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Hormone-Sensitive Adenylate Cyclase

Mutant Phenotype with Normally Regulated *Beta*-Adrenergic Receptors Uncoupled from Catalytic Adenylate Cyclase

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SUMMARY

BOURNE, H. R., D. KASLOW, H. R. KASLOW, M. R. SALOMON, AND V. LICKO. Hormone-sensitive adenylate cyclase: mutant phenotype with normally regulated *beta*-adrenergic receptors uncoupled from catalytic adenylate cyclase. *Mol. Pharmacol.* **20**:435–441 (1981).

Adenylate cyclase in H21a, a recently isolated S49 mutant cell line, fails to synthesize cyclic AMP in response to hormones, cholera toxin, guanine nucleotides, and NaF. H21a membranes contain M_r = 42,000 and 52,000 peptide subunits of the guanine nucleotide-binding regulatory component (*N* protein) of adenylate cyclase. These peptides are ADP-ribosylated by cholera toxin in H21a, as in wild-type S49. Nonetheless, extracts of H21a membranes do not complement the functional defect of *N*-deficient *cyc*$^-$ S49 membranes in vitro [M. R. Salomon and H. R. Bourne, *Mol. Pharmacol.* **19**:109–116 (1981)]. We asked whether *N* in H21a can perform functions observed in wild-type S49 but absent or deficient in *cyc*$^-$ and in another S49 mutant, *unc*. The *N* lesion in *unc* is thought to prevent interaction with receptors and allow normal interaction with the catalytic unit. Like wild-type, but unlike *cyc*$^-$ and *unc*, membranes of H21a contained *beta*-adrenergic receptors that exhibited high affinity for binding an agonist (isoproterenol) in the absence, but not in the presence, of GTP. In addition, exposure of H21a cells to isoproterenol for 16 hr caused an 85% decrease in density of *beta*-adrenergic receptors. Agonist-induced down-regulation of *beta*-adrenergic receptors occurred to a similar extent in wild-type, but not in *unc*. These results suggest that the *N* protein in H21a can interact with hormone receptors, but not with catalytic adenylate cyclase. Two-dimensional gel electrophoresis showed that the size and charge of the M_r = 42,000 and 52,000 cholera toxin substrates are similar in wild-type and H21a membranes. The H21a and *unc* lesions appear to affect the same protein, because extracts of each type of mutant membrane failed to complement the adenylate cyclase defect of the other in vitro.

INTRODUCTION

Ross and Gilman (1) observed that the adenylate cyclase defect in membranes of the *cyc*$^{-2}$ mutant S49 lymphoma cell could be repaired by the addition of detergent extracts from membranes of wild-type S49 and other cells. This observation led to discovery (2), characterization (3, 4), and purification (5) of a guanine nucleotide-binding regulatory protein that is essential for functional coupling of hormone receptors to stimulation of catalytic adenylate cyclase in membranes of animal cells.

Specific regulatory functions of this coupling protein, termed *N* in the present report, can be inferred from their absence in the *N*-deficient S49 *cyc*$^-$ mutant (6, 7). *N* mediates (a) stimulation of adenylate cyclase by *beta*-adrenergic agonists and prostaglandins; (b) regulation by guanine nucleotides of the affinity of *beta*-adrenergic receptors for agonists; (c) down-regulation of the number of *beta*-adrenergic receptors caused by *beta*-adrenergic agonists (8, 9); (d) stimulation of adenylate cyclase by guanine nucleotides and fluoride ion; and (e) stimulation of adenylate cyclase by cholera toxin, which catalyzes ADP-ribosylation of the *N* protein. The *cyc*$^-$ cells are resistant to stimulation of adenylate cyclase by the toxin (10), and *cyc*$^-$ membranes lack the M_r = 42,000 and 52,000 peptide subunits of *N* that are ADP-ribosylated by the toxin in wild-type S49 (11).

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[2] The abbreviations used are: *cyc*$^-$, mutant phenotype of S49 lymphoma that is deficient in the guanine nucleotide-binding regulatory component of adenylate cyclase (*N*); *R*, hormone receptor; *HR*, hormone-receptor complex; *C*, catalytic component of adenylate cyclase; *unc*, mutant S49 phenotype in which receptors are uncoupled from *N* and adenylate cyclase; IHYP, iodohydroxybenzylpindolol.

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The first three of these functions are absent or markedly impaired in the S49 *unc* mutant (12), a cell line in which hormones fail to stimulate cyclic AMP synthesis. Cholera toxin does activate adenylate cyclase in *unc*, however, and the M_r = 42,000 and 52,000 subunits of the *N* protein are altered in electrical charge with respect to wild-type (13). The three functions altered in *unc* appear to require interaction of *N* with hormone receptors (*R*). Functions that require interaction of *N* with catalytic adenylate cyclase (*C*)—stimulation of cyclic AMP synthesis by guanine nucleotides, NaF, and cholera toxin—remain intact in *unc*. Thus the *unc* lesion specifically affects interactions of *N* with *R*, but not with *C*.

We recently described isolation and preliminary characterization of a novel S49 mutant phenotype, in cells of a clone termed H21a (14). H21a cells and membranes, like those of *cyc*$^-$, were unresponsive to stimulation of cyclic AMP synthesis by hormones, cholera toxin, and other agents that act by mechanisms involving *N*. In contrast to *cyc*$^-$, however, the toxin specifically catalyzed ADP-ribosylation of M_r = 42,000 and 52,000 peptides in H21a membranes to an extent similar to that observed in wild-type. Here we report further studies of the H21a phenotype in comparison with those of wild-type and *unc*. The results indicate that H21a cells bear a lesion that specifically prevents the *N* protein from interacting with *C*, but allows it to interact with *R*.

MATERIALS AND METHODS

Chemicals. [^{125}I]IHYP was purchased from New England Nuclear Corporation, Boston, Mass. We found that it was necessary to filter this radioligand through a Whatman 2.4 cm GF/C filter before using it in binding assays. The prefiltering step removed approximately 10% of the radioactivity and dramatically improved reproducibility of binding assays.

Cell culture. S49 cells were propagated as described (10). The lines used included wild-type (clone 24.3.2) (10); a bromodeoxyuridine-resistant *cyc*$^-$ line (M3B1) (15); *unc* [obtained from Dr. A. G. Gilman (12)]; and H21a, isolated by virtue of resistance to cholera toxin (14).

Membranes. Partially purified membranes were prepared by the method of Ross *et al.* (16), modified as previously described (11).

Binding assay. As described previously (17), binding of [^{125}I]IHYP to *beta*-adrenergic receptors was measured by incubation of partially purified membranes (10–15 μg) for 60 min at 30° in a volume of 100 μl containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8), 4 mM $MgSO_4$, various concentrations of the radioligand, and other compounds as indicated. Incubations were terminated by filtering over 2.4-cm GF/C filters which were washed with 20 ml of a solution containing 1 mM $MgSO_4$ and 20 mM potassium phosphate (pH 8) at a temperature of 37°. Specific binding, defined as the difference between radioligand bound in the absence and presence of 1 μM (−)-propranolol, was always greater than 80% of total binding.

Analysis of competition binding experiments. Experimental data were analyzed for competition at one or at two sites (hyperbolic binding components) by nonlinear least-squares search for the parameters P_i of the function

$$B = \frac{P_1}{P_2 + C} + \frac{P_3}{P_4 + C}$$

where *B* is the amount of radioligand bound at steady state in the presence of competitor at concentration *C*.

In the case of competition at one site, parameters P_3 and P_4 were not considered and the other parameters had the following significance:

$$P_1 = R_0 K_L (A^*/K_A^*)$$
$$P_2 = K_L (1 + A^*/K_A^*)$$

where R_0 is the capacity (total number of available binding sites), K_L is the dissociation constant of the competitor, A^* is the concentration of the radioligand, and K_A is the dissociation constant of the radioligand.

When competition at two sites was considered, the parameters had the following significance:

$$P_1 = R_L K_L (A^*/K_A^*)$$
$$P_2 = K_L (1 + A^*/K_A^*)$$
$$P_3 = R_H K_H (A^*/K_A^*)$$
$$P_4 = K_H (1 + A^*/K_A^*)$$

where R_L and R_H represent, respectively, capacities of sites that bind the competitor with dissociation constants K_L (low affinity) and K_H (high affinity), and A^* and K_A^* refer to the concentration and dissociation constant of the radioligand, as before.

For curve fitting, all experimental values were weighted equally.

In vitro complementation. Complementation experiments, using cholate extracts of donor membranes and various recipient membranes, were performed exactly as described by Sternweis and Gilman (18). Absolute values for adenylate cyclase activities in the reconstituted membranes were lower in our experiments than in those reported (18), but the results were qualitatively identical (see Results) in that wild-type extracts conferred on *unc* and *cyc*$^-$ membranes the capacity to synthesize cyclic AMP in reponse to isoproterenol plus GTP.

Down-regulation of beta-adrenergic receptors. Cells in the logarithmic phase of growth were exposed to 1 mM ascorbic acid plus or minus 1 μM isoproterenol and cultured for 16 hr. Then cells were harvested by centrifugation (150 × *g* for 8 min), washed twice in Puck's G saline (without divalent cations) at 4°, and resuspended at a density of 5 × 10^7 cells/ml in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM $MgCl_2$, and 1 mM EDTA (pH 8) (membrane buffer). After incubating on ice for 30 min, cells were subjected to sonication for 5 sec with a Biosonik IV sonicator set at 60 W. Sonicates were centrifuged at 225 × *g* for 3 min and the supernatant fraction was centrifuged at 27,000 × *g* for 30 min. The pellet was suspended in membrane buffer, immediately frozen in Dry Ice and stored at −70° for subsequent assay of [^{125}I]IHYP binding and protein. Protein was measured by the method of Lowry *et al.* (19). [^{125}I]IHYP binding was assessed as described above, with 150 μg of particulate protein and a saturating concentration (700 pM) of radioligand.

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Two-dimensional gel analysis. Two dimensional gel analysis of peptides ADP-ribosylated in the presence of cholera toxin and [^{32}P]NAD$^+$ was performed as described previously (20).

RESULTS

Beta-adrenergic receptors in membrane preparations. [^{125}I]IHYP bound with high affinity to a single class of *beta*-adrenergic receptors in membranes of H21a cells (Fig. 1). Density (mean = 250, range 138–314 fmoles/mg in multiple membrane preparations) and affinity of these receptors for [^{125}I]IHYP (K_D = 73 pM) were similar to corresponding values for wild-type and *unc* S49 membranes previously reported (12, 16, 17) or determined in the course of this work (not shown).

We compared the abilities of a representative *beta*-adrenergic antagonist, propranolol, and an agonist, isoproterenol, to compete for [^{125}I]IHYP binding sites of wild-type, H21a and *unc* membranes, either in the presence or in the absence of exogenous GTP (Fig. 2). As previously reported (12, 16), the antagonist competed with similar apparent affinities for *beta*-adrenergic receptors in wild-type and *unc*, and these affinities were not altered by addition of GTP. The same results were obtained with H21a membranes (Fig. 2).

In contrast, *beta*-adrenergic agonists such as isoproterenol bind with higher affinity in the absence than in the presence of exogenous guanine nucleotides to receptors of many cell types, including wild-type S49 (16). In S49 mutants where *N* is functionally absent (*cyc*$^-$) or "uncoupled" from interaction with receptors (*unc*), however, isoproterenol binds with the same affinity whether or not GTP is present; this affinity is similar to the low affinity

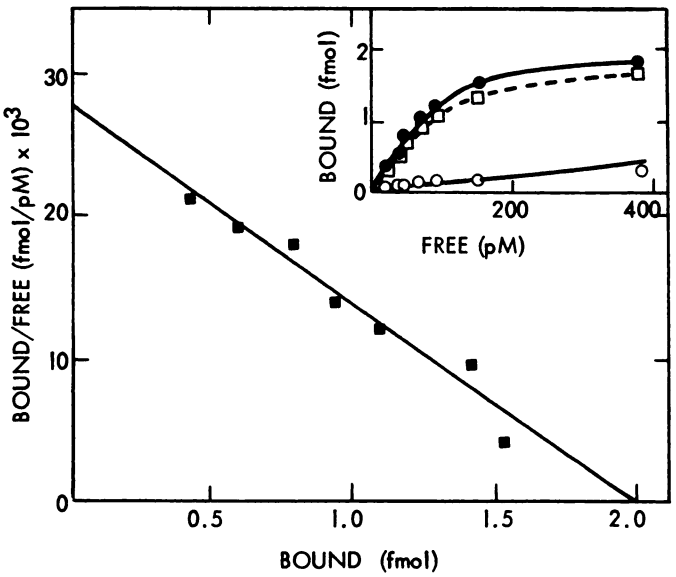

FIG. 1. *Binding of [^{125}I]IHYP to H21a membranes*

Binding was performed as described under Materials and Methods, with 12 μg of H21a membrane protein per tube. Scatchard analysis (■) indicated a K_D of 73 pM and a binding capacity of 167 fmoles/mg of protein. The inset provides direct binding data used for the Scatchard analysis: ●, total radioligand bound; ○, radioligand bound in the presence of 1 μM (−)-propranolol ("nonspecific" binding); □, calculated "specific" binding (difference between total and nonspecific binding).

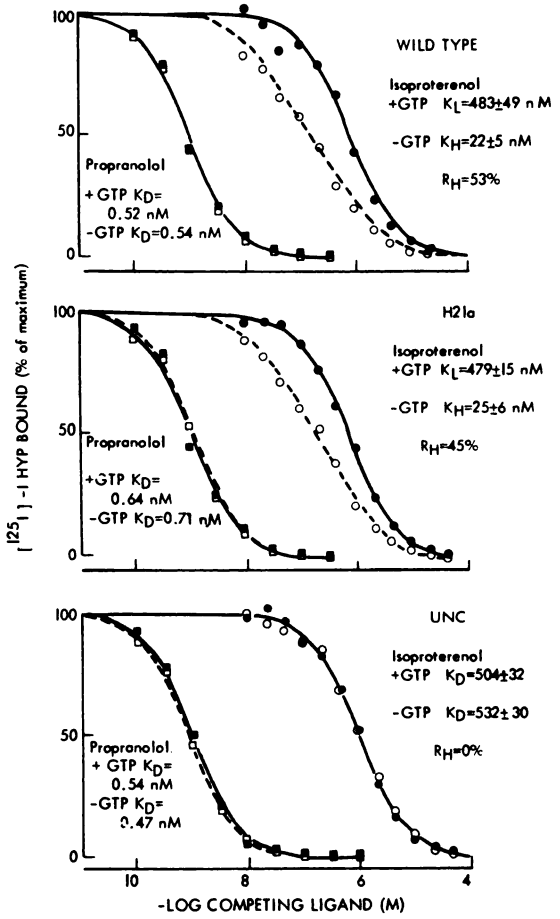

FIG. 2. *Competition by isoproterenol or propranolol for [^{125}I]IHYP binding sites in membranes of wild-type (top), H21a (middle), and unc (bottom) cells*

Membranes were incubated with 72 pM [^{125}I]IHYP in competition with increasing concentrations of isoproterenol (○, ●) or propranolol (□, ■) in the presence or absence of 300 μM GTP (*filled* or *open symbols*, respectively). Maximum binding represents the binding of radioligand in the absence of competing ligands. Each *data point* represents the mean of duplicate determinations in a single representative experiment. *Lines* are computer-generated curves fitting the observed data points (——, +GTP; - - -, −GTP; where the two curves coincide, only a *solid line* is shown). All of the propranolol competition curves and all of the isoproterenol competition curves in the presence of GTP modeled to single states of receptor affinity with dissociation constants shown. In the absence of GTP, isoproterenol competition curves in wild-type and H21a modeled to two binding states,with K_L assumed to be equal to the K_I calculated for isoproterenol in the presence of GTP (see text). K_H and percentage of receptors in the high-affinity form (% R_H) are shown.

observed in wild-type membranes in the presence of GTP (12, 16). Our experiments confirmed this difference between wild-type and *unc* membranes. Receptors in H21a membranes behaved like wild-type, rather than like *unc* (Fig. 2, Table 1).

In addition to showing a higher affinity (shift to the left), the isoproterenol competition curves in wild-type and H21a membranes are less steep in the absence of GTP than in its presence (Fig. 2). As observed for *beta*-adrenergic receptors of wild-type S49 (12, 16) and many other cell types, isoproterenol competition curves in wild-type and H21a consistently showed slope factors

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TABLE 1

Competition by isoproterenol for [^{125}I]IHYP binding sites in S49 clones

Parameters were calculated by a computerized curve-fitting program, as described under Materials and Methods. Each value represents the mean ± standard error of the mean for three experiments.

Cell type	+GTP, 1-state model, K_D (=K_L)	−GTP, 1-state model, K_D	−GTP, 2-state model	
			K_H	R_H
	nM	nM	nM	%
Wild-type	322 ± 80[a]	54 ± 11[b]	11.5 ± 5	50 ± 1.7
H21a	342 ± 72[a]	108 ± 19[b,c]	16.5 ± 5[a]	41 ± 2.0[c]
unc	622 ± 98[a]	592 ± 35[d]	—	—

[a] These values are not different from the corresponding parameter(s) listed for other clones ($p > 0.05$).

[b] Different from K_D in the presence of GTP ($p < 0.01$).

[c] Different from corresponding value for wild-type ($p < 0.05$).

[d] Value not different from K_D in the presence of GTP ($p > 0.05$).

("pseudo" Hill coefficients) less than 1.0 in the absence of GTP (0.55–0.65) and close to 1.0 in its presence (0.94–0.99).

De Lean and his co-workers (21) and Kent *et al.* (22) explicitly formulated one interpretation of the differences in slopes of agonist competition curves, based upon the idea that the receptors exist in either of two interconvertible states, R_L and R_H, characterized by low or high affinities for binding agonist (K_L and K_H, respectively). We used their formulation to analyze the competition binding data for S49 cell lines (Table 1), using a similar computerized curve-fitting procedure (see Materials and Methods). According to this formulation, antagonists do not discriminate between the two receptor states (i.e., $K_L = K_H$), and thus antagonist competition curves should be adequately described in terms of a single K_D value, which should not depend upon the presence or absence of guanine nucleotides. For propranolol this was the case for all three S49 cell lines tested: computer-fitted curves closely corresponded to the data (Fig. 2), and K_D values were not affected by the addition of GTP.

Similarly, according to the De Lean model (21, 22), saturating concentrations of guanine nucleotides convert all of the agonist binding sites into the R_L state. Accordingly, isoproterenol competition curves in the presence of 300 μM GTP should be adequately described by a one-state binding model with a single K_D (which is equal to K_L). This was the case the three types of S49 membranes (Fig. 2, Table 1).

In the absence of guanine nucleotides, some of the receptors may be in the R_H state. This can produce a shift to the left in isoproterenol competition curves, as was observed for wild-type and H21a membranes (Fig. 2). We fitted these curves to a two-state model, assuming that K_L was equal to the K_D value determined in the presence of GTP and allowing the computer program to determine appropriate values for K_H and fraction of receptors in the R_H state. For wild-type and H21a the computer-drawn curves correlated closely with binding data (Fig. 2). Calculated values of K_H were similar in the two types of membranes, although the percentage of receptors in the R_H form was somewhat greater in wild-type than in H21a membranes (Table 1).[3]

Isoproterenol competition curves in *unc* membranes were not affected by the absence of GTP, and a one-state model with a single K_D adequately described the binding data obtained (Fig. 2, Table 1). A one-state model could also be used to calculate K_D for isoproterenol competition in the absence of GTP for wild-type and H21a membranes (Table 1); such a "composite" K_D was consistently

TABLE 2

Beta-adrenergic receptor density following incubation of S49 cells with isoproterenol

Cells were incubated with 1 μM isoproterenol and 1 mM sodium ascorbate for 16 hr. *Beta*-adrenergic receptor density in particulate preparations was determined as described under Materials and Methods. Data are expressed as percentage of receptor density in particulates of paired control cells incubated for 16 hr in the absence of isoproterenol.

Cell type	*Beta*-receptors[a]
	% control
Wild type	21.3 ± 3.5 (3)
H21a	15.1 ± 3.6 (3)[b]
unc	62 (1)

[a] Mean ± standard error of the mean for number of experiments shown in parentheses.

[b] Not statistically different from wild-type ($p > 0.05$).

TABLE 3

Complementation of N defects in vitro

The complementation procedure was performed exactly as described previously (18) by mixing 120 μl of acceptor membrane (1.5 mg/ml) with 72 μl of donor extract [2 mg/ml solubilized protein in 1% (w/v) cholate]. After reconstitution and subsequent centrifugation the membranes were resuspended and assayed for adenylate cyclase activity as described previously (4) with 40 μg of membrane protein for 25 min at 30° and at 0.4 mM ATP. The results are means of duplicate determinations, which differed from one another by less than 10%. This experiment was repeated twice, with nearly identical results each time.

Donor extract	Acceptor membrane	Adenylate cyclase activity		
		GTP	Isoproterenol[a] + GTP	NaF[a]
		pmoles cyclic AMP/tube		
Wild type	—	0.17	0.22	0.72
unc	—	0.20	0.20	0.94
H21a	—	0.29	0.29	0.20
Wild type	Wild type	3.87	27.4	44.8
Wild type	*unc*	2.34	7.69	59.2
Wild type	H21a	1.92	8.67	24.9
unc	*unc*	2.38	2.30	78.9
unc	H21a	1.28	1.48	21.2
H21a	*unc*	2.01	2.03	52.0
H21a	H21a	1.21	1.62	1.04

[a] Activators were present in the adenylate cyclase assay at the following concentrations: GTP, 100 μM; isoproterenol, 100 μM; NaF, 10 mM.

[3] High-affinity binding of agonists is thought to reflect binding of hormones to receptors complexed with the *N* protein (see Discussion). If so, the lower percentage of R_H in H21a, as compared with wild-type, may indicate that the interaction of *N* with *R* in H21a is slightly impaired, although not so drastically as in *unc*, where all receptors appear to be in the R_L state.

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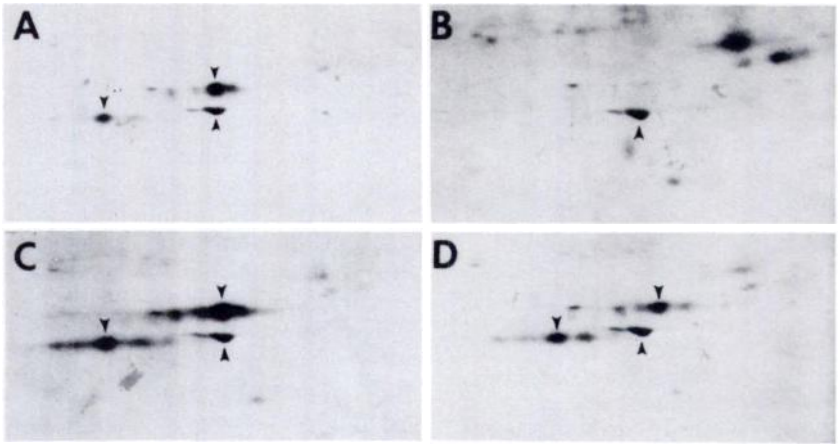

FIG. 3. *Two-dimensional gel analysis of cholera toxin substrates*

Autoradiograms of S49 cell membranes incubated with [^{32}P]NAD$^+$ and cholera toxin and then subjected to equilibrium 2-dimensional gel electrophoresis. Membranes were from wild type (*A*), *cyc*$^-$ (*B*), H21a (*C*), and *unc* (*D*) cells. In each *panel* the first (isoelectric focusing) dimension runs from basic (*left*) to acidic (*right*), whereas the second dimension shows migration (from *top* to *bottom*) in polyacrylamide gel electrophoresis in sodium dodecyl sulfate. To provide electrophoretic markers, extracts from cells metabolically labeled with [^{35}S]methionine were added to each sample before electrophoresis. The *upward-pointing arrowhead* indicates ^{35}S labeled actin (M_r = 45,000). Spots corresponding to the M_r = 42,000 and 52,000 peptide subunits of *N* are indicated by *downward-pointing arrowheads*. The spots in the *upper right* of *panel B* were variably detected in preparations of all S49 cell types tested, and are not related to the *N* protein.[4]

lower than the K_D (= K_L) derived from competition curves performed in the presence of GTP, for both membrane types. "Goodness of fit" of such curves (not depicted in Fig. 2) was clearly inferior to that of curves calculated using the two-state model, both by inspection and by calculation of residual variance, according to De Lean *et al.* (21, 22).

Down-regulation of receptor number. Previous studies showed that exposure of wild-type S49 to isoproterenol causes a time- and concentration-dependent decrease in number of *beta*-adrenergic receptors, as assessed by the capacity of particulate preparations to bind [^{125}I]IHYP (8, 9). This isoproterenol-induced "down-regulation" of receptor number does not occur in *cyc*$^-$ cells (8, 9), and is less extensive in *unc* than in wild-type (9). Table 2 shows that 16-hr incubation of H21a cells with isoproterenol caused a reduction in number of *beta*-adrenergic receptors comparable to that observed in wild-type, and greater than was observed in *unc*.

In vitro complementation of membranes with defective N activity. Because H21a and *unc* appear to have lost different functions of *N*, we asked whether extracts of either mutant membrane could complement the other's defective response to stimulators of adenylate cyclase. To do so, we used the complementation procedure of Sternweiss and Gilman (18), which allows *N* in cholate extracts to interact with *unc* or *cyc*$^-$ membranes in a fashion that is stable to dilution.

Table 3 shows that cholate extracts of H21a membranes did not restore responsiveness of adenylate cyclase in *unc* membranes to isoproterenol, although wild-type extracts did so. Conversely, although cholate extracts of wild-type membranes conferred on H21a the ability to synthesize cyclic AMP in response to isoproterenol and NaF, cholate extracts of *unc* allowed H21a membranes to respond only to NaF. Thus, H21a × *unc* complementation procedures resulted in the *unc* phenotype, no matter which membrane served as the donor extract, whereas both wild-type × *unc* and wild-type × H21a complementation procedures produced a pattern of response similar to that of wild-type. In this respect, H21a membranes or extracts behaved exactly like those of *cyc*$^-$ (18).

In other experiments (not shown), cholate extracts of H21a failed to complement the defect of *cyc*$^-$, confirming previous results (14) with H21a extracts in a non-ionic detergent, Lubrol 12A9.

Two-dimensional gel electrophoresis of toxin substrates. Two-dimensional gel analysis of proteins from membranes radiolabeled in the presence of cholera toxin and [^{32}P]NAD$^+$ (Fig. 3) showed that the toxin substrates of H21a and wild-type S49 membranes are similar or identical in charge and size. Figure 3 also confirms the observation (13) that the toxin substrate peptides of *unc* are more acidic than those of wild-type. Such peptides were not detected in 2-dimensional gels of *cyc*$^-$.

DISCUSSION

H21a cells have lost functions of the *N* protein that are intact in *unc*, and vice versa. Figure 4 depicts an interpretation of the functional defects in the two mutants, according to a model of hormone-sensitive adenylate cyclase proposed by several laboratories (6, 7, 9, 21). According to the model, guanine nucleotides regulate separate binding interactions of the *N* protein with catalytic adenylate cyclase (*C*) and with hormone-receptor (*HR*) complexes. Using [^{32}P]ADP-ribosylated *N* proteins in soluble detergent extracts, Pfeuffer (23) and Limbird *et al.* (24) have presented evidence for the binding of *N* to *C* and to *HR*, respectively.

In the first of these interactions, intact in *unc* but apparently impaired in H21a, N_{GTP} activates cyclic AMP synthesis by binding to *C*. Adenylate cyclase of the N_{GTP}:*C* complex is terminated by loss of the γ-phosphate of

[4] H. R. Kaslow, in preparation.

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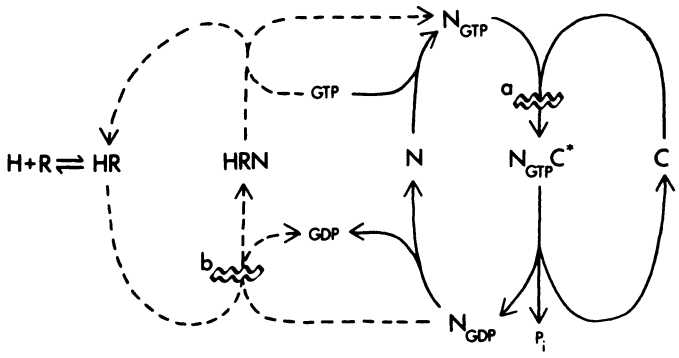

FIG. 4. *Model of component interactions in hormone-sensitive adenylate cyclase, showing postulated defects in H21a and unc.*

Principal features of the model are outlined in the text. The H21a lesion (*a*) prevents formation of the N_{GTP}:C* complex that synthesizes cyclic AMP from ATP. Interactions of *N* with guanine nucleotides and hormone-receptor (*HR*) complexes (– – –) occur normally with H21a, as in wild-type. The lesion in *unc* (*b*) prevents binding of *N* (or N_{GDP}) to *HR* and therefore prevents *HR* from catalyzing accelerated formation of N_{GTP} to increase synthesis of cyclic AMP.

GTP and dissociation of (inactive) *C* from N_{GDP}. Cyclase activation by this pathway occurs at a slow rate, because the availability of N_{GTP} is limited. According to the model, *HR* stimulates cyclic AMP synthesis by increasing the rate of formation of N_{GTP} via a second interaction of *N* (Fig. 4, – – –): *HR* binds to *N* and facilitates exchange of GDP for GTP at a guanine nucleotide-binding site. The interaction of *HR* with *N* is apparently impaired in *unc* membranes, which synthesize little cyclic AMP in response to hormones.

In contrast, *N* can bind to *HR* in both H21a and wild-type membranes, as shown by the ability of *beta*-adrenergic receptors in both types of membranes to bind isoproterenol with high affinity (Fig. 2). High-affinity binding of agonists is thought to involve a ternary complex composed of *H*, *R*, and *N* (6, 7, 9, 21). Thus *cyc*$^-$ and *unc* membranes, in which *N* is functionally absent or defective, respectively, do not show high-affinity binding of isoproterenol (12, 16), although this defect can be repaired by complementation *in vitro*, using *N*-containing extracts of wild-type (18).

The ability of isoproterenol to reduce the number of *beta*-adrenergic receptors in H21a cells (Table 2) is additional evidence of the ability of *N* in H21a to interact with receptors. Isoproterenol causes no decrease in receptor number in *cyc*$^-$ cells (8, 9), and a smaller decrease in *unc* than in wild-type (ref. 9, confirmed in Table 2).[5] The H21a phenotype thus shows that an agonist-induced decrease in receptor number may be dependent upon the presence of an *N* protein that can interact with receptors, but does not require cyclic AMP accumulation [which does not occur in H21a cells exposed to isoproterenol (14)]. Similar conclusions were derived from study of HTC cells (9), which are phenotypically almost devoid of *C* activity but which contain *beta*-adrenergic receptors that are reduced in number by exposure to isoproterenol and that exhibit high-affinity binding of agonist in membrane preparations.

In models like that in Fig. 4, it is tacitly assumed that *N*, *R*, and *C* are mobile entities that associate and dissociate in the plane of the membrane under the influence of water-soluble hormones, guanine nucleotides, and ions. Although these entities are separable in detergent-soluble preparations and can be shown to bind to one another in ways that conform to the model (23, 24), knowledge of their states of aggregation and dissociation in the cell membrane is necessarily limited and indirect (25, 26). In this context, the H21a and *unc* phenotypes show that the postulated interactions of *N* with *C* and *R* are functionally separable. Thus, either regulation of cyclase activity or of receptor affinity and number can occur separately in membranes and intact cells. If so, *N* may act as an intramembrane "messenger" for hormones, shuttling back and forth between *HR* and *C* (or other effector molecules). Thus, it is not necessary to imagine that *R*, *N*, and *C* bind to one another simultaneously, and it is possible that such a complex rarely occurs in S49 membranes.

Presumably the H21a lesion affects a portion of the *N* molecule that binds to or regulates *C*. The lesion could be located in the cholera toxin substrate peptides, both of which are affected in *unc* (13). Alternatively, because these peptides in H21a are similar in charge and size to those of wild-type, the H21a defect could reside in another peptide subunit of the protein, such as the M_r = 35,000 subunit of *N* in rabbit liver membranes, which is not a substrate for cholera toxin (5). If so, it may be possible to construct a normally functioning molecule from unaffected subunits derived from *unc* and H21a, either *in vitro* or in somatic hybrids. Such hydridization experiments are currently in progress in our laboratory.

Finally, we have so far assumed that the H21a lesion is confined to the *N* molecule alone. This seems likely because the defect in cyclase stimulation is complemented *in vitro* by *N*-containing extracts of wild-type membranes, partially complemented by *unc* extracts, and not affected by *cyc*$^-$ extracts (Table 3). The demonstration (5) that a pure protein complements both the *cyc*$^-$ and *unc* lesions established that these mutations affect the function of a single protein. Our complementation experiments establish that the same protein is affected by the H21a lesion, but do not formally rule out the possibility that H21a membranes bear a second defect as well. Although an additional defect is neither likely nor required by the data, it can only be completely ruled out by showing that homogeneous *N* protein complements the H21a lesion *in vitro*.

[5] The fact that isoproterenol reduces the number of *beta*-adrenergic receptors in *unc*, but not in *cyc*$^-$, probably indicates that "uncoupling" of *unc* receptors is not complete. Isoproterenol and prostaglandin E_1 do cause slight stimulation of adenylate cyclase and cyclic AMP accumulation in *unc* (12, 15). Because down-regulation of receptors is time-dependent and virtually irreversible (9), a small residual capacity of *N* in *unc* to interact with *R* could have allowed a substantial reduction in receptor number in response to isoproterenol.

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