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Adenylate cyclase permanently uncoupled from hormone receptors in a novel variant of S49 mouse lymphoma cells

(β-adrenergic receptor/catecholamines/prostaglandins/guanine nucleotides)

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ABSTRACT **A novel variant of the S49 mouse lymphoma has been selected from wild-type cells by growth in medium containing the β-adrenergic agonist terbutaline and inhibitors of cyclic nucleotide phosphodiesterase. In contrast to the situation in the wild-type clone, synthesis of adenosine 3′:5′-monophosphate (cyclic AMP) is not stimulated by β-adrenergic agonists or by prostaglandin E_1 either in intact variant cells or in membrane preparations of such clones. However, basal and NaF-stimulated activities of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] are normal, enzyme activity is stimulated by guanyl-5′-yl imidodiphosphate [Gpp(NH)p], and intact cells accumulate cyclic AMP when exposed to cholera toxin. Furthermore, variant cell membranes possess ligand-binding activity consistent with the conclusion that a normal or an excessive number of β-adrenergic receptors is present. Thus, interaction between the hormone-binding and the catalytic moieties of the adenylate cyclase system is lost. This variant phenotype, designated as uncoupled (UNC), has been stable for more than 100 generations without exposure to the drugs used for selection. Such cells should be useful for the elucidation of mechanisms of transmission of information from hormone receptors to adenylate cyclase.**

Many of the biochemical and physiological effects of catecholamines result from the intracellular action of 3′:5′-cyclic AMP. The synthesis of this mediator is stimulated by β-adrenergic agonists, and current models of the membrane-bound synthetic enzyme, adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], depict a catalytic moiety that is distinct from receptors for catecholamines or other hormones that regulate enzymatic activity. Several types of evidence are consistent with the independence of the ligand-binding and the catalytic components of the adenylate cyclase system. Kinetic studies suggest that receptors for different hormones can regulate the activity of a common catalytic unit (1, 2), and receptor function or catalytic activity can be selectively perturbed—chemically or enzymatically (3, 4). Furthermore, genetic manipulation has suggested that the β-adrenergic receptor and adenylate cyclase are the products of separate genes (5) and that cells can modify their level of response to β-adrenergic agonists by regulation of receptor concentration (6).

Once the independence of the hormone-binding component and adenylate cyclase is assumed, the question of the mechanism of their interaction becomes paramount. Several studies have suggested that the lipid environment is important for this interaction (3, 7, 8), and it has been proposed that the lipid bilayer may itself act as a "transducer" coupling the receptor and the enzyme (9). Current data also suggest that a guanine-nucleotide-binding component may be vitally important for communication between receptors and adenylate cyclase (10).

We have attempted to explore the relationship between hormone receptors and adenylate cyclase by genetic manipulation of cultured mammalian cells (11). The S49 lymphoma cell is killed by cyclic AMP or by agents that enhance its accumulation (12), and variant cells can thus be selected that are defective in the hormone-response pathway; adenylate cyclase-deficient clones have been described previously (13). Herein we report the selection and characterization of an S49 lymphoma clone with a phenotype that, to our knowledge, is novel for any adenylate cyclase system: while these cells possess both β-adrenergic receptors and adenylate cyclase, β-adrenergic agonists fail to alter enzymatic activity. Interaction between the ligand-binding and the catalytic functions thus appears to be lost, and the response system is permanently uncoupled.

MATERIALS AND METHODS

Cell Culture and Subcellular Fractionation. Methods for culture of S49 mouse cells have been described.* Growth medium was Dulbecco's modified Eagle's medium supplemented with 7.5–10% heat-inactivated horse serum. We have recently reported techniques for the rapid preparation of a relatively crude but hormone-responsive particulate fraction from these cells (6) and for the preparation of purified membranes in which specific activities of adenylate cyclase and concentrations of the β-adrenergic receptor are enriched 20- to 40-fold over those of the homogenate.*

Isolation of Variants. It must be admitted that the first isolation of clones with the phenotype to be described (designated as uncoupled, UNC) was the result of relatively uncontrolled "pilot" studies. Wild-type S49 cells were placed in stationary suspension culture in growth medium containing terbutaline (0.1 mM; a β-adrenergic agonist chosen for its resistance to oxidative degradation) and the cyclic nucleotide phosphodiesterase inhibitors 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724) (0.03 mM) and 1-methyl-3-isobutylxanthine (0.1 mM); the mixture with the three drugs is designated *TRM medium*. After approximately 1 month in this medium, cells that began to grow were cloned (14) and studied as described. All data presented were obtained on clones of cells that had not been exposed to TRM medium for at least 40 generations. Data on three such clones (UNC 1, 2, and 3) are presented; these clones were derived from a common pool and their relationship to each other is thus not known.It can be emphasized, however, that several independent clones of the same phenotype have been isolated under more controlled conditions by selection of wild-type S49 cells according to the

Abbreviations: cyclic AMP, adenosine 3′:5′-cyclic monophosphate; TRM medium, growth medium containing terbutaline (0.1 mM), R020-1724 (0.03 mM), and 1-methyl-3-isobutylxanthine (0.1 mM); R020-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; IHYP, iodohydroxybenzylpindolol [hydroxybenzylpindolol is (±)-3-indoloxy-1-(2-*p*-hydroxybenzylpropyl-2-amino)isopropanol]; UNC, uncoupled phenotype; AC^-, adenylate-cyclase-deficient phenotype; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; PGE_1, prostaglandin E_1.

* E. M. Ross, M. E. Maguire, T. W. Sturgill, R. L. Biltonen, and A. G. Gilman, (1977) *J. Biol. Chem.*, in press.

Table 1. Parameters of cell growth and karyology of wild-type and variant S49 cells

	Cloning efficiency,* %		Generation time,† hr			Chromosome no.‡
	Control	TRM	Control	TRM	Cholera toxin	Mean (range)
Wild type	63	<1	17	§	§	40 (39–40)
AC⁻	70	70	16	23	19	40 (39–40)
UNC 1	81	91	17	19	§	40 (39–40)
UNC 2	ND	ND	17	19	§	40 (39–40)

ND, not determined.

* One-hundred cells were plated in each of three to six dishes in 0.25% agarose (Seakem; see ref. 14) in growth medium containing 10% horse serum and drugs as indicated. Colonies were counted after 2 weeks. Data are the means of colony counts from individual dishes.

† Cells (10^5)/ml were cultured in 50 ml stationary suspension cultures of the composition indicated. Medium designated as "cholera toxin" contained the purified toxin at 1 μg/ml and R020-1724 (0.03 mM). Cells were exposed to the toxin for 30 min before the addition of serum. Cells were counted daily.

‡ Chromosomes were counted from 20 metaphases of each clone. All chromosomes of the S49 cell are telocentric.

§ Cell death; there was no accumulation of cells during 5 days of culture.

method of Coffino *et al.* (14) in soft agar and TRM growth medium.

Assays. Adenylate cyclase activity was quantified by the procedure of Salomon *et al.* (15), exactly as described.* The ligand-binding properties of the β-adrenergic receptor were assessed with the specific ligand ^{125}I-labeled iodohydroxybenzylpindolol ([^{125}I]IHYP) as described (16), except that 0.1 mM ascorbic acid was included in incubation media. Binding equilibrium was obtained by 60 min at 30°. Specific binding is defined as the difference in [^{125}I]IHYP bound in the absence and the presence of 1 μM (−)-propranolol. In purified membrane fractions, specific binding represents more than 95% of the total at the ligand concentrations routinely utilized.

For determination of intracellular concentrations of cyclic AMP, approximately 10^7 cells per ml were incubated in Dulbecco's modified Eagle's medium containing 20 mM sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (NaHepes) (instead of $NaHCO_3$) and 0.2 mM 1-methyl-3-isobutylxanthine. After 15 min at 37°, potential stimulators of cyclic AMP synthesis were added for the times indicated. Incubations were terminated by addition of 0.2 ml of 1 M HCl. Following addition of approximately 0.2 pmol of cyclic [^{3}H]AMP (5000 cpm) to monitor recovery, neutralization with NaOH, and purification of cyclic AMP on columns of Dowex-1-Cl, samples were lyophilized and finally assayed for cyclic AMP by a protein-binding technique (17).

The sources of all materials have been listed elsewhere.*

RESULTS

The cloning efficiency of wild-type S49 lymphoma cells is markedly reduced when they are plated in medium containing a β-adrenergic agonist and inhibitors of cyclic nucleotide phosphodiesterase. Two types of stable phenotypic variants have been isolated by virtue of their resistance to these inhibitory conditions (TRM medium). One is the same as that described by Bourne *et al.* (13)—cells that are deficient in adenylate cyclase activity (AC⁻). The other variant is the subject of this report and is designated as uncoupled (UNC). Table 1 summarizes cloning efficiencies in control and TRM medium, generation times in these media and in the presence of cholera toxin (a stimulator of adenylate cyclase activity), and the karyotypes of wild-type cells and variant clones of each type. Deficiency of adenylate cyclase does confer resistance to TRM medium, supporting the hypothesis that the inhibitory action of the drugs is exerted via the adenylate cyclase system. It is thus a suitable medium with which to attempt the isolation of other variants that are defective in the response to hormone. UNC cells are similarly resistant. However, UNC clones are not resistant to cholera toxin; this suggests the presence of functional adenylate cyclase (see below) and distinguishes them from AC⁻ cells.

The mechanism of occurrence and the incidence of the AC⁻ and UNC variations are not known. AC⁻ clones arise in soft agar/TRM medium at a rate of approximately 1 per 10^3

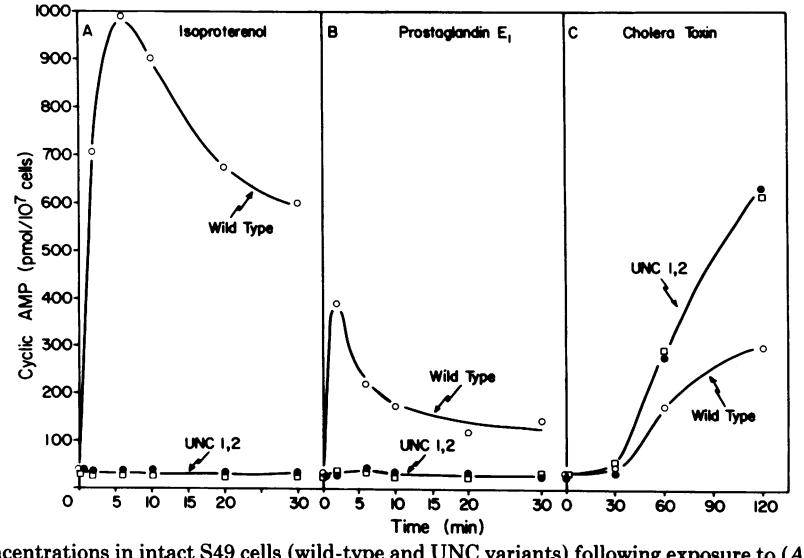

FIG. 1. Cyclic AMP concentrations in intact S49 cells (wild-type and UNC variants) following exposure to (*A*) isoproterenol (10 μM), (*B*) prostaglandin E_1 (10 μM), or (*C*) cholera toxin (1 μg/ml). All incubation media also contained 1-methyl-3-isobutylxanthine (0.2 mM).

Table 2. Adenylate cyclase activities in wild-type and in UNC clones

Clone	Enzyme activity,* pmol/min per mg protein							
	Basal	NaF	Gpp(NH)p	GTP	Isoproterenol	GTP + isoproterenol	PGE_1	GTP + PGE_1
UNC 1	11	310	150	22	11	19	11	29
UNC 2	16	415	205	31	16	83	16	45
UNC 3	15	350	175	27	15	29	15	43
Wild type	10	365	250	20	10	290	10	200

* Adenylate cyclase was assayed as described under *Materials and Methods*. Purified membrane preparations of each clone were used. Assay incubation time was 20 min and protein concentration was approximately 100 μg/ml. Other concentrations were NaF, 10 mM; Gpp(NH)p, 0.1 mM; GTP, 0.1 mM; isoproterenol, 10 μM; prostaglandin E_1, 10 μM.

wild-type cells plated. UNC variants are apparently only somewhat less frequent.

The effects of isoproterenol, prostaglandin E_1(PGE_1), and cholera toxin on intracellular concentrations of cyclic AMP in UNC and wild-type cells are shown in Fig. 1. Wild-type cells show large responses to all three agents, and the effect of cholera toxin is characteristically delayed. In contrast, UNC cells fail to respond to hormones—not only to the β-adrenergic agonist isoproterenol, but also to prostaglandin E_1. However, the UNC clones have a response to cholera toxin that is even greater than that of wild-type cells, consistent with the data of Table 1. The low intracellular concentration of cyclic AMP in AC^- cells is not altered by any of these agents (13).

Adenylate cyclase activities in purified membrane fractions of wild-type and UNC clones are shown in Table 2. Wild-type cell membranes display low basal activities of the enzyme and marked responses to NaF, guanyl-5′-yl imidodiphosphate [Gpp(NH)p], isoproterenol, and prostaglandin E_1. Responses to the latter two agents are *dependent* on the presence of GTP, Gpp(NH)p, or certain other purine nucleotides.* GTP itself has only a slight ability to stimulate catalysis, while Gpp(NH)p activates the enzyme irreversibly. Hormones only accelerate the rate at which activation by Gpp(NH)p occurs; the extent of activation by Gpp(NH)p is the same in the presence or absence of isoproterenol.

UNC membranes have basal and NaF-stimulated adenylate cyclase activities that are indistinguishable from those of wild-type cells. However, there is no effect of isoproterenol in the presence of GTP, and the effect of prostaglandin E_1 is less than 10% of that observed in wild-type cells. By contrast, the average response to Gpp(NH)p is approximately two-thirds of that observed in the control clone; this effect is thus obviously present.

The reduced effect of Gpp(NH)p apparently reflects a change in the maximal capacity of the system to respond, rather than an alteration in the kinetics of the activation or in the affinity for the nucleotide. Although synthesis of cyclic AMP in the presence of Gpp(NH)p is not a linear function of time (10), the temporal pattern of response is identical in UNC clones (Fig. 2) and in wild-type cells.* Furthermore, the concentration of Gpp(NH)p that produces half-maximal activity (at 20 min) was 0.4 μM in both UNC and wild-type cells (not shown). Not expected was a small stimulatory effect of isoproterenol on the rate of enzyme activation by Gpp(NH)p (Fig. 2). This is the only effect of isoproterenol that has been observed on the adenylate cyclase activity of UNC cells; it is qualitatively identical but quantitatively much reduced when compared to similar experiments with wild-type cells.*

Stimulatory effects of isoproterenol in the presence of GTP were not observed in whole homogenates or in membranes at any stage of purification. Distribution of basal or of NaF- or Gpp(NH)p-stimulated adenylate cyclase activities among subcellular fractions of UNC was the same as that observed for wild-type cells.* These data and those on intact cells make it unlikely that labile factors necessary for the response to hormone were lost during homogenization or preparation of membranes.

Considerable data indicate that the specific binding capacity of S49 cell membranes for [^{125}I]IHYP is a valid measure of their concentration of β-adrenergic receptors (16, 18,*). Such binding of [^{125}I]IHYP to UNC and wild-type membranes is shown in Fig. 3. The density of β-adrenergic receptors in UNC membranes is at least as great and is possibly as much as twice that of wild-type cells. The K_D for [^{125}I]IHYP determined from such equilibrium binding studies varied between 20 and 60 pM in different experiments, but there was no difference in this value when UNC and wild-type membranes were compared. The kinetics of binding of [^{125}I]IHYP to UNC membranes (not shown) was identical to that previously described for wild-type preparations.*

Both β-adrenergic agonists and antagonists compete for [^{125}I]IHYP-binding sites stereoselectively and at concentrations that reflect their ability to stimulate or to prevent stimulation of the adenylate cyclase of S49 cells. We have previously shown that guanine nucleotides increase both the K_D and the apparent Hill coefficient for the binding of agonists to the β-adrenergic receptor (19,*); the binding of antagonists is not altered by such nucleotides. In AC^- cells this interaction between β-adrenergic agonists and guanine nucleotides is lost.* Competition for [^{125}I]IHYP-binding sites by agonists in AC^- cells in the presence or absence of nucleotides is identical to that seen in wild-type cells in the presence of nucleotide. At the simplest level of hy-

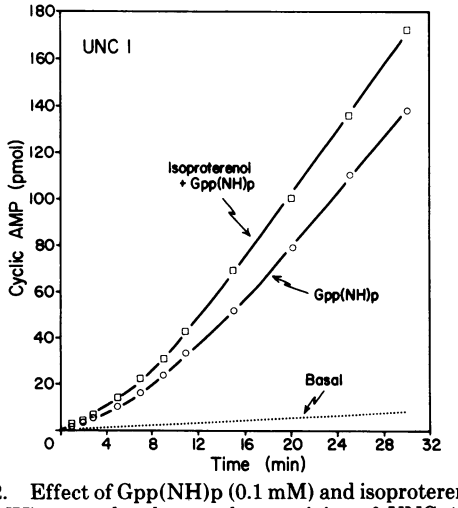

FIG. 2. Effect of Gpp(NH)p (0.1 mM) and isoproterenol (2 μM) + Gpp(NH)p on adenylate cyclase activity of UNC 1 (purified membrane preparation). The protein concentration was 130 μg/ml. Aliquots (100 μl) of an adenylate cyclase reaction mixture were sampled at the times indicated.

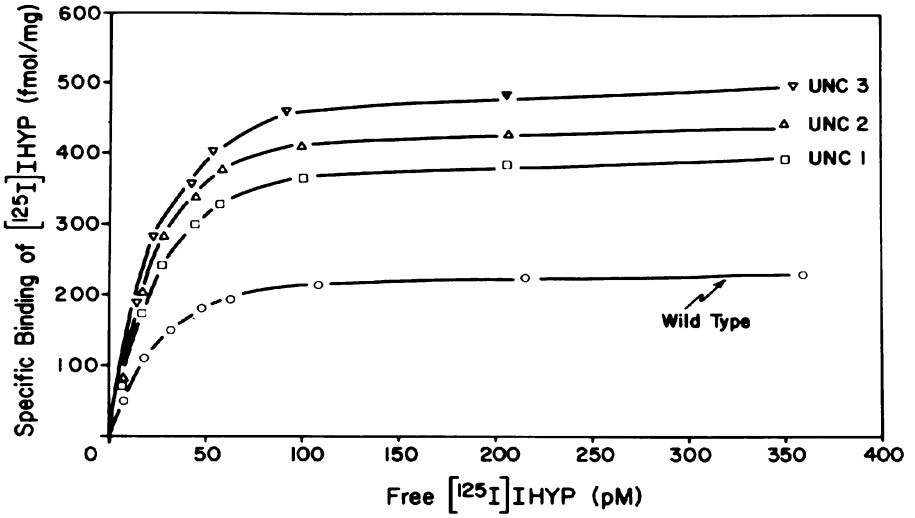

FIG. 3. Specific binding of [^{125}I]IHYP to purified membrane preparations from wild-type cells and from UNC 1, 2, and 3. Binding was determined as described at the ligand concentrations indicated in the presence and absence of 1 μM (−)-propranolol. Membrane protein concentrations were approximately 100 μg/ml.

pothesis, this alteration in binding properties could be due to an inability of receptor and enzyme to interact in AC$^-$ cells; in this case the same phenomenon might be expected in UNC cells. This was in fact observed (Fig. 4). Competition by the antagonists (−)- and (+)-propranolol is identical in wild-type and UNC membranes. However, the affinity for the agonist (−)-isoproterenol is reduced in UNC membranes, the apparent Hill coefficient is increased (from 0.55 to 0.9), the effect of guanine nucleotides is lost, and binding of isoproterenol is identical to that seen in wild-type cells in the presence of nucleotide.

Finally, to test the stability of the UNC phenotype, UNC cells were recloned, and crude particulate preparations from 10 subclones were characterized (Table 3); all remained uncoupled. Because cells that have been cloned twice and that have not been exposed to TRM medium for 120 generations remain uncoupled, the stability of the phenotype seems assured. Of interest, the ratio of Gpp(NH)p- to NaF-stimulated adenylate cyclase activity in UNC cells appears to be lower in the crude particulate fractions shown in Table 3 than in purified membranes (compare Table 2). Because this is not the case when wild-type cells are examined, this observation may provide insight into the molecular mechanism of the UNC variation.

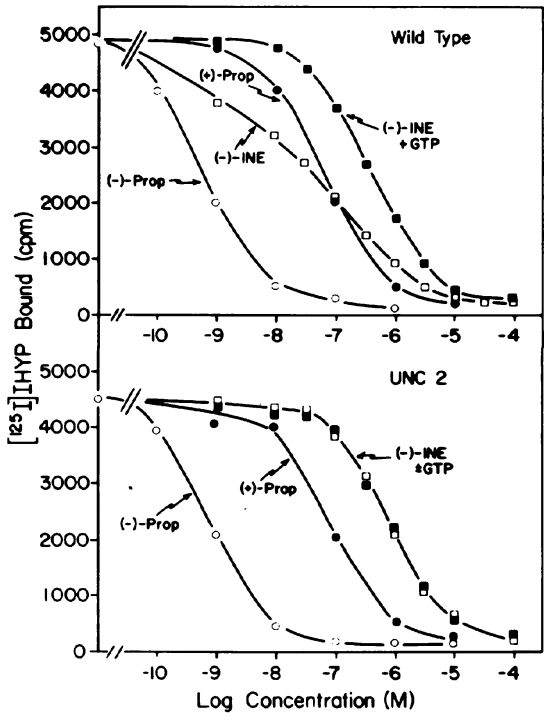

FIG. 4. Competition for [^{125}I]IHYP-binding sites in purified membrane preparations from wild-type and UNC clones. Competition by (−)-propranolol (Prop) (○) and by (+)-propranolol (●) is unaltered by GTP (not shown). The effects of (−)-isoproterenol (INE) (□) and (−)-isoproterenol in the presence of 100 μM GTP (■) are shown. The [^{125}I]IHYP concentration was 25 pM and protein was approximately 100 μg/ml.

DISCUSSION

Exposure of S49 lymphoma cells to a combination of terbutaline and two phosphodiesterase inhibitors has allowed the selection of a unique variant. These new UNC clones have a heritable and stable alteration that results in uncoupling of the hormone binding and the catalytic function of the β-adrenergic receptor–adenylate cyclase system. Of note, despite the fact that a β-adrenergic agonist was utilized in the selection procedure, responsiveness to prostaglandin E_1 was also largely lost. We have not yet determined the status of the high affinity binding sites for prostaglandin E_1 in UNC cells (6, 20).

The mechanism by which UNC variants arise is unknown. The frequency of the AC$^-$ variation is high, and that of the UNC variation may not be much lower. We do not know if these phenotypic variations represent mutations and/or if there is some specific component(s) of TRM medium that is essential for the occurrence or isolation of UNC (in addition to AC$^-$) variants. The UNC phenotype was not detected in previous protocols that utilized isoproterenol and one inhibitor of phosphodiesterase (13). Also to be noted is the fact that a β-adrenergic-receptor-deficient variant has yet to be isolated; this is presumably a problem of the relative frequencies of the different lesions.

While we have described the UNC phenotype in this report, the molecular lesion that accounts for these properties is unknown. Current data suggest that guanine nucleotides are intimately involved in coupling the binding of hormone to the activation of adenylate cyclase (10). For example, purified wild-type S49 cell membranes fail to respond to isoproterenol or prostaglandin E_1 unless GTP or a related nucleotide is present, while the response to NaF is independent of nucleotide. Thus, an alteration or deletion of a hypothetical coupling factor that binds (and hydrolyzes?) GTP would be a logical first hypothesis to explain the UNC phenotype. Most probably, the relatively normal response to Gpp(NH)p in UNC cells rules out

Table 3. Adenylate cyclase activities and [^{125}I]IHYP binding in UNC 1 subclones*

Clone	[^{125}I]IHYP bound,† fmol/mg	Enzyme activity,‡ pmol/min per mg protein					
		Basal	NaF	Gpp(NH)p	GTP	GTP + isoproterenol	GTP + PGE_1
UNC 1-B	125	8	230	65	8	10	7
C	120	7	200	90	9	10	11
D	130	6	157	43	7	7	8
E	145	7	195	70	8	8	11
G	190	5	195	55	8	8	12
H	115	8	200	55	10	10	12
I	115	6	230	55	10	10	9
J	155	6	140	45	6	6	8
Wild type	55	10	100	130	10	200	160

* Enzyme and binding activities were measured as described with crude membrane fractions from eight subclones of UNC 1 (isolated in the absence of drugs) and from wild-type cells.
† [^{125}I]IHYP binding was determined at a saturating ligand concentration of 270 pM.
‡ Assay incubation time was 20 min and protein concentration was approximately 200 μg/ml. Other concentrations were as given in Table 2.

this conjecture. While it is not certain that GTP and Gpp(NH)p act at the same site, one would tend to assume that any guanine-nucleotide-related coupling component is at least present, if not unaltered.

There are an unfortunately great number of other logical possibilities to explain the UNC phenotype. Among these, any one of the known components of the system could be present but defective and incapable of interaction with certain others. An unknown component that is specifically required for the response could be missing or altered. Components of the membrane, protein or lipid, that are not integral parts of the system, but that impinge upon and influence it, could also be at fault. To be noted in this regard are the experiments of Rubalcava and Rodbell (7). These investigators found that treatment of rat hepatic plasma membranes with a preparation of phospholipase C that hydrolyzes acidic phospholipids caused a loss of glucagon-stimulated adenylate cyclase activity; enzyme activity stimulated by NaF was unaltered. Furthermore, there was a decreased affinity of binding of the agonist to such membranes, and the effect of GTP to enhance the rate of dissociation of bound glucagon was lost. Binding of the antagonist des-His^1-glucagon was unaltered. The similarity of these properties to those reported here is striking. This similarity may extend to the status of the phospholipids of the membranes.

These UNC clones and others that may be derived should serve as useful tools for the study of coupling of hormone receptor to adenylate cyclase. In addition, they might be a valid model of certain forms of transient refractory states that result from prolonged exposure to catecholamines or other hormones (21). Finally, with the availability of UNC clones, we now have two types of variants with different defects in the hormone-sensitive adenylate cyclase system (UNC and AC^-). It is hoped that techniques will be found that will allow reconstitution of a responsive system by complementation of such lesions. This would be an important step in learning how to resolve and to reconstitute a hormone-sensitive adenylate cyclase system.

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