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Beta Adrenergic Receptors and Adenylate Cyclase: Products of Separate Genes?

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SUMMARY

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Wild-type S49 mouse lymphoma cells produce adenosine 3',5'-monophosphate when exposed to the *beta* adrenergic agonist isoproterenol; treatment with this agent in the presence of a phosphodiesterase inhibitor is cytocidal. Particulate preparations from such cells have adenylate cyclase activity and bind [^{125}I]iodohydroxybenzylpindolol, a potent *beta* adrenergic antagonist that can be used to examine *beta* adrenergic receptors. The binding of this ligand is rapid and reversible; Scatchard analysis gives results compatible with a single class of binding sites with a K_D of 33 pM. *Beta* adrenergic agonists and antagonists compete for radioactive ligand binding sites stereoselectively and at concentrations that correlate closely with those required for activation or inhibition of activation of adenylate cyclase. A clone has been isolated from wild-type cells that is resistant to isoproterenol-induced cyclic AMP killing. This clone has no detectable adenylate cyclase activity in response to isoproterenol, prostaglandin E_1, or sodium fluoride. Nevertheless, the characteristics of binding of [^{125}I]iodohydroxybenzylpindolol by this adenylate cyclase-negative clone resemble those of wild-type preparations. The similarity of binding by these two clones extends to the kinetics of binding, the affinity for ligand, and the potency of competitors for binding sites. Both clones have 200-300 receptor sites/cell. Another clonal cell line, HC-1 (a rat hepatoma), is phenotypically similar to the adenylate cyclase-negative lymphoma cell; enzyme activity is not detectable, although binding activity characteristic of the *beta* adrenergic receptor is present. The existence of *beta* adrenergic receptor activity in cells that appear to lack adenylate cyclase suggests that the *beta* adrenergic recognition (receptor) site is a product of a gene different from that coding for the enzymatic component that generates cyclic AMP.

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INTRODUCTION

Catecholamines, histamine, prostaglandins, and multiple polypeptide hormones presumably regulate their specific actions by interacting with plasma membrane receptors. Activation of the membrane-bound enzyme adenylate cyclase (EC 4.6.1.1) then occurs, and increased cellular concentrations of adenosine 3′,5′-monophosphate result. Numerous studies support the concept that the receptors for such hormones or autacoids and adenylate cyclase are intimately linked (1–4). Yet the two functions can be separated, as has been demonstrated by the selective effects of enzymatic treatment or hormone withdrawal on specific hormone activators of adipocyte adenylate cyclase (5, 6), the apparent dissociation of hormone binding and activation of adenylate cyclase by alterations in calcium concentrations (7) or by enzymatic (6, 8) or antibiotic (9) treatment of membranes, and the uncoupling of hormone binding and adenylate cyclase that is seen in solubilized membrane preparations (10). However, to date the drug or hormone receptor and the cyclic AMP-generating portion of adenylate cyclase have not been demonstrated to be separate molecules.

We have recently isolated a variant clone of S49 mouse lymphoma cells that is defective in its ability to generate cyclic AMP, both in intact cells and in particulate preparations (11). This clone appears to lack adenylate cyclase, since particulate preparations will not produce cyclic AMP in the presence of hormones (prostaglandin E_1 or catecholamines) or of sodium fluoride, all of which are potent activators of the enzyme in wild-type cells. This variant clone offered a novel means to examine whether or not receptor function is independent of a functional or structural change in the catalytic activity of adenylate cyclase. [^{125}I]Iodohydroxybenzylpindolol, a potent *beta* adrenergic antagonist of high specific activity, has been used as a ligand to study binding to *beta* adrenergic receptors in several types of cells (12, 13). Preparations of the wild-type cells and the adenylate cyclase-negative clone are shown here to have similar binding characteristics for [^{125}I]IHYP[7] with respect to the kinetics of the process, stereoselectivity, affinity, and the number of receptors per cell. Another clonal cell line, HC-1, which has undetectable adenylate cyclase activity, is also shown to have binding sites for [^{125}I]IHYP that display the characteristics required of a *beta* adrenergic receptor. These results indicate that the *beta* receptor and the cyclic AMP-generating components of the adenylate cyclase system may be products of separate genes.

MATERIALS AND METHODS

Cells. S49 mouse lymphoma tissue culture cells (14), obtained from the Salk Institute, were propagated in Dulbecco's modified Eagle's medium with 10% heat-inactivated horse serum, as previously described (15). The adenylate cyclase-negative clone described here was selected from a population of wild-type cells that were cloned in soft agar that contained 1 μM isoproterenol and 60 μM Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone], a potent inhibitor of cyclic AMP phosphodiesterase (16).

HC-1 cells, a 6-thioguanine-resistant clone derived from the HTC rat hepatoma cell line (17), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Particulate preparations. Cells were counted, washed in Puck's saline G at 4° (without divalent cations), and suspended in 5 mM HEPES (Na^+, pH 8.0) and 1 mM $MgSO_4$. HC-1 cells were washed as above, scraped from the dish, and suspended in the same buffer. Cells were left undisturbed for 5 min and then disrupted in a Dounce homogenizer. The broken cell

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[7] The abbreviations used are: [^{125}I]IHYP, [^{125}I]iodohydroxybenzylpindolol; HEPES, *N*-2-hydroxyethyl-*N*′-2-ethanesulfonic acid.

preparation was centrifuged at 20,000 × *g* for 30 min, and the supernatant was discarded. The pellet was resuspended in 5 mM HEPES (pH 8.0) and 1 mM $MgSO_4$, and aliquots of particulate material were then rapidly frozen in a Dry Ice–acetone bath and stored at −70°. [^{125}I]IHYP binding and adenylate cyclase activity were stable under such conditions for at least 4 weeks; some preparations included 2 mM dithiothreitol, which further enhanced the stability of the adenylate cyclase activity.

Adenylate cyclase assay. Particulate adenylate cyclase activity was determined by a previously published procedure (13, 18). In the present studies the procedure was modified as follows. ATP was used as the substrate, and the product, cyclic AMP, was measured by the competitive protein binding method (19). The total volume of assay incubations was 100 μl. Each tube contained 30–60 μg of enzyme protein, 4 mM $MgSO_4$, 100 μg of bovine serum albumin, 50 mM HEPES (pH 8.0), 0.2 mM 1-methyl-3-isobutylxanthine, and 1 mM ATP. Incubations were initiated by adding the enzyme and continued for 20 min at 30°. The reactions were terminated by adding acetic acid (50 μl, 150 mM). The amount of cyclic AMP was determined by comparing unknown samples with standards prepared in the same reagents. Adenylate cyclase activity was linear with respect to time of incubation and protein concentration under these conditions.

[^{125}I]IHYP binding. Binding of [^{125}I]-IHYP was determined by incubating particulate membrane protein (20–150 μg), 50 mM HEPES (pH 8.0), 4 mM $MgSO_4$, 20,000–60,000 cpm of [^{125}I]IHYP (approximately 10–30 pM), and other compounds, as specified, in a final volume of 500 μl, as previously described (13). Binding reactions were continued for at least 30 min at 30°. Samples were then filtered and washed over 25-mm Gelman type A/E glass filters with 25 ml of 20 mM K_2HPO_4 (pH 8.0)–1 mM $MgSO_4$ buffer at 37°, as previously described (13). The radioactivity on the filters was measured either in a γ counter or in a liquid scintillation spectrometer. Specific binding was defined as the difference between [^{125}I]IHYP bound by preparations incubated in the absence and presence of 10 μM (−)-propranolol. Data reported here were derived from duplicate to quadruplicate samples. Specific binding was linear with respect to protein concentrations under the conditions used in these experiments.

Protein. The concentration of protein was determined by the method of Lowry *et al.* (20), with bovine serum albumin standards dissolved in 5 mM HEPES and 1 mM $MgSO_4$.

Materials. Hydroxybenzylpindolol was a gift from Dr. G. D. Aurbach, National Institutes of Health, or Dr. D. Hauser, Sandoz Pharmaceuticals, Basel. [^{125}I]IHYP was prepared as described previously (13). All other chemicals were obtained from standard sources, with the exception of the following drugs, which were gifts: (+)- and (−)-propranolol, from Ayerst Research Laboratories; (±)-pindolol, from Sandoz, Inc.; (+)-isoproterenol, from Sterling-Winthrop Research Institute; and Ro 20-1724, from Dr. H. Sheppard, Hoffmann-La Roche.

RESULTS

Adenylate cyclase activity. Intact wild-type S49 cells increase their concentration of cyclic AMP by more than 50-fold after exposure to isoproterenol; they respond similarly to cholera toxin, with prostaglandin E_1 producing a smaller response (11, 16). Adenylate cyclase activity in particulate preparations from such cells is stimulated by *beta* adrenergic agonists, prostaglandin E_1, or sodium fluoride. Stereoselectivity of the *beta* adrenergic receptor, with preference for the (−) isomer of isoproterenol, is readily demonstrated (Fig. 1 and Table 1); propranolol inhibits isoproterenol-stimulated adenylate cyclase activity with a similar degree of discrimination between isomers (Table 1). In contrast, adenylate cyclase activity of the variant clone cannot be observed in particulate preparations in the presence of any of the compounds that stimulate wild-type cells (11). Similarly, intact cells from this variant clone do not accumulate cyclic AMP when incubated with isoproterenol, cholera toxin, or prostaglandin E_1.

Kinetics of [^{125}I]IHYP binding. As has been found in other cultured cell lines (13), nonspecific binding in the presence of 10 μM (−)-propranolol is independent of time of incubation, but the specific binding of [^{125}I]IHYP reached equilibrium by 30 min under the conditions described in MATERIALS AND METHODS. All subsequent incubations were continued for at least that length of time. Wild-type and adenylate cyclase-negative cells appeared to have identical rates of binding (data not shown). The rate of release of bound ligand from receptor was measured by adding 10 μM (−)-propranolol to the particulate preparations after equilibration with [^{125}I]IHYP for 30 min (Fig. 2). Wild-type and adenylate cyclase-negative cells had

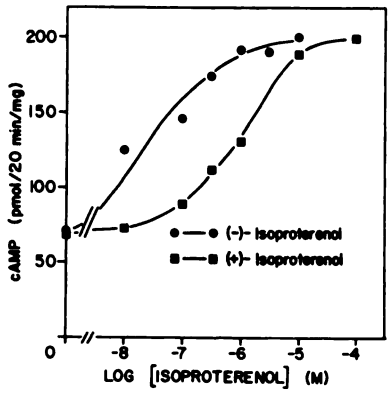

FIG. 1. *Stimulation of wild-type S49 adenylate cyclase by (+)- and (−)- isoproterenol*

The protein concentration was 570 μg/ml. Data are expressed as picomoles of cyclic AMP (cAMP) generated per 20 min per milligram of protein. This preparation, which was used to maximize the yield of *beta* receptors, produced a specific activity of adenylate cyclase that was lower than previously reported (11).

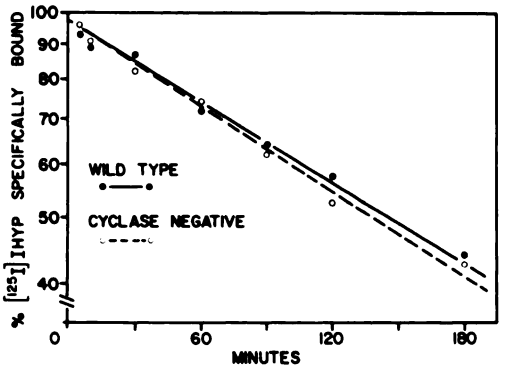

FIG. 2. *Reversal of [^{125}I]IHYP binding in wild-type and adenylate cyclase-negative particulate preparations*

Binding was reversed by adding 10 μM (−)-propranolol after 30 min of incubation with 20 pM [^{125}I]IHYP. The data shown are the means of at least three experiments (standard deviations are all within 11% of the means).

TABLE 1

Comparison of adenylate cyclase and [^{125}I]IHYP binding in wild-type and adenylate cyclase-negative S49 lymphoma clones

The data shown are the means of at least two experiments for each determination, with a range of less than 3-fold for K values. Adenylate cyclase activity was measured as described in MATERIALS AND METHODS. The concentrations of isoproterenol are those required to give 50% activation. The propranolol concentrations are the K_I values calculated from the concentration required for 50% inhibition of activity in the presence of 0.3 μM *l*-isoproterenol (I_{50}): $K_I = I_{50} \cdot K_D/(S + K_D)$, where K_D is the activation constant for isoproterenol (20 nM) and S is the concentration of isoproterenol (0.3 μM). The K_I values for inhibition of [^{125}I]IHYP binding were determined from the concentration of drug required for 50% inhibition of specific [^{125}I]IHYP binding (I_{50}) as above, where K_D is 33 pM for wild-type and 34 pM for adenylate cyclase-negative clones and S is the concentration of [^{125}I]IHYP in each experiment (10–40 pM).

Compound	Adenylate cyclase activation and inhibition (wild-type)	Inhibition of [^{125}I]IHYP binding (K_I)	
		Wild-type	Adenylate cyclase-negative
	M	*M*	*M*
(−)-Isoproterenol	2×10^{-8}	2×10^{-8}	
(+)-Isoproterenol	1×10^{-6}	1×10^{-6}	
(−)-Propranolol	5×10^{-10}	6×10^{-10}	6×10^{-10}
(+)-Propranolol	4×10^{-8}	1×10^{-7}	1×10^{-7}
Hydroxybenzylpindolol		6×10^{-10}	6×10^{-10}
Pindolol		7×10^{-10}	5×10^{-10}

similar half-times of dissociation (approximately 2 hr). Although data from some experiments indicated that an additional, small (less than 10%), rapidly reversible component of specific binding was present, it was difficult to quantify and was less than that noted for C6TG1A cells (13).[8]

Stereoselectivity of binding and competition for receptor. Since determinations of the effect of activation of *beta* adrenergic receptors indicate that it is selective for (−) isomers of both agonists and antagonists, similar selectivity is a necessary requirement for a radiolabeled ligand that is used to demonstrate binding to the *beta* adrenergic receptor. In S49 cells, (−)-propranolol competes with [^{125}I]IHYP with 80 times the affinity of (+)-propranolol; this is true in both wild-type and adenylate cyclase-negative cells (Fig. 3). Similarly, in wild-type cell preparations, (−)-isoproterenol inhibited [^{125}I]IHYP binding by 50% at 50 times less than the concentration required for (+)-isoproterenol (Table 1). These values for propranolol and isoproterenol agree well with those required for agonist activation and antagonist inhibition of adenylate cyclase in these wild-type cells (Table 1). Similarity of binding sites in wild-type and adenylate cyclase-negative cells was also shown by essentially identical patterns of inhibition of binding of [^{125}I]IHYP in the two clones by both hydroxybenzylpindolol and pindolol (Table 1).

Concentration studies with [^{125}I]IHYP (Fig. 4). In four experiments, when [^{125}I]IHYP was incubated with wild-type and adenylate cyclase-negative cells, the apparent dissociation constants of binding were 33 and 34 pM, respectively. The maximal binding capacity was 26 fmoles/mg of protein in wild-type preparations and 30 fmoles/mg for adenylate cyclase-negative cells. The average yield of protein in this preparation of S49 cells is 144 μg/10^7 cells. When this value is used, the total number of binding sites per cell is calculated to be 230 for wild-type and 260 for the mutant clone. Thus both the affinity for ligand and the number of receptor sites of these two clones are very similar.

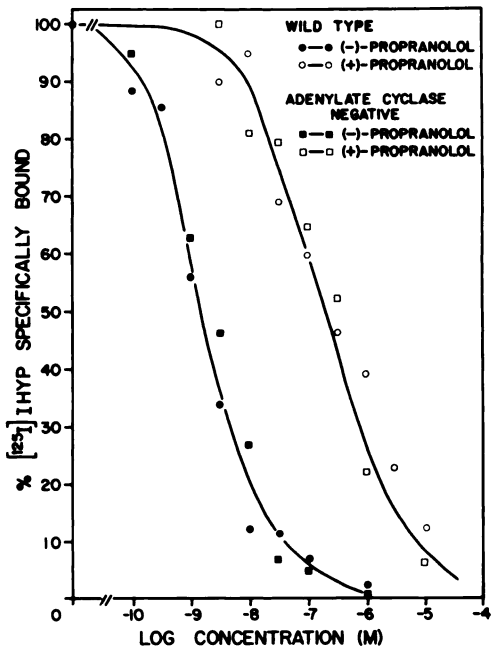

FIG. 3. *Competition for [^{125}I]IHYP binding sites by (+)- and (−)-propranolol in wild-type and adenylate cyclase-negative particulate preparations*

Protein concentrations were 153 μg/ml for wild-type and 109 μg/ml for adenylate cyclase-negative preparations. The [^{125}I]IHYP concentration was 23 pM. The data are plotted as the percentage of specific [^{125}I]IHYP binding (see MATERIALS AND METHODS).

Beta receptors in clone HC-1. Clone HC-1 has no detectable adenylate cyclase activity (less than 0.5 pmole of cyclic AMP generated per minute per milligram of protein, which is the limit of sensitivity of the assay), although intact cells do have trace concentrations of cyclic AMP (less than 1 pmole/mg of protein). However, these cells exhibit [^{125}I]IHYP binding that suggests the presence of a *beta* receptor. The K_d for [^{125}I]IHYP is about 35 pM (Fig. 5), and inhibition of binding by isoproterenol or propranolol is stereoselective and occurs at concentrations similar to those observed above (Fig. 6). The specific activ-

[8] The rate of association observed in S49 cells is somewhat faster than predicted from the data obtained in concentration and reversal experiments (predicted half-time of association, 60–70 min). This apparent discrepancy might result either from the variability in individual determinations of K_d and k_{-1} or, hypothetically, from the time-dependent formation of a second species of ligand-receptor complex with greater affinity for ligand and a slower rate of dissociation.

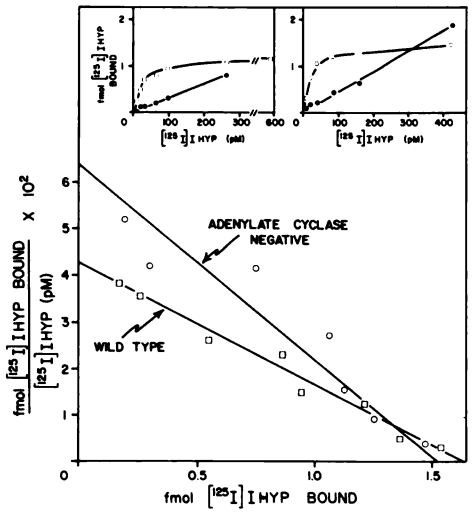

FIG. 4. *Concentration dependence of binding of [125I]IHYP to wild-type and adenylate cyclase-negative particulate preparations*

The insets depict femtomoles of [125I]IHYP bound as a function of [125I]IHYP concentration. □ and ○, specific binding (see MATERIALS AND METHODS); ●, binding in the presence of 10 μM (−)-propranolol. The main figure is a Scatchard plot of specific [125I]IHYP binding. The protein concentrations were 125 μg/ml for wild-type and 60 μg/ml for adenylate cyclase-negative incubations. The means of four such experiments were 33 and 34 pM for the dissociation constant and 26 and 30 fmoles/mg of maximal binding capacity for wild-type and adenylate cyclase-negative clones, respectively.

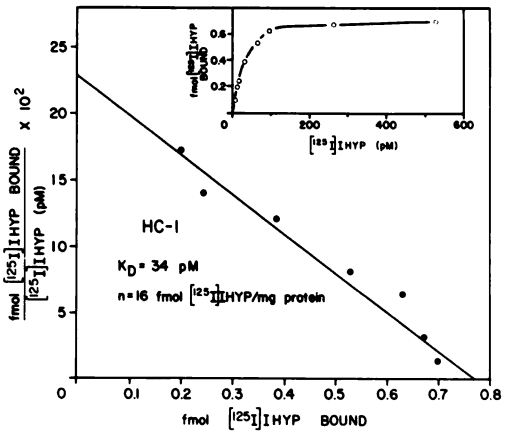

FIG. 5. *Concentration dependence of binding of [125I]IHYP to particulate preparations of HC-1*

The inset shows femtomoles of [125I]IHYP specifically bound vs. concentration of [125I]IHYP. The main figure is a Scatchard plot of specific [125I]IHYP binding. The protein concentration was 97 μg/ml.

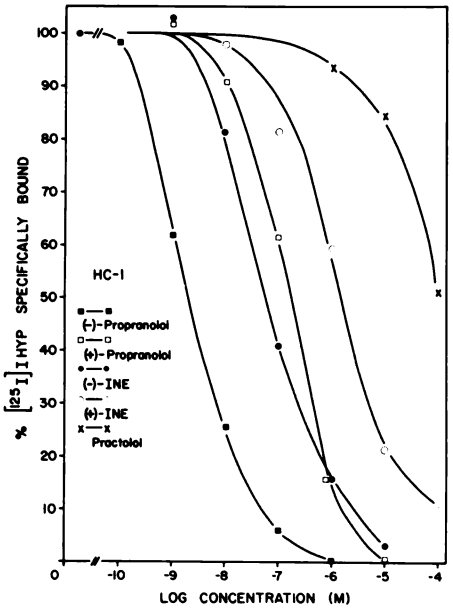

FIG. 6. *Competition for [125I]IHYP binding sites in HC-1 particulate preparations*

The protein and [125I]IHYP concentrations were 106 μg/ml and 35 pM, respectively. The data are plotted as the percentage of specific [125I]IHYP binding (see MATERIALS AND METHODS).

ity of [125I]IHYP binding sites is similar to that determined for the lymphoma cells, although the larger HC-1 cell appears to contain nearly 1000 such sites per cell. Binding studies thus suggest that these cells possess *beta* adrenergic receptors. While the function of these binding sites cannot be monitored in this adenylate cyclase-deficient clone, cell hybridization studies support the hypothesis that these binding sites are in fact capable of acting as functional receptors.[9]

DISCUSSION

These findings further demonstrate the usefulness of [125I]IHYP binding as an assay for *beta* adrenergic receptors. The time course, stereoselectivity, and close correlation of [125I]IHYP binding with activation of adenylate cyclase in S49 cells are similar to results noted in previously studied cell lines (13). Thus specific binding sites for [125I]IHYP would appear to be identical

[9] L. L. Brunton, M. E. Maguire, H. J. Anderson, and A. G. Gilman, manuscript in preparation.

with functional *beta* adrenergic receptors, although the information available to date is only indirect.

The results in S49 cells demonstrate that such *beta* adrenergic receptors are present both in wild-type cells and in a clone with undetectable adenylate cyclase activity. These observations are complemented by those with the HC-1 cell, a hepatoma clone that was fortuitously discovered to be phenotypically similar to the adenylate cyclase-deficient S49 cell. Although parent HTC cells have been shown to possess catecholamine-responsive adenylate cyclase activity under certain conditions (21), HC-1, a 6-thioguanine-resistant clone, has not exhibited such enzyme activity. The absence of [^{125}I]IHYP binding from other tissue culture lines, such as RAG, B82, and N4TG1 (13), indicates that such binding is not simply a property common to all cultured cells.

The most straightforward explanation of these findings is that the receptor for *beta* adrenergic amines and the component of adenylate cyclase that generates cyclic AMP are coded for by separate genes, since the adenylate cyclase-negative S49 clone was derived from its wild-type parent in a single-step selection procedure. This is plausible, since the clone lacks cyclase catalytic activity and presumably arose by gene mutation. The observed findings, however, do not distinguish between the hypothesis of distinct genes for receptor and catalytic cyclase and other explanations for the persistence of receptors despite loss of cyclase activity. For example, both functions could be mediated by a protein, the product of a single gene, that is structurally altered in such a way as to affect catalytic, but not binding, function. Alternatively, the loss of adenylate cyclase catalytic activity could result from a nonspecific change in membrane structure or function, from a post-translational alteration in catalytic adenylate cyclase, from production of a modulator substance, or from loss of a necessary, but currently unknown, cofactor for the enzyme. Previous work demonstrated no soluble modulator produced by the adenylate cyclase-negative clone (11). These and other hypotheses must await further clarification of the lesion(s) in that clone. By analogy, however, with results from other S49 mutant clones that are resistant to the cytocidal effect of dibutyryl cyclic AMP and that have structurally altered cyclic AMP-dependent protein kinase (15, 16, 22, 23), a reasonable hypothesis is that the adenylate cyclase-negative clone may be the result of a single-step mutation affecting its catalytic cyclase component.

This conclusion is partially dependent on the observation that such a clone does indeed lack adenylate cyclase. In previous experiments in which we used the protein binding assay, we detected no cyclic AMP production in broken cell preparations in either particulate or supernatant fractions (11); more recently, we have found no hormone-, sodium fluoride-, or guanylylimidodiphosphate (alone or with hormone)-stimulated accumulation of cyclic AMP, even when the more sensitive acetylation-radioimmunoassay was used (24). Nevertheless, this clone does contain "basal" cyclic AMP at a concentration that is approximately 30% of wild-type values, as determined both by the protein binding assay and by acetylation-immunoassay. The cyclic AMP in the adenylate cyclase-negative clone is eluted as cyclic AMP from Dowex columns and is destroyed by phosphodiesterase (11). If this clone does lack adenylate cyclase, the origin of the cyclic AMP remains unknown. In membrane preparations made from multiple tissues, adenylate cyclase is usually rather labile. While this may explain our inability to detect such activity in broken cells, even in the presence of sodium fluoride, it would not explain the lack of response of intact cells to appropriate stimuli. However, such a cell could possess functionally inactive adenylate cyclase and still have a functional receptor component. A measure of catalytic adenylate cyclase components independent of the generation of cyclic AMP (e.g., an antibody to the enzyme) would be necessary to prove such a hypothesis; this is not currently available.

Definitive proof of the hypothesis that the receptor and the catalytic moiety are separately coded genetic entities will re-

quire the isolation and purification of the two postulated membrane components. Thus far, biochemical procedures have not yet resolved and characterized the putative proteins that function to recognize hormonal stimuli and to generate cyclic AMP. Using the genetic approach, we hope to isolate a mutant that lacks *beta* adrenergic receptors but that maintains responsiveness of adenylate cyclase to other agents. Studies with such a mutant would further substantiate our findings that the receptor and catalytic cyclase components have separate genetic origins.

ACKNOWLEDGMENTS

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