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Reconstitution of catecholamine-sensitive adenylate cyclase activity: Interaction of solubilized components with receptor-replete membranes

(S49 lymphoma/L cell/complementation *in vitro*/somatic cell variants)

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ABSTRACT **Membranes of mouse L cells that contain adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] but lack β-adrenergic receptors have been solubilized with Lubrol 12A9. Addition of such adenylate cyclase-containing extracts to β-adrenergic receptor-replete membranes from adenylate cyclase-deficient S49 lymphoma cells results in the production of a catecholamine-sensitive adenylate cyclase system. The effects of β-adrenergic agonists and antagonists on the reconstituted system reproduce those that are characteristic of the wild-type S49 lymphoma cell. The uncoupled variant of the S49 lymphoma contains adenylate cyclase, but donor extracts from this clone fail to reconstitute the hormone-sensitive enzyme activity when added to adenylate cyclase-deficient membranes. Thus, the uncoupled and adenylate cyclase-deficient variants of the S49 cell are not complementary.**

Many actions of catecholamines, prostaglandins (PGs), and polypeptide hormones result from their effects on the intracellular concentration of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in target cells. The initial step in these processes seems to be the binding of hormone to a specific receptor on the outer surface of the plasma membrane; unknown intermediate events then result in the stimulation of the activity of the enzyme adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. Adenylate cyclase and hormone binding activities appear to reside on separate, integral membrane proteins. We have recently reviewed the interaction of the β-adrenergic (catecholamine) receptor and adenylate cyclase (1), and available data indicate that these two entities can be resolved genetically (2, 3) or physically (4, 5).

Given the independence of hormone receptor and adenylate cyclase, the mechanism of their interaction within the membrane becomes a major question. Unfortunately, purified preparations of either receptors or enzyme are not readily available for study. In addition, it is likely that there are one or more "coupling factors" involved in the process—i.e., components that neither bind hormone nor catalyze the formation of cyclic AMP but that are required for the proper interaction of receptor and enzyme or for the regulation of catalytic activity (3, 6–8). Even if all the purified protein components of the system were available, it further seems likely that they would have to be properly integrated into a suitable membrane in order to interact correctly, because hormone responses are lost upon solubilization of the plasma membrane or perturbation of the membrane lipids (9–12).

We are attempting to study the mechanism of regulation of catecholamine-sensitive adenylate cyclase by the stepwise resolution of its components from intact membranes. To investigate the role of these components, we hope to assay them by the reconstitution of hormone-sensitive adenylate cyclase activity in membranes that have been depleted of one or more factors by genetic or chemical manipulation. Here we report on a procedure for the solubilization of adenylate cyclase components from membranes of one cell type and their reconstitution with membranes from other cells. These experiments demonstrate that the β-adrenergic receptor in the acceptor membranes can modulate the activity of at least one component, hypothetically the adenylate cyclase, from the donor extract.

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MATERIALS AND METHODS

Cell Lines and Growth Conditions. The origins and descriptions of all cell lines used in this study have appeared previously. Wild-type S49 murine lymphoma cells (13) and an adenylate cyclase-deficient variant strain (AC^-) (14) were obtained from H. Bourne and P. Coffino, University of California, San Francisco. Another S49 variant denoted UNC (uncoupled) retains adenylate cyclase and the β-adrenergic receptor, but hormone binding does not stimulate enzyme activity (3). The murine clone designated as B82 is a bromodeoxyuridine-resistant clone of L cells; it has been characterized with regard to cyclic AMP metabolism in this laboratory (15, 16). S49 cells were grown in suspension as described (17), and B82 cells were grown in suspension in spinner-modified Eagle's medium containing 5% heat-inactivated horse serum.

Isolation and Solubilization of Plasma Membranes. S49 cell plasma membranes were isolated as described (17). This procedure yields a relatively pure preparation in which exogenous purine nucleotides (e.g., GTP) are required for hormonal stimulation of adenylate cyclase activity (17). This procedure was also used for B82 cells, but it yields membranes contaminated by some mitochondria and only partially dependent on added nucleotides for hormonal stimulation. Adenylate cyclase was solubilized from cell membrane preparations by suspension in 2.5 mM Na *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (Hepes)/0.25 mM $MgCl_2$/0.125 mM EDTA/0.7% Lubrol 12A9/0.5 mM GTP, pH 8.0. After 60 min at 4°, the suspension was centrifuged for 30 min in a Spinco 50Ti rotor (maximum relative centrifugal force = 104,000 × *g*).

Reconstitution of Catecholamine-Sensitive Adenylate Cyclase. Appropriate detergent extracts were added slowly (2–5 min) to suspensions of acceptor membranes and stirred in

Abbreviations: PG and PGE_1, prostaglandin and prostaglandin E_1, respectively; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; AC^-, adenylate cyclase-deficient phenotype; UNC, uncoupled phenotype; B82, bromodeoxyuridine-resistant clone of L cells; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; INE, (−)-isoproterenol.

Table 1. Recovery of activities on reconstitution in AC⁻ membranes*

Source of donor extract	Preparation	Adenylate cyclase activity, pmol/min per mg original donor protein				
		Gpp(NH)p	NaF	GTP	GTP + INE	GTP + PGE_1
B82	Donor membranes	76	83	11	12	107
	Membranes + detergent	41	28	5	5	6
	Donor extract	49	28	4	5	6
	Reconstituted system	160	116	14	41	16
S49, wild-type	Donor membranes	281	338	38	326	ND†
	Membranes + detergent	336	182	19	21	ND
	Donor extract	316	170	20	20	ND
	Reconstituted system	514	460	39	105	ND
S49, UNC	Donor membranes	57	160	11	11	ND
	Membranes + detergent	61	37	5	4	ND
	Donor extract	67	44	5	5	ND
	Reconstituted system	160	136	16	16	ND

ND, not determined.

* Donor membranes (with or without detergent) and donor extracts were assayed after dilution into 20 mM NaHepes/2 mM $MgCl_2$/1 mM EDTA, pH 8.0, according to the protocol for reconstitution, except that AC⁻ membranes were included only where "reconstituted system" is indicated. Enzyme activity per mg of original donor protein is given; the percentages of membrane protein in the donor extracts were: B82, 30%; wild-type, 34%; and UNC, 33%. Concentrations of effectors were Gpp(NH)p or GTP, 100 μM; NaF, 10 mM; INE, 1 μM; and PGE_1, 10 μM.

20 mM NaHepes/2 mM $MgCl_2$/1 mM EDTA, pH 8.0; the acceptor membrane concentration was usually about 0.5–1.0 mg/ml. The mixtures were used after incubation for at least 20 min at 4°.

Other Procedures. Adenylate cyclase activity was measured exactly according to Ross *et al.* (17), and the product was isolated by the method of Salomon *et al.* (18). The identity of the reaction product as cyclic [^{32}P]AMP was confirmed further by chromatography on Dowex 1-(Cl), on cellulose thin-layer plates in chloroform/methanol/concentrated NH_4OH (2:2:1, vol/vol) (19), and in isobutyric acid/2 M NH_4OH (2:1, vol/vol) (20). Protein was assayed, following trichloroacetic acid precipitation of samples, by the method of Lowry *et al.* (21). Gel exclusion chromatography and sucrose density gradient centrifugation were performed as described by Haga *et al.* (5), except that 0.1 M NaCl was omitted from the density gradient solutions.

Materials. Lubrol 12A9, $(ethylene\ oxide)_{9.5}$ (dodecyl, tetradecyl)ether, equivalent to Lubrol PX, was a gift of I.C.I., Ltd. It was deionized with Dowex 501 before use. The sources of other reagents have been listed (15, 17).

RESULTS

Cell clones with presumably complementary phenotypes were selected as candidates for attempts to reconstitute a response to hormone by interaction between solubilized factors and appropriately depleted acceptor membranes. Adenylate cyclase from wild-type S49 lymphoma was sensitive to β-adrenergic agonists and to PGE_1 (17) (Table 1). The AC⁻ S49 variant served as an acceptor membrane that was depleted of adenylate cyclase catalytic activity. This clone does, however, contain β-adrenergic receptors (2). The ideal hypothetical complement would be a β-adrenergic receptor-deficient S49 cell. Because this variant has yet to be isolated, B82 was chosen as a substitute. It is a murine clone with an active PG-sensitive adenylate cyclase system; however, it failed to respond to β-adrenergic agonists (16) (Table 1) and it does not contain binding sites characteristic of β-adrenergic receptors (15). UNC cells have both β-adrenergic receptors and adenylate cyclase, but enzyme activity is not sensitive to β-adrenergic agonists (3). The nature of the lesion is unknown. The NaF-stimulated adenylate cyclase activity of this clone is similar to that of wild-type cells, but the guanyl-5′-yl imidodiphosphate [Gpp(NH)p]-stimulated activity appears to be somewhat reduced (3).

When plasma membranes from wild-type S49 lymphoma cells or from B82 fibroblasts were exposed to the nonionic detergent Lubrol 12A9, adenylate cyclase lost its responsiveness to stimulatory hormones. Enzyme activity measured in the presence of GTP (equivalent to basal activity) or NaF was decreased considerably under this condition, as was the Gpp(NH)p-stimulated activity from B82 cells (Table 1). With all clones, the activity that remained after detergent treatment could be recovered completely in a 100,000 × *g* supernatant. The adenylate cyclase in the supernatant appeared to be truly soluble, because it behaved as a monodisperse species when studied by sucrose density gradient centrifugation and gel exclusion chromatography (data not shown). Solubilization of S49 adenylate cyclase under the conditions in *Materials and Methods* yielded a preparation similar to that described by Haga *et al.* (5), whereas the B82 cell enzyme appeared to have a slightly higher sedimentation coefficient.

Reconstitution of Hormone-Stimulated Adenylate Cyclase Activity. Once membranes had been solubilized and responses to hormones had been lost, we were unable to restore the response by removal of the detergent, with or without concomitant addition of phospholipids. However, as shown in Fig. 1, the addition of a detergent extract of B82 membranes (which never responded to catecholamines) to a suspension of plasma membranes from the AC⁻ S49 cell yielded a preparation that was stimulated up to 6-fold by (−)-isoproterenol (INE). The formation of this responsive system was dependent on both the amount of B82 extract and the amount of AC⁻ membranes used in the initial mixture. The acquisition of the response was also time-dependent, although at 4° the process was nearly complete in 10 min and an accurate time course has been difficult to determine.*

The hormone response of the reconstituted system meets the pharmacological criteria of agonist and antagonist specificity that are characteristic of the β-adrenergic receptor (Fig. 2). Both

* Despite the fact that AC⁻ membranes may have receptors for PGE_1 (16), this response is not restored in the reconstituted system. This is due at least in part to the extreme sensitivity of this response to the presence of detergent, presumably because of interaction between PGE_1 and detergent micelles.

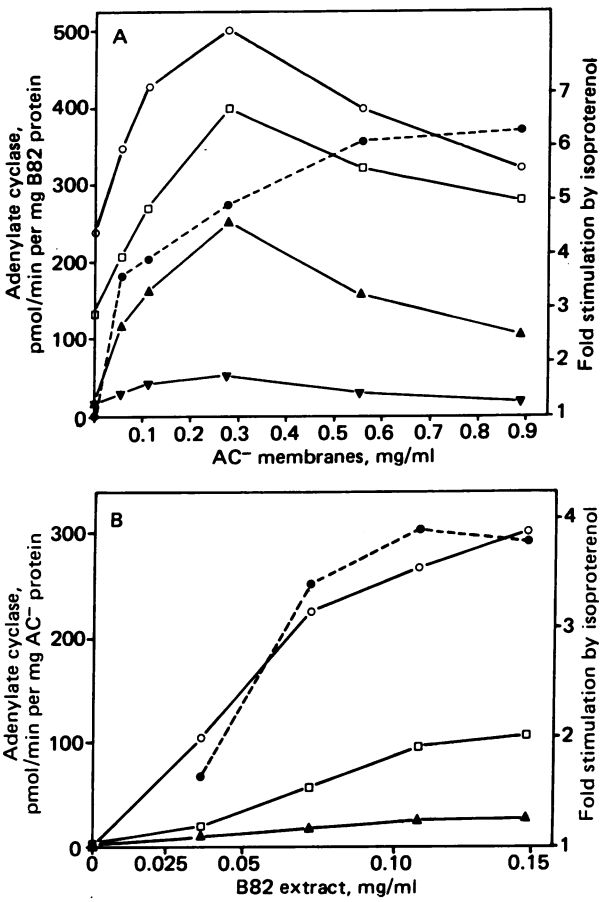

FIG. 1. Dependence of enzymatic activities on the concentrations of donor extract and acceptor membranes. (*A*) Adenylate cyclase was assayed in the presence of 100 μM GTP (▼), 100 μM GTP + 1 μM (−)-INE (▲), 10 mM NaF (□), or 100 μM Gpp(NH)p (○). Stimulation by INE over that observed with GTP alone is also shown (●) (scale on right). Aliquots of Lubrol supernatant from B82 membranes (35 μl; 2.2 mg/ml) were added to suspensions of increasing concentrations of AC⁻ membranes in 400 μl of 20 mM NaHepes/2 mM $MgCl_2$/1 mM EDTA, pH 8.0. (*B*) Increasing volumes of B82 extract (2.2 mg/ml) were added to 300 μl of AC⁻ membranes (1.3 mg/ml). Component concentrations are as in *A*. Symbols: GTP (▲); GTP + INE (□); NaF (○); and stimulation by INE over that observed with GTP alone (●). Concentrations shown on the abscissa are those in the enzyme assay and represent a 1:2.5 dilution of the reconstituted mixture.

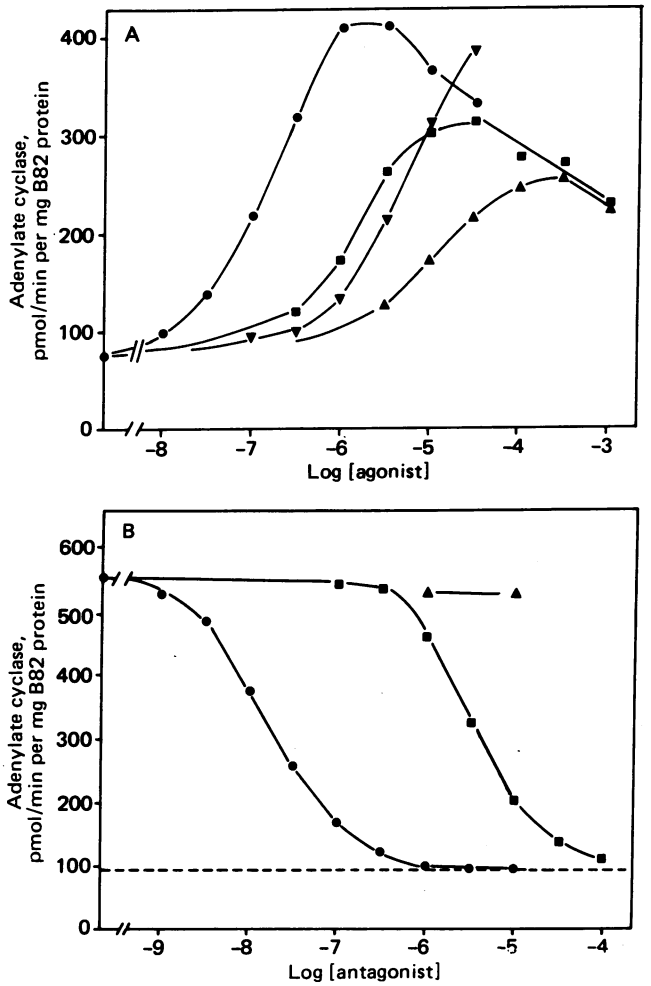

FIG. 2. Concentration dependence for effects of agonists and antagonists on the reconstituted B82–AC⁻ adenylate cyclase system. (*A*) Response to agonists in the presence of 100 μM GTP. Symbols: (▲) (−)-Norepinephrine; (▼) terbutaline; (■) (+)-INE; and (●) (−)-INE. (*B*) Effect of antagonists on the response to 1 μM (−)-INE + 100 μM GTP. Symbols: (- - -) Basal (GTP); (●) (−)-propanolol; (■) (+)-propanolol; (▲) phentolamine.

(−)-INE and the noncatechol agonist terbutaline stimulated enzyme activity with a concentration dependence similar to that observed in wild-type S49 cells (17). The (+)-isomer of INE was characteristically less potent than the (−)-isomer, and it also seemed to act only as a partial agonist. (−)-Norepinephrine was a partial agonist in this system and displayed the same $K_{activity}$ in the reconstituted system as it did in wild-type membranes. Stereoselectivity for antagonists was also observed (Fig. 2*B*); (−)-propranolol had a K_I of 1.3 nM, equal to its K_D for binding to the β-adrenergic receptor (17), and the (+)-isomer was about 1% as potent. The α-adrenergic antagonist phentolamine was without effect.

Because B82 cells have neither a catecholamine-responsive adenylate cyclase system (16) nor a ligand-binding activity characteristic of the β-adrenergic receptor (15) and because AC⁻ S49 cells lack any detectable adenylate cyclase activity (2, 14), it seems plausible that the reconstituted system just described represents the interaction of adenylate cyclase from the donor extract with β-adrenergic receptors in the acceptor membranes. However, other interpretations will be discussed below. It is consistent with this proposal that a wild-type S49 cell membrane extract can also serve as an apparent donor of catecholamine-sensitive adenylate cyclase activity to an AC⁻ acceptor membrane (Table 1). Because we have not yet been able to restore hormone-stimulated activity to B82 membranes with receptor-containing soluble extracts from wild-type, AC⁻, or UNC cells (data not shown), it seems unlikely that wild-type soluble extracts are contributing both receptor and enzyme to AC⁻ membranes to reconstitute the response to INE.

The need for an extract containing active adenylate cyclase is supported by other control experiments. B82 or wild-type S49 plasma membranes treated with *N*-ethylmaleimide to inactivate their adenylate cyclase (22) could no longer function as donors (data not shown). Nor did the introduction of buffer containing the detergent and GTP to AC⁻ membranes activate any masked endogenous activity. However, these data do not prove that the adenylate cyclase of the soluble extract is necessary or sufficient, and data obtained with soluble extracts of UNC plasma membranes, which contain both Gpp(NH)p- and NaF-stimulated adenylate cyclase activity, indicated that the situation was more complex. When UNC extract and AC⁻ membranes were combined, the resulting mixture *failed* to display catecholamine-stimulated adenylate cyclase activity

(Table 1). By this criterion, UNC and AC$^-$ variants are not complementary.

Similarly, a β-adrenergic receptor-replete acceptor membrane appears to be required for the reconstitution of a catecholamine-responsive enzyme. The use of β-adrenergic receptor-deficient B82 membranes or of heat-treated AC$^-$ membranes was unsuccessful, as was the simple dilution of the donor extract (B82 or wild-type S49) into buffer or buffer containing bovine serum albumin or polystyrene beads (to adsorb excess detergent) (data not shown). We have also noted that treatment of acceptor membranes with *N*-ethylmaleimide abolished the restoration of hormone response, although the binding of the β-adrenergic ligand [^{125}I]iodohydroxybenzylpindolol was unaltered.

Effect of Reconstitution on Hormone-Independent Adenylate Cyclase Activity. Fig. 1*A* indicates that the reconstitution procedure also appeared to increase the "total activity" of adenylate cyclase in the system [assayed in the presence of NaF or Gpp(NH)p]. This activation required a suitable acceptor membrane: a heat-treated or *N*-ethylmaleimide-treated membrane was relatively ineffective.

Other characteristics of this phenomenon are evident in Table 1. With B82 as the donor, NaF- and Gpp(NH)p-stimulated activities decreased when membranes were exposed to detergent. Upon reconstitution, the total NaF-stimulated activity returned to a level near that of the donor membrane. The Gpp(NH)p-stimulated activity also declined upon solubilization, but the reconstituted system contained activity in excess of the starting material. With S49 lymphoma donors, the pattern with NaF was similar; the Gpp(NH)p-stimulated activity appeared not to decline upon solubilization, and the level of reconstituted activity was clearly in excess of that of the donor membranes.

These phenomena will be discussed below, but a crucial point can be made at this time. The increase in total activity *cannot* account for the reconstitution of the hormone response that is observed. Thus, a similar increase in total activity was seen when UNC extract was added to AC$^-$ membranes, but, as mentioned, there was no hormone response with this combination (Table 1). The two phenomena are thus dissociated.

Physical Nature of the Reconstituted System. We are unable as yet to demonstrate the stable attachment of the adenylate cyclase activity in donor extracts to the acceptor membranes. As shown in Table 2, centrifugation of the reconstituted system yielded a largely enzyme-free pellet and a hormone-unresponsive but enzymically active supernatant. The increases in NaF- and Gpp(NH)p-stimulated activities that were seen when extract was combined with membranes were lost when membranes and supernatant were separated. The recombination of these two fractions restored some of the hormone response that was originally observed and restored the enhanced total catalytic activity. These experiments rule out the possibility that the donor extract has caused a stable activation of a latent adenylate cyclase in the acceptor membrane.

Table 2. Centrifugation of the reconstituted system*

Fraction	Adenylate cyclase activity, pmol/min per ml†			
	NaF	Gpp(NH)p	GTP	GTP + INE
Total mixture	45	70	9	24
Supernatant	14	27	2	2
Pellet	4	6	1	2
Pellet + supernatant	32	61	6	12

* AC$^-$ membranes were reconstituted with B82 donor extract as described under *Materials and Methods*. Two aliquots were centrifuged for 20 min at 81,000 × *g*. Supernatant and pellet (resuspended in an equal volume of 20 mM NaHepes/2 mM $MgCl_2$/1 mM EDTA, pH 8.0) from one tube were assayed separately. In the other, the pellet was resuspended in its own supernatant.

† Activity is expressed relative to the volume of original reconstituted mixture used.

DISCUSSION

Because of the difficulties encountered in attempts to purify adenylate cyclase and ignorance of what components may make up the hormone-sensitive enzyme complex, many investigators have directed their efforts toward the analysis of partially fractionated systems or to the study of partial activities thought to make up that complex. For example, Pfeuffer and Helmreich (7) have described a guanine nucleotide-binding protein thought to be related to the activity of adenylate cyclase, and Cassel and Selinger (8) have studied a catecholamine-stimulated GTPase which could reflect an activity of the same protein. In another approach, Orly and Schramm (23) are attempting to classify the components of the adenylate cyclase system by analysis of heterokaryons formed between cells with different lesions in enzyme activity.

We have begun an analysis of the components of hormone-sensitive adenylate cyclase by the development of a reconstituted system in which components solubilized from an enzyme-containing membrane can interact with a β-adrenergic receptor-replete membrane to yield catecholamine-stimulated enzyme activity. The procedure described herein is reproducible, and a relatively large proportion of the adenylate cyclase in the mixture appears to be available for regulation by receptor. Approximately 30 experiments have been performed without a failure to create a hormone response; the extent of the response has varied from about 3-fold to 6-fold. The system should be useful in the complementation analysis of different cells and, more importantly, should give us a starting point for stepwise dissection of various adenylate cyclase components.

It is necessary, however, to consider critically the mechanism by which the reconstitution occurs. The data of Figs. 1 and 2 provide strong evidence for the formation of adenylate cyclase activity subject to true β-adrenergic stimulation. In the simplest case, in which a B82 extract is added to membranes of AC$^-$ lymphoma cells, it is plausible to assume that B82 adenylate cyclase is influenced by the β-adrenergic receptors in the AC$^-$ membranes. This assumption rests largely on the phenotypes of the cells involved: B82 cells show no hint of a catecholamine response or any ligand-binding activity characteristic of the β-adrenergic receptor, whereas AC$^-$ cells retain the β-adrenergic receptor but lack any measurable adenylate cyclase activity. A similar mechanism could apply for the addition of a wild-type S49 extract to AC$^-$ membranes.

This simple mechanism may be correct but seems to be insufficient to deal with all the data. Extracts of membranes from the UNC variant do not complement AC$^-$ even though they contain active adenylate cyclase, suggesting that B82 and wild-type S49 membranes contain some factor necessary for the coupling of hormone binding and enzyme activation. One would then assume that AC$^-$ cells lack this coupling factor (or factors) in addition to one or more components of the catalytic machinery of adenylate cyclase. We find the possibility provocative that the loss of a coupling factor may be causally related to the basic AC$^-$ lesion, perhaps, for example, because of failure of a coupling factor to bind appropriately to a truly AC$^-$ membrane.

A second complicating feature of the reconstituted system lies in the modulation of hormone-independent enzyme ac-

tivities during solubilization and reconstitution. Enzyme activity measured in the presence of either NaF or Gpp(NH)p increases when B82 or wild-type S49 extracts are added to AC^- membranes (Table 1 and Fig. 1A). It is conceivable that the increase in activity is in fact due to the activation of inactive enzyme in the AC^- membranes by other necessary components in the donor extract. However, this cannot be a stable activation (Table 2). In the most critical analysis, we must state our ignorance of the number of discrete components necessary for the expression of enzyme activity (subunits, activators, etc.) and say that we cannot be sure if we are adding the catalytic moiety or some equally necessary entity. Our development of this system should allow us to attack questions such as these.

An alternative and more attractive explanation for the increase in hormone-independent activity in the reconstituted system lies in a probable increase in donor enzyme activity when it is recombined with proteins and/or lipids in S49 membranes. This explanation is at least consistent with the similarity of the NaF-stimulated activities in the reconstituted system and in the original membranes from which the donor extracts were prepared. One must also remember that during the reconstitution procedure the concentration of detergent and its location (in detergent micelles, in detergent–lipid mixed micelles, and dissolved in membranes) change drastically. Nonionic detergents can activate adenylate cyclase activity up to 4-fold or can completely inhibit the enzyme, depending on such variables as detergent concentration, detergent–protein ratio, other activators, or ionic strength. The Gpp(NH)p-stimulated activity is particularly difficult to evaluate. This activity is prone to marked stimulation by detergent, is not a linear function of time, and likely results from an interaction between proteins (7), certain of which may be in the extract and certain of which may be in the membrane. Careful accounting of total activities may be foolhardy under these circumstances, or perhaps even impossible. In any event, it can be noted again that this increase in hormone-independent activity has been dissociated from the reconstitution of the hormone response by the use of an UNC donor extract (Table 1).

The physical state of the reconstituted system poses another question of mechanism. Clearly, some sort of interaction between components of the acceptor membrane and donor extract must take place, and in wild-type cells all of these necessary components are firmly anchored to the membrane. The weak binding of the adenylate cyclase components of the donor extract to the acceptor membrane implies some alteration in the physical arrangement of these species, but we can only guess at their exact orientation at this time. We have suggested previously that adenylate cyclase binds a significant amount of detergent (5). If the enzyme binds to the membrane via a particularly hydrophobic area of the protein's surface, a once-solubilized enzyme might reinsert into the membrane very poorly or slowly because that surface is tightly bound to detergent. This enzyme may still be free, however, to engage in regulatory interactions with proteins at the surface of the membrane.

The reconstituted hormone-responsive adenylate cyclase system that we have described is clearly not obvious in its mechanism, but no one who studies adenylate cyclase remains sufficiently naive to expect this. It does serve as a method for the assay of individual components of the cyclase system, and we are proceeding to use it for the study of donor extracts and depleted membranes of increasing definition. The data presented here already suggest to us a view of the S49 cell adenylate cyclase system that minimally includes five proteins. Components already defined include the catalytic protein and the receptors for catecholamines and PGs. The existence of NaF- and Gpp(NH)p-stimulated activities and their enhancement in extracts combined with AC^- membranes suggest the existence of at least another distinct component necessary for enzymic activity. Such a component might be related to the guanine nucleotide-binding protein studied by Pfeuffer and Helmreich (7). The lack of complementation between AC^- and UNC provides evidence for the existence of an additional specific factor that couples hormone binding to the stimulation of adenylate cyclase activity.

Note Added in Proof. Recently, we have noted the stable association of adenylate cyclase activity with the AC^- membranes during the course of the enzyme assay incubation.

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