detergent shells with Triton X-100 at 37°C released the largest macromolecular complex; treatment of the plasma membranes with 0.1 mM EDTA (pH 7.6) at 37°C produced the smallest. Solubilization of plasma membranes with Triton X-100 at 0°C resulted in the release of an intermediate-size molecular species. This material was also produced by treatment with cholate or 1 M KCl in the presence of Triton X-100 at 0°C. Incubation of the material corresponding to the intermediate-size peak at 37°C for 30 min did not yield either of the the other two peaks, implying that the differences in the molecular complexes were not due solely to differences in the temperature of extraction. p[NH]ppG, but not NaF, treatment followed by detergent extraction induced a shift to a molecular form with a smaller sedimentation coefficient (Fig. 5). A similar p[NH]ppG-induced shift in sedimentation properties has been noted by others (2, 3). A 26-ml AcA-34 column was used to characterize this p[NH]ppG effect further. Solubilization of p[NH]ppG-pretreated membranes with Triton X-100 at 0°C produced a labeled molecular entity that eluted 1.6 ml after the labeled material solubilized from control membranes (data not shown).

DISCUSSION

The development of specific reconstitution (16–19) and labeling (8, 18, 20) techniques has resulted in a better understanding of the adenylate cyclase system. We had previously detected both a particulate and a soluble guanyl nucleotide-binding protein (N-protein) which mediated the activation of adenylate cyclase by guanyl nucleotides in several rat tissues (16, 17). In addition, we also demonstrated a reversible interaction between this protein and p[NH]ppG, leading to a conformational change in the N-protein which altered its sensitivity to proteolysis and thermal inactivation (17). This activated N-protein formed a high-affinity complex with a catalytic component, resulting in the activation of adenylate cyclase (17). Affinity chromatography (18) and somatic cell variants (19) have also been used to isolate and characterize the N-protein. Moreover, a multisubunit form of the protein (M_r 130,000) has recently been detected (21) and purified (15). In this communication we characterized the interaction of the N-protein with other membrane components and provide evidence for the possible functional significance of these interactions.

Several findings suggest a functional interaction between the N-protein and the cytoskeleton: (*a*) the protein is associated with Triton and digitonin shells and is solubilized with 0.1 mM EDTA; (*b*) cholate and 1 M KCl (in the presence of Triton X-100), which solubilize the M_r 45,000 polypeptide efficiently, are also known to solubilize certain cytoskeletal components (22, 23); (*c*) the N-protein has been shown to bind detergent poorly and to be largely exposed to the inner aspect of the plasma membrane (21, 24). Furthermore, the present results provide evidence for the occurrence of a specific proteinaceous site on the cytoplasmic surface of erythrocyte plasma membranes which binds the N-protein in a divalent cation-dependent reaction. This binding site may be involved in the association between the N-protein and the cytoskeleton.

The cytoskeletal interactions of the N-protein seem to correlate with its state of activation. The effects of p[NH]ppG, GTP, and NaF are consistent with a weakening of this interaction. In contrast, $MnCl_2$, which uncouples the N-protein from the catalytic component (7), promotes this interaction. The aforementioned uncoupling may also be due to the effect of $MnCl_2$ on other membrane proteins which are also rendered more resistant to detergent solubilization by $MnCl_2$.

The separation of three molecular species that contain the ADP-ribosylated M_r 45,000 polypeptide indicates that this polypeptide is a constituent of a multicomponent molecular complex. The largest molecular form derived from Triton shells may include one or more cytoskeletal elements. The intermediate molecular entity appears to be similar in size to that reported earlier (21) and purified subsequently (15). p[NH]ppG seems to facilitate the dissociation of the cytoskeletal complex and the intermediate molecular species, resulting in a smaller molecular entity. This dissociative reaction may be required for the effect of p[NH]ppG on the affinity of several hormone receptors (25) and on the activity of adenylate cyclase. Two recent reports indicate that the molecular weight of adenylate cyclase increases by about 40,000 (26) or 50,000 (27) after its activation by p[NH]ppG. This may be due to the binding of a smaller p[NH]ppG-dependent activated form of the N-protein to the catalytic component of adenylate cyclase. The same molecular change of the N-protein may also account for its altered sensitivity to proteolysis and thermal inactivation in the presence of p[NH]ppG (17, 28). The recent determination of the subunit structure of the N-protein (15) should facilitate further elucidation of these molecular changes.

Based on the foregoing discussion, the following sequence of reactions that leads to the activation of adenylate cyclase by p[NH]ppG is suggested:

$$i.\quad N\text{–}M + G \rightarrow G\text{–}N' + M$$

$$ii.\quad G\text{–}N' + E \rightarrow G\text{–}N'\text{–}E$$

$$iii.\quad G\text{–}N'\text{–}E + M \rightarrow G\text{–}N'\text{–}E\text{–}M.$$

The N-protein (N) is bound to the cytoskeletal matrix (M). p[NH]ppG (G) binds to N and releases it from M, which leads to the formation of a smaller, activated form of N (G–N'). The G–N' complex dissociates readily after the removal of free G (17). However, G–N' binds tightly to the catalytic component of adenylate cyclase (E) to produce the active state of the enzyme (17). The velocity sedimentation and gel permeation results indicate that N' is larger than the M_r 45,000 polypeptide. Accordingly, it is possible that N' dissociates further to the M_r 45,000 polypeptide prior to interacting with the catalytic component. This possibility is consistent with the observed change in the size of adenylate cyclase after its activation by p[NH]ppG (26, 27). In addition to its role in activating adenylate cyclase,

G–N′ may also be the entity responsible for the effect of guanyl nucleotides on receptor affinity. Furthermore, the activated enzyme binds to M to form a relatively high-affinity complex (6). This may serve to sequester an active enzyme pool or it may play a role in the inactivation limb (not shown).

Molecular interactions involving the cytoskeleton also appear to regulate the β-adrenergic receptor. We recently found an association between a subpopulation of this receptor and digitoning shells of rat erythrocytes (unpublished data). Moreover, agonists, but not antagonists, seem to release the receptor from the cytoskeletal constraints, suggesting that this reaction may be required for hormone action (29). Thus, activation of both the N-protein and the β-adrenergic receptor may involve the dissociation of each of these components from its cytoskeletal binding site.

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