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Isolation and Characterization of S49 Lymphoma Cells Deficient in β-Adrenergic Receptors: Relation of Receptor Number to Activation of Adenylate Cyclase

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SUMMARY

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Variant S49 lymphoma cells that have a diminished response to *beta*-adrenergic amines were selected from wild-type clones, using the *beta*-adrenergic agonist terbutaline in the presence of phosphodiesterase inhibitors. Variants (termed $beta_p$ variants) having approximately 50% of wild-type *beta*-adrenergic receptors and 50% catecholamine-stimulated adenylate cyclase activity relative to wild-type were isolated in a single step selection. When these $beta_p$ variants were mutagenized and subjected to a second round of selection with terbutaline and phosphodiesterase inhibitors, receptor deficient variants (termed $beta_d$) expressing 10–30% of wild-type *beta*-adrenergic receptors and responsiveness to isoproterenol were isolated. Two independent $beta_p$ clones and the respective $beta_d$ clones selected from them were characterized with respect to their adenylate cyclase and receptor properties. Both $beta_p$ and $beta_d$ variant cells demonstrated a diminished response to isoproterenol and an increased response to PGE_1 relative to wild-type. Membranes from all four receptor-deficient clones responded normally to Gpp(NH)p, NaF, and PGE_1.

The density of *beta*-adrenergic receptors in membranes measured by the specific binding of [^{125}I]IHYP was between 40 and 65%, relative to wild-type, for clones $beta_{p1}$ and $beta_{p2}$ and from 10 to 30% in $beta_{d1}$ and $beta_{d2}$. The dissociation constant for agonist and antagonist binding was the same for receptor-deficient and wild-type membranes. A direct relationship was obtained between *beta*-adrenergic receptor number and maximal activation of adenylate cyclase, indicating that *beta*-adrenergic receptor number limits the maximal activation of adenylate cyclase by catecholamines. These results conflict with the finding that isoproterenol stimulates S49 adenylate cyclase at concentrations well below those required to produce an equivalent fractional occupancy of receptors. The $beta_p$ and $beta_d$ phenotypes rule out an explanation of this discrepancy that depends on a number of receptors in excess of that required for maximal stimulation.

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INTRODUCTION

Interaction of catecholamines with *beta*-adrenergic receptors causes stimulation of adenylate cyclase (E.C. 4.6.1.1) activity in membranes of many animal cells (1). Hormone receptors and catalytic adenylate cyclase have been shown to be physically distinct entities (2, 3), but the functional stoichiometry of receptors and cyclase remains obscure. *Beta*-adrenergic receptors can be enumerated using specific radio-ligand binding assays (4–6), but no method currently exists for determining the number of adenylate cyclase molecules. The number of *beta*-adrenergic receptors of cells that respond to catecholamines varies from approximately 400 to 80,000 per cell (7–10). The efficacy of catecholamines to stimulate adenylate cyclase of intact cells or plasma membrane preparations varies over a similar 200-fold range (9, 11–14). In several systems, however, the concentration of hormone required to maximally activate adenylate cyclase is significantly less than that required for maximal occupation of receptors (15–17). This discrepancy between receptor occupation and enzyme activation exists even when receptor number is relatively low and hormone-stimulated adenylate cyclase activity is high (for review, see reference 18). One interpretation of this discrepancy is that cells possess a large number of receptors, only a fraction of which are required for maximal activation of adenylate cyclase. The classic concept of excess or "spare" receptors was first suggested in measurement of contractile responses to histamine or muscarinic agents, such as acetylcholine (19–21).

The possibility that receptors are present in excess of the number required to give a maximal response can be tested by reducing the number of receptors without altering other components of the adenylate cyclase system. If the discrepancy between receptor occupation and enzyme activation persists in cells with decreased receptor number, then this discrepancy cannot be explained only by an excess of receptors. Cultured S49 mouse lymphoma cells provide a system in which we can determine whether receptor number limits maximal response to hormone. The fact that S49 cells are killed by cAMP[3] allows clonal selection by agents that elevate intracellular cAMP levels (22). We have used *beta*-adrenergic agonists to isolate S49 variants deficient in *beta*-adrenergic responsiveness. All of the variants having a decreased number of *beta*-adrenergic receptors also have a diminished adenylate cyclase activation by *beta*-adrenergic agonists. The characterization of such variants permits a novel approach to studying the functional relationship between receptor number and activation of adenylate cyclase.

MATERIALS AND METHODS

Cell culture. Clonal wild-type S49 lymphoma cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (23).

Mutagenesis. Mutagenic changes in S49 cells were produced by exposure to ICR 191 (0.8 μg/ml) for 24 hr or nitrosoguanidine (2 μg/ml) for 3 hr as described previously (24). Cells were exposed to mutagens during logarithmic growth of cultures and maintained 6 to 8 days before being subjected to selective pressure.

Selection of variants. Clones deficient in *beta*-adrenergic responsiveness were isolated from wild-type S49 cells by a modification of the procedures described for the isolation of adenylate cyclase deficient (25) and uncoupled (26) S49 variant phenotypes. Wild-type cells were incubated for 24 hr in the presence of 0.5 mM dbcAMP, 50 μM 1-methyl-3-isobutylxanthine and 50 μM RO 20-1724, washed twice by dilution and low speed centrifugation, and resuspended in growth medium containing 0.1 mM terbutaline and 50 μM 1-methyl-3-isobutylxanthine and 50 μM RO 20-1724 (designated TRM medium as described by Haga *et al.* [26]). The cells were maintained in TRM

[3] Abbreviations used are cAMP, adenosine 3′:5′-monophosphate; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [^{125}I]IHYP, [^{125}I]iodohydroxybenzylpindolol ([±]-iodo-3-indoloxyl-1-[2-p-hydroxybenzylpropyl-2-amino]-isopropanol); PGE_1, prostaglandin E_1; RO20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imido-zolidinone; TRM, terbutaline (0.1 mM) + RO20-1724 (0.05 mM) + 1-methyl-3-isobutylxanthine (0.05 mM).

for 24 hr and then cloned in agarose containing TRM. Clones were picked 8 to 10 days later and grown in the absence of TRM for at least 10 generations before cells were screened for cAMP accumulation or [^{125}I]IHYP binding.

cAMP accumulation. Cells grown in suspension culture to a density of approximately 10^6 cells per ml were washed and suspended in Dulbecco's modified Eagle's medium containing 0.1% heat-inactivated horse serum, 20 mM NaHEPES (pH 7.4) and 0.5 mM 1-methyl-3-isobutylxanthine at a density of 10^6 cells per ml. The cells were allowed to equilibrate for 10 min at 37° and then challenged with isoproterenol or PGE_1 for 10 min or cholera toxin for 90 min. Basal values were determined in the presence of 0.5 mM 1-methyl-3-isobutylxanthine. After the appropriate incubation period the cells were centrifuged at 1,300 × *g* for 2 min, the media aspirated and the cells suspended in 0.5 ml 50 mM sodium acetate, pH 4.0, containing 0.2 mM 1-methyl-3-isobutylxanthine, and immediately placed in a boiling water bath for 5 min. The samples were then centrifuged at 1300 × *g* for 5 min and 100 μl aliquots were removed for the measurement of cAMP by the method of Gilman (27).

Plasma membrane preparation. Plasma membranes were prepared by the method of Ross *et al.* (15), with modification as described previously (28). Adenylate cyclase in membrane preparations was activated by cholera toxin as described elsewhere (28).

Adenylate cyclase. Adenylate cyclase was measured exactly as described previously (28). Briefly described, ATP (1 mM) was used as substrate in a reaction mixture of 50 mM NaHEPES, 4 mM $MgCl_2$, 0.2 mM 1-methyl-3-isobutylxanthine, 5 mM 2-mercapto-ethanol, 0.2 mg/ml bovine serum albumin, pH 8.0, at 30° in a final volume of 100 μl. Incubations were initiated by addition of membranes (approximately 10 μg membrane protein) continued for 10 min at 30° with constant shaking and terminated by the addition of 50 μl 150 mM acetic acid. Cyclic AMP was then measured by the method of Gilman (27). Under these conditions NaF-stimulated activity was linear with time and protein concentration.

[^{125}I]IHYP assay. [^{125}I]IHYP binding was measured by a filtration assay described elsewhere (7). For membranes, incubations were conducted for either 30 or 60 min at 30° in the same medium used for adenylate cyclase measurements except that 0.1 mg/ml Na ascorbate was present. The Na ascorbate had no effect when present in the adenylate cyclase assay. Generally, 10 μg membrane protein was used in a final volume of 100 μl. For intact cells, incubations were conducted in Dulbecco's modified Eagle's medium containing 0.1% heat-inactivated horse serum and 0.1 mg/ml Na ascorbate. Cells (10^6) were incubated at 37° for 60 min in a final volume of 100 μl. A detailed examination of the intact cell binding assay will be reported elsewhere (29). Specific binding was defined for both membrane preparations and intact cell binding studies as the difference between bound [^{125}I]IHYP in the absence and presence of 1 μM (—)-propranolol.

Protein determinations. Protein was measured by the method of Lowry *et al.* (30) using bovine serum albumin as standard.

Materials. Hydroxybenzylpindolol was a gift from Dr. G. D. Aurbach of the National Institutes of Health; (—)-propranolol was provided by Ayerst Research Laboratories; RO 20-1724 was a gift from Dr. H. Sheppard, Hoffman-La Roche; PGE_1 was supplied by Dr. J. Pike, Upjohn. [^{125}I]IHYP was synthesized as described previously (31).

RESULTS

Isolation of variants deficient in beta-adrenergic responsiveness. When S49 cells are exposed to *beta*-adrenergic agonists the elevation of cAMP levels is transient even in the presence of maximally effective concentrations of agonist and phosphodiesterase inhibitors (32). Because of this transient response, generally termed refractoriness or desensitization, *beta*-adrenergic agonists are relatively inefficient killing agents for S49 cells. A further complication of the isolation of *beta*-adrenergic receptor-deficient variants is the high frequency of occurrence of S49 variants that are deficient

in adenylate cyclase activity (cyc$^-$) or uncoupled (UNC) (26) with respect to the ability of hormones to activate adenylate cyclase.

In an attempt to increase the killing ability of *beta*-adrenergic agonists, wild-type S49 cells were first exposed to dbcAMP. The death of S49 cells exposed to dbcAMP is preceded by a reversible G_1 growth arrest (33). When S49 cells are first exposed to dbcAMP for 24 hours and the drug is then removed by washing, a significant percentage of the cells survive. However, if S49 cells are exposed to a *beta*-adrenergic agonist in the presence of phosphodiesterase inhibitors immediately after removal of the dbcAMP, the killing of wild-type cells is significantly greater than that caused by exposure to either dbcAMP or the *beta*-adrenergic agonist alone. Table 1 shows the cloning efficiencies of wild-type, protein kinase deficient (34), cyc$^-$ and UNC variant cells treated according to this protocol. The selective pressure exerted by dbcAMP and subsequent exposure to TRM is specific for cells that are both capable of responding to *beta*-adrenergic agonists with a rise in cAMP levels and possess a functional cAMP-dependent protein kinase.

TABLE 1

Cloning Efficiency (%) of S49 Cells

Cells were incubated for 24 hours in the presence of RO 20-1724 and 1-methyl-3-isobutylxanthine (50 μM each) in the presence or absence of dibutyryl cAMP (0.5 mM). The cells were then washed and aliquots incubated an additional 24 hours with terbutaline (0.1 mM) and RO 20-1724 and 1-methyl-3-isobutylxanthine (TRM) or in the presence of the phosphodiesterase inhibitors alone. The cells were then cloned in soft agarose. Cells exposed to TRM in suspension were also cloned in TRM. Cloning efficiency was determined 8 days later.

Cell Type	Selective Agents			
	Control	Dibutyryl cAMP only	TRM only	dibutyryl cAMP – TRM
Wild-type	18	3	2	.004
Protein kinase deficient	50	50	55	44
cyc$^-$[a]	13	4	12	2
UNC[b]	27	10	36	2

[a] Deficient in adenylate cyclase activity.

[b] Uncoupled with respect to the ability of hormones to activate adenylate cyclase.

To reduce the high background of cyc$^-$ variants, a screening procedure was developed to distinguish potential variants deficient in *beta*-adrenergic responsiveness from cyc$^-$. Cholera toxin, an effector known to activate adenylate cyclase independent of *beta*-adrenergic receptors (35), kills S49 cells that have a functional adenylate cyclase. Of the clones that survive dbcAMP-TRM selection, only those that are also susceptible to killing by cholera toxin are of potential interest as variants specifically deficient in *beta*-adrenergic stimulation.

Figure 1 summarizes the selection procedures used to isolate variants deficient in *beta*-adrenergic responsiveness. Clones picked from the dbcAMP-TRM selection procedure were grown in small flasks and aliquots used to measure their susceptibility to killing by cholera toxin. Only the clones susceptible to cholera toxin killing were screened for their cAMP accumulations in response to isoproterenol, PGE_1 and cholera toxin. Measurement of the number of *beta*-adrenergic receptors relative to wild-type was determined using a whole cell binding assay with [^{125}I]IHYP (50 pM) for intact S49 cells (29). The majority (80 to 90%) of the clones screened for cAMP accumulation and [^{125}I]IHYP binding appeared to be indistinguishable from wild-type. However, three clones from experiment 1 and 4 clones from experiment 2 were found to have approximately 50% fewer [^{125}I]IHYP binding sites and a 50% decrease of maximal cAMP accumulation in response to isoproterenol. The variants partially deficient in *beta*-adrenergic responsiveness (termed $beta_p$) were subjected to a second round of selection because we suspected that they might be heterozygous for a normal *beta*-adrenergic receptor gene. Because mutations specifically decreasing *beta*-adrenergic responsiveness appeared to be rare, the cells were first mutagenized with ICR 191, a known frameshift mutagen in prokaryotes (36) that has been demonstrated to increase the frequency of specific mutations in S49 cells (23). The mutagenized $beta_p$ variants were then cloned in TRM medium. The cloning efficiency in

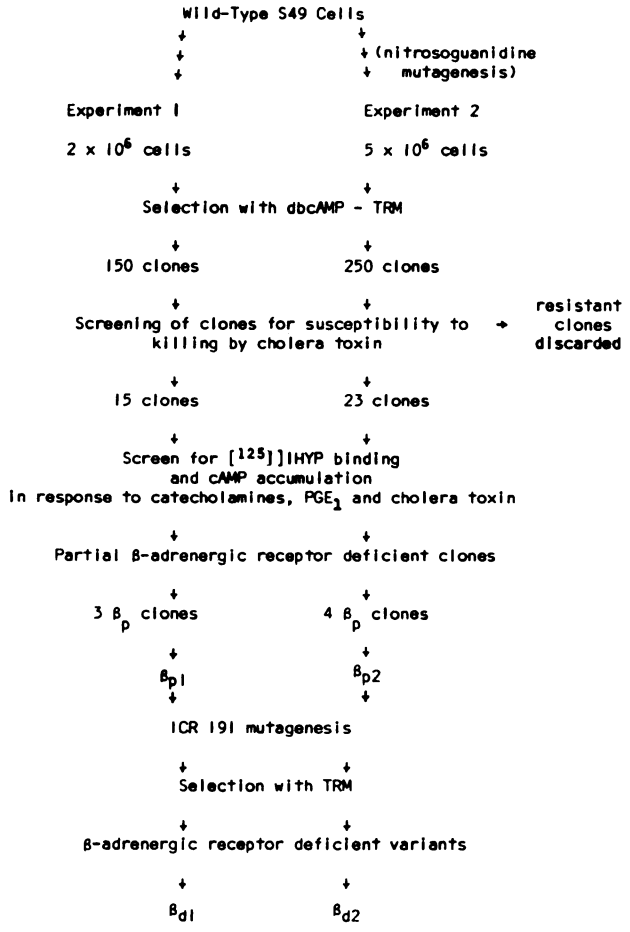

FIG. 1. *Protocol for selection of variant clones deficient in beta-adrenergic receptors* See text for explanation.

TRM medium was approximately 10% of that in dishes containing the control medium with only the phosphodiesterase inhibitors. Fifty clones were isolated from the second selection for screening with [^{125}I]-IHYP. Five clones were isolated that had 25% or fewer of wild-type [^{125}I]IHYP specific binding sites (referred to as *beta*$_d$ clones). Two *beta*$_d$ clones derived from the second TRM selection were further characterized.

Table 2 compares the cAMP accumulation in response to isoproterenol and PGE_1 of wild-type cells and the four clones deficient in *beta*-adrenergic responsiveness. All four clones demonstrate a diminished response to isoproterenol (approximately 50% for *beta*$_{p1}$ and *beta*$_{p2}$ and 20% for *beta*$_{d1}$ and *beta*$_{d2}$). With respect to wild-type, the 4 clones also exhibit a significantly increased cAMP accumulation in response to a maximally effective concentration of PGE_1. An estimate of the variation in these responses within each clone is demonstrated by the standard errors in Table 2; values were obtained from five flasks of each clone grown independently for seven days (approximately ten generations).

The diminished *beta*-adrenergic responsive phenotype is clonally stable, as demonstrated by the responses of $beta_{d1}$ and $beta_{d2}$ subclones to isoproterenol and PGE_1 (Table 3). All eight subclones of both variants show a greatly diminished isoproterenol response and an elevated PGE_1 response compared to the wild type. The diminished *beta*-adrenergic responsive phenotype has remained stable after propagation of $beta_p$ and $beta_d$ clones for at least 75 generations in the absence of selective pressure.

Adenylate cyclase activity. The lesion affecting the 4 variants deficient in *beta*-adrenergic responsiveness results in a loss in maximal responsiveness of membrane adenylate cyclase to isoproterenol, and not a decreased molar potency of the agonist (Fig. 2). Maximal responsiveness to isoproterenol varied from one membrane preparation to the next. However, the response of variant membranes to a maximally effective concentration of isoproterenol always fell within the following ranges (as % of wild-type response): 35 to 60% for $beta_{p1}$, 25 to 50% for $beta_{p2}$, and 10 to 25% for both $beta_{d1}$ and $beta_{d2}$. Despite the variation, these values closely reflect the differences in maximal cAMP accumulations in response to isoproterenol of intact cells (Table 2).

The concentration-effect relationship for PGE_1 indicates little difference in potency or maximal stimulation of adenylate cyclase from the receptor-deficient clones as compared to that of wild-type cells (Fig. 3). Thus, the increased PGE_1 response observed in intact $beta_d$ or $beta_p$ cells (Table 2) was not detectable in membranes pre-

TABLE 2

cAMP accumulation in wild-type and beta-*receptor deficient clones*

Values are ± S.E.M. where (N = 5). Each clone was subcultured in 5 separate flasks one week prior to measurement of response to the various agonists. The cAMP accumulation in the cells from each of the flasks was determined as described in the METHODS section.

Clone	Basal	Isoproterenol		PGE_1
		0.3 μM	10 μM	10 μM
		pmol/10⁶ cells		
Wild-type	5 ± 0.3	213 ± 19	246 ± 21	55 ± 5
$Beta_{p1}$	3 ± 0.2	108 ± 12	129 ± 21	164 ± 18
$Beta_{d1}$	3 ± 0.4	40 ± 6	48 ± 11	136 ± 32
$Beta_{p2}$	6 ± 1	77 ± 9	102 ± 12	312 ± 46
$Beta_{d2}$	3 ± 0.1	31 ± 2	49 ± 1	271 ± 14

TABLE 3

cAMP accumulation in wild-type and beta-*receptor deficient subclones*

The *beta*-receptor deficient clones $beta_{d1}$ and $beta_{d2}$ were subcloned in the absence of any selective pressure and these subclones tested for their cAMP accumulation (pmol/10^6 cells) in response to isoproterenol and PGE_1, as described in MATERIALS AND METHODS. Values represent the mean of triplicate determinations.

Clone	Basal	Isoproterenol	PGE_1	Clone	Basal	Isoproterenol	PGE_1
	pmol/10⁶ cells				*pmol/10⁶ cells*		
Wild-type	7	305	75	Wild-type	9	298	53
$Beta_{d1}$—A	4	47	203	$Beta_{d2}$—A	6	127	160
B	6	118	107	B	6	71	170
C	4	45	298	C	14	66	153
D	4	46	116	D	9	76	189
E	6	68	233	E	8	39	194
F	5	39	191	F	6	75	219
G	8	98	319	G	7	96	178
H	6	39	164	H	7	58	146

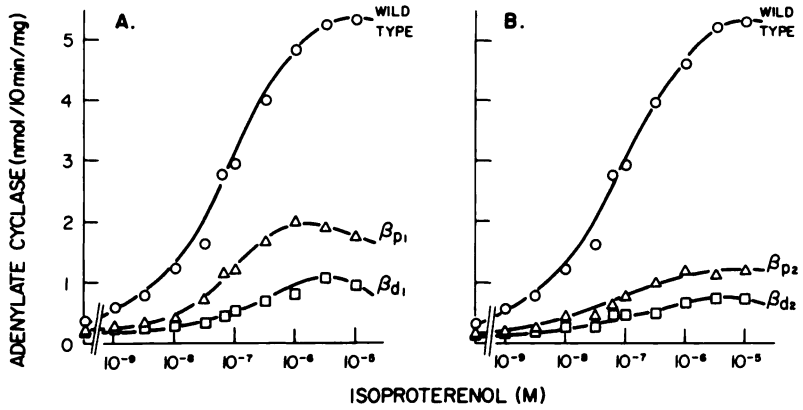

FIG. 2. *Concentration-effect relationship for isoproterenol stimulation of adenylate cyclase activity of wild-type and beta$_p$ and beta$_d$ receptor deficient membranes*

Enzyme activity was measured for 10 minutes in the presence of 100 μM GTP and increasing concentrations of isoproterenol. Reaction was initiated by the addition of 10 μg membrane protein. Values represent the means of duplicate determinations, which differed by less than ±10%.

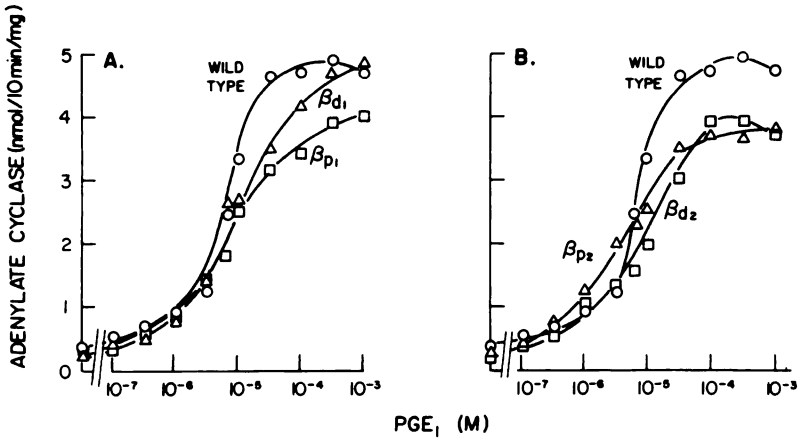

FIG. 3. *Concentration-effect relationship for PGE_1 stimulation of adenylate cyclase activity of wild-type and beta$_p$ and beta$_d$ receptor deficient membranes*

Enzyme activity was measured in the presence of 100 μM GTP and increasing concentrations of PGE_1. Initiation of the reaction was by addition of 10 μg membrane protein. Values represent the mean of duplicate determinations which varied by less than ±15%.

pared from these clones. These results indicate that the adenylate cyclase system of the *beta*-adrenergic deficient variants is comparable to that of wild-type cells for a hormone whose action is mediated by a receptor distinct from the *beta*-adrenergic receptor.

Basal, NaF- and Gpp(NH)p-activated adenylate cyclase activities are shown in Table 4. Basal activity measured in the presence of GTP is low with both wild-type cells and *beta*-adrenergic deficient clones. The responsiveness to NaF and Gpp(NH)p is similar in membranes from wild-type cells and *beta$_d$* and *beta$_p$* variants, indicating that the adenylate cyclase catalytic activity in the presence of a hydrolysis-resistant GTP analogue and NaF is similar to wild-type in these variants.

[^{125}I]IHYP binding. A representative experiment measuring the binding of [^{125}I]-IHYP to diminished *beta*-adrenergic responsive and wild-type membranes is shown in Figure 4. The density relative to

that of wild-type membranes, of *beta*-adrenergic receptors varied between 40 and 65% in membranes from clones $beta_{p1}$ and $beta_{p2}$ and from 10 to 30% in $beta_{d1}$ and $beta_{d2}$, depending on the membrane preparations used for comparison. The K_d for [^{125}I]IHYP varied from 30 to 100 pM in different experiments and membrane preparations for receptor-deficient and wild-type membranes. Similar variations have been reported by us (7) and others (15). No consistent difference in K_d for [^{125}I]IHYP was observed for any of the receptor-deficient clones. Similarly, both agonists and antagonists competed for [^{125}I]IHYP binding sites with no detectable differences in apparent K_d for any of the *beta*-adrenergic deficient clones compared to wild-type. Figure 5 demonstrates the competition for binding between [^{125}I]IHYP and *l*-isoproterenol. The apparent K_d for *l*-isoproterenol in such experiments is approximately 1 μM for diminished *beta*-adrenergic responsive and wild-type membranes. These experiments are complicated somewhat by the low binding capacity of the $beta_{d1}$ and $beta_{d2}$ receptor-deficient clones, causing difficulty in precise calculations of K_d for the competing ligand. Similar results were found with [^{125}I]IHYP binding studies using intact cells (not shown). Figure 6 illustrates the direct correlation between *beta*-adrenergic receptor density and maximal isoproterenol responsiveness in receptor-deficient and wild-type membranes.

TABLE 4

NaF activated adenylate cyclase activity of wild-type and clones deficient in beta-adrenergic responsiveness

Values represent the means of quadruplicate determinations for two independent membrane preparations of each clone. Adenylate cyclase activity was determined in the presence of 100 μM GTP, 10 mM NaF, or 100 μM Gpp(NH)p.

Clone	Adenylate Cyclase		
	Basal	NaF	Gpp(NH)p
	(nmol/10 min/mg)		
Wild-type	0.33	1.56	0.69
	0.13	1.21	0.54
$Beta_{p1}$	0.21	1.51	0.56
	0.16	1.22	0.36
$Beta_{d1}$	0.13	1.31	0.29
	0.14	1.26	0.44
$Beta_{p2}$	0.15	1.86	0.77
	0.12	1.40	0.40
$Beta_{d2}$	0.14	1.78	0.32
	0.21	3.01	0.58

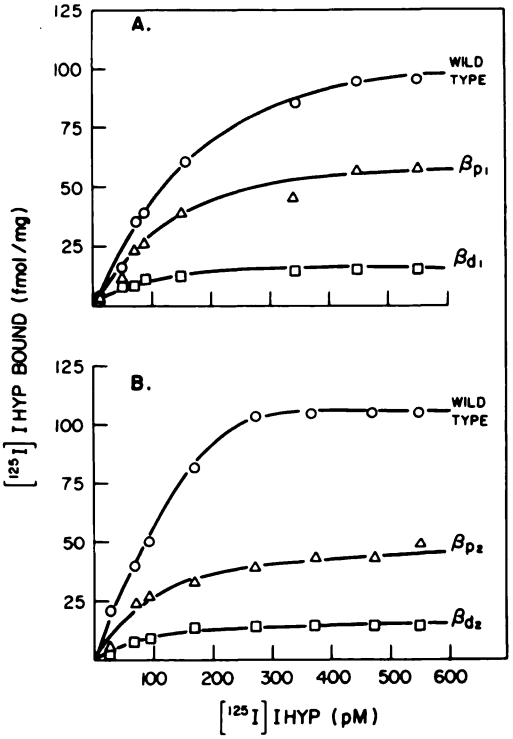

FIG. 4. *Specific binding of [^{125}I]IHYP to plasma membranes of wild-type, $beta_p$ and $beta_d$ receptor deficient clones*

Specific binding was determined by the difference between the amount of [^{125}I]IHYP bound in the presence and absence of 1 μM (—)-propranolol for each ligand concentration indicated. Incubations were for 60 min and values represent the mean of triplicate determinations which differed by less than ±15%.

DISCUSSION

We have isolated stable variant clones of S49 cells deficient in both *beta*-adrenergic receptors and responsiveness to *beta*-adrenexgic amines. In order to draw inferences regarding the relation of adrenergic receptors and adenylate cyclase, it is necessary to consider the possible nature and genetic 1 13basis of the lesion or lesions that produced basis of the lesion or lesions that produced the variant phenotype.

The simplest explanation is that expression of *beta*-adrenergic receptors is dimin-

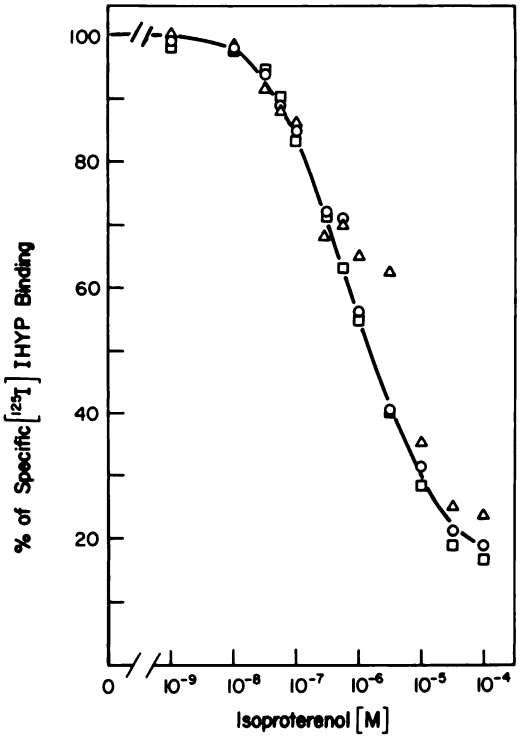

FIG. 5. *Competition for binding between [^{125}I] IHYP and (—)-isoproterenol*

Incubations were for 60 min in the presence of 50 pM [^{125}I]IHYP and 100 μM GTP. Membrane concentrations were 10 μg per assay tube. The K_d for [^{125}I] IHYP in these membrane preparations was about 100 pM. Specific binding was determined by the difference between the amount of [^{125}I]IHYP bound in the presence and absence of 1 μM (—)-propranolol. Specific [^{125}I]IHYP binding in the absence of (——)-isoproterenol for wild-type, (○); $beta_{p2}$ (□); and $beta_{d2}$ (△) membranes was 49, 23, and 16 fmol/mg respectively. Values represent the mean of triplicate determinations which differed by less than ±15%.

ished in the variant cells, and this produces a coordinate decrease in the ability of isoproterenol to activate adenylate cyclase. This explanation is consistent with the observation that loss of [^{125}I]IHYP binding sites closely paralleled loss of isoproterenol responsiveness in independently selected clones, and also with the finding that remaining *beta*-adrenergic receptors in the variants are similar to those in wild-type, in their apparent affinities for [^{125}I]IHYP and isoproterenol. The variants also exhibit an increased responsiveness to PGE_1 in intact cells, relative to wild-type, but this difference is lost in membrane preparations. It is possible that the increased PGE_1 response in intact cells results from depletion of *beta*-adrenergic receptors, but we do not know why this effect should be lost when plasma membranes are prepared. The variants' decreased response to isoproterenol, however, is observed with both intact cells and isolated membranes.

If the primary lesion producing the variant phenotype is a decreased expression of *beta*-adrenergic receptors, its genetic basis is not clear. A single selection produced $beta_p$ clones expressing 50% of wild-type [^{125}I]IHYP binding sites. Our hypothesis that $beta_p$ cells were heterozygous for the *beta*-receptor gene was apparently incorrect, since the second round of selection failed to produce clones devoid of *beta*-adrenergic receptors. Instead, the $beta_d$ phenotypes expressed approximately 25% of wild-type receptors.

Alternatively, the primary lesion(s) in $beta_p$ and $beta_d$ variants may not lie in expression of *beta*-adrenergic receptors per se, but rather in some other process that coordinately regulates *beta*-receptor number and maximal response to *beta*-agonists without altering fluoride-stimulated adenylate cyclase activity. This possibility would imply that receptor number need not

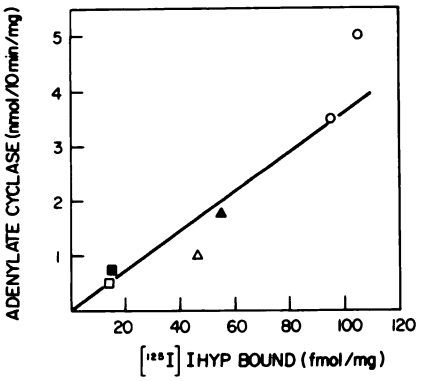

FIG. 6. *Relationship between beta-adrenergic receptor density and maximal adenylate cyclase activity*

Adenylate cyclase activity in response to 10 μM isoproterenol + 100 μM GTP versus the number of [^{125}I]IHYP specific binding sites of the same membrane preparation is plotted for wild-type (○, two separate membrane preparations); $beta_{p1}$ (▲) and $beta_{p2}$ (△); $beta_{d1}$ (■) and $beta_{d2}$ (□).

directly limit maximal hormone responsiveness. We cannot rigorously exclude such a possibility, since variant clones were selected on the basis of decreased cAMP accumulation in response to a *beta*-adrenergic agonist, rather than on the basis of decreased numbers of [^{125}I]IHYP binding sites.

The first explanation appears more likely, in part because of its simplicity, but also because the implication that maximal adrenergic responsiveness is limited by the number of available receptors is consistent with other observations in S49 cells and other systems. Insel and Stoolman (29) reported that occupancy of half the *beta*-adrenergic receptors of wild-type S49 cells by the slowly reversible antagonist, [^{125}I] IHYP, produced a 50% decrease in maximal stimulation of cAMP accumulation by isoproterenol, while higher occupancy by the antagonist caused still further decreases in catecholamine responsiveness. The notion that a full complement of *beta*-adrenergic receptors is required for maximal catecholamine activation of adenylate cyclase is supported by indirect evidence in two other systems. Brown *et al.* (8, 39) found a one-to-one relationship between binding of *beta*-adrenergic antagonists and adenylate cyclase inhibition in turkey erythrocytes. Lucas and Bockaert (40), studying [^{3}H]alprenolol binding to C6 rat glioma membranes, found that the relationship between binding and activation of adenylate cyclase by isoproterenol was nonlinear, but that 100% occupation of receptors was required for maximal stimulation. The findings of Maguire *et al.* (31), studying the time course of reversal of [^{125}I]IHYP binding from C6 rat glioma membranes and the associated stimulation of adenylate cyclase by isoproterenol, also are consistent with the notion of a linear relation between receptor occupancy and activation.

In the glucagon-sensitive hepatic adenylate cyclase system maximal glucagon stimulation occurs when only a fraction of the receptors is occupied (41, 42). However, in the presence of GTP only a small fraction of the total glucagon receptors—postulated to represent active receptor-enzyme complexes—show high affinity for hormone (42). Further work is required to explain possible differences between glucagon- and catecholamine-sensitive adenylate cyclases in the different preparations.

In apparent contrast to the evidence that receptor number limits maximal response to *beta*-adrenergic catecholamines, isoproterenol stimulates adenylate cyclase of S49 and several other cell types at concentrations well below those required to produce equivalent fractional occupation of receptor binding sites (c.f. Figs. 2 and 5). In these systems the activation constant for agonists (K_{act} = concentration for half-maximal stimulation of cAMP synthesis) may be 5 to 30-fold lower than the dissociation constant (K_d) for the same agonist, determined by receptor binding assays (15–17, 29). Spare receptors, in the sense that only a fraction of the total receptors need be occupied for maximal stimulation, cannot explain the K_d/K_{act} discrepancy observed in the *beta*-adrenergic receptor-deficient phenotypes since receptor number limits maximal response. However, for both wild-type and *beta*-adrenergic receptor-deficient phenotypes the K_d for isoproterenol is at approximately 10 to 50-fold higher concentrations than the K_{act}.

How then can the K_d/K_{act} discrepancy be explained? We suggest the following working hypothesis as suggested previously by others (18): If receptors and cyclase are separate molecules (2, 3), the activation of a cyclase molecule may persist in time beyond occupancy of a receptor by hormone. If so, the total number of receptors present could limit the number of cyclase molecules activated per unit time, but the activating hormone-receptor complex will dissociate, on the average, before the activated cyclase molecule becomes inactive. This would shift the K_{act} for an agonist toward a lower concentration than the K_d, but all receptors must be present for maximal activation. Therefore, receptor number limits responsiveness, and in this sense, receptors are not spare.

This general hypothesis is supported by current knowledge of the role of GTP in regulating adenylate cyclase. As suggested by Cassel and Selinger (43, 44), the turn-off mechanism for hormonal stimulation of

adenylate cyclase may be the activation of a GTPase, resulting in the hydrolysis of GTP at the GTP regulatory site and inactivation of hormone-stimulated adenylate cyclase activity. Thus, the GTP-liganded state of the enzyme could persist longer than does the hormone-receptor complex. This hypothesis predicts that any perturbation of the hormone-sensitive adenylate cyclase system that prolongs the GTP-liganded active state will increase the K_d/K_{act} discrepancy. This is, in fact, observed in cells or membranes treated with cholera toxin. Cholera toxin inhibits a catecholamine-stimulated GTPase (44), has no effect on agonist binding to *beta*-adrenergic receptors (16, 45), and decreases the K_{act} for isoproterenol (15, 16, 45).

It should be noted that the S49 variants reported here do not rule out the existence of spare receptors in the activation of adenylate cyclase for other systems, especially those in which extremely large numbers of receptors have been detected (10). Genuinely spare receptors would be expected to augment the K_d/K_{act} discrepancy to a level beyond that produced by a difference between the duration of adenylate cyclase activation and occupancy of receptor.

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