MOLECULAR PHARMACOLOGY, 22:258–266

Characterization of Coexisting *Alpha*$_1$- and *Beta*$_2$-Adrenergic Receptors on a Cloned Muscle Cell Line, BC3H-1

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Received October 12, 1981; Accepted May 10, 1982

SUMMARY

In order to determine whether or not a single cell type can express different adrenergic receptors simultaneously, we have characterized *alpha*- and *beta*-adrenergic receptors on a cloned muscle cell, BC3H-1. *Alpha*- and *beta*-adrenergic receptors were investigated using [^{3}H]prazosin and [^{125}I]cyanopindolol ([^{125}I]CYP), respectively. [^{125}I]CYP bound specifically to a membrane preparation of these cells at a density of 87 fmoles/mg of membrane protein (approximately 9000 receptors/cell), with a dissociation constant of 105 pM. The rank order of potency of agonists in competing for [^{125}I]CYP binding sites was characteristic of a *beta*$_2$-adrenergic receptor, i.e., (−)-isoproterenol > (−)-epinephrine ≫ (−)-norepinephrine. The concentration dependence for competing ligands in displacing [^{125}I]CYP indicated that agonists were binding to both low- and high-affinity sites and that GTP reduced the over-all apparent affinity. Ligands selective for receptor subtypes, practolol (*beta*$_1$) and zinterol (*beta*$_2$), each bound to only one class of [^{125}I]CYP binding sites in a manner consistent with a single population of *beta*$_2$-adrenergic receptors. The *alpha*$_1$-adrenergic ligand [^{3}H]prazosin bound specifically to a distinct single class of receptors at a density of 137 fmoles/mg of membrane protein (approximately 14,000 receptors/cell) with a dissociation constant of 80 pM. The rank order of potency of agonists in competition for [^{3}H]prazosin binding sites was characteristic of an *alpha*-adrenergic receptor, i.e., (−)-epinephrine ≥ (−)-norepinephrine ≫ (−)-isoproterenol. The binding data could be described by a single class of binding sites, and GTP had no effect on the (−)-epinephrine competition curve. *Alpha*$_1$- and *beta*$_2$-adrenergic receptors were expressed in a stable manner by subclones of the parent BC3H-1 cell. Functional studies measuring cyclic AMP accumulation and $^{45}Ca^{2+}$ efflux confirmed the presence of *alpha*$_1$- and *beta*$_2$-adrenergic receptors on BC3H-1 cells. In addition, we found a striking difference in the turnover rate of the two classes of adrenergic receptor of these cells. These data indicate that BC3H-1 cells possess *alpha*$_1$- and *beta*$_2$-adrenergic receptors which have distinct binding properties, turnover, and linkage to separate second-messenger systems.

INTRODUCTION

More than three decades ago, Ahlquist (1) classified the adrenergic receptors into two groups; namely, *alpha* and *beta*. This he did on the basis of differing potencies of epinephrine, norepinephrine, and isoproterenol in eliciting cellular responses. Since that time, additional studies have indicated that these two groups of receptors can be further subdivided into *alpha*$_1$, *alpha*$_2$, *beta*$_1$, and *beta*$_2$ subtypes on the basis of agonist and antagonist specificity and the involvement of distinct intracellular second messengers (2–5). Thus, these four classes of adrenergic receptors are distinguished on the basis of ligand specificity and the nature of cellular response to receptor activation. Tissues, even those which appear to be morphologically uniform, are found to contain at least two classes of adrenergic receptors (5–8). To date, however, the relative populations of more than one class of adrenergic receptor on a single cloned cell have never been directly quantitated.

BC3H-1 is a nonfusing muscle cell line which has been reported to possess nicotinic-cholinergic and *alpha*- and *beta*-adrenergic responses. Detailed biochemical and pharmacological characterizations have been reported for nicotinic receptors on these cells (9, 10). Agonist-elicited changes in ion permeability indicate that these cells also possess adrenergic receptors (11, 12). Other evidence indicates that [^{3}H]dihydroalprenolol can be used to detect *beta*-adrenergic receptors on BC3H-1 cells (12, 13) and that these receptors are linked to activation

This work was supported in part by Grant 79-680 from the American Heart Association and Grant HL-25457 from the National Institutes of Health.

[1] Recipient of a postdoctoral fellowship from the American Heart Association, California Affiliate (80-F14).

[2] Recipient of a Postdoctoral Fellowship from the National Institutes of Health.

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0026-895X/82/050258-09$02.00/0

of adenylate cyclase (12–14). Since these cells offer the opportunity to examine the simultaneous regulation and functional properties of both adrenergic receptor types, we have ascertained the subtype of receptor on these cells using both radioligand binding studies and measurements of functional response and have measured a difference in the rate of turnover of these receptors.

EXPERIMENTAL PROCEDURES

Materials. The following drugs were received as generous gifts: clonidine hydrochloride and phentolamine mesylate from Ciba-Geigy Corporation (Summit, N. J.); CYP[4] from Dr. G. Engel, Sandoz Pharmaceuticals (East Hanover, N. J.); (−)-epinephrine, (+)-epinephrine, (−)-isoproterenol, (+)-isoproterenol, and (−)-norepinephrine as the (+)-bitartrate salts from Sterling-Winthrop Research Institute (New York, N. Y.); (−)-propranolol, (+)-propranol, (±)-propranolol and practolol from Ayerst Research Laboratories (New York, N. Y.); [^{3}H]prazosin (33 Ci/mmole) from Pfizer Laboratories (New York, N. Y.); zinterol from Mead Johnson & Company (Evansville, Ind.); and WB4101 from Dr. S. Snyder, Johns Hopkins University (Baltimore, Md.). Phenylephrine and GTP were purchased from Sigma Chemical Company (St. Louis, Mo.) and yohimbine from United States Biochemical Corporation (Cleveland, Ohio). Carrier-free Na^{125}I (>350 mCi/ml) and $^{45}Ca^{2+}$ (20–30 mCi/mg) and [^{3}H] leucine (55 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). Dulbecco's modified Eagle's medium and fetal calf serum were obtained from Grand Island Biological Company (Grand Island, N. Y.); 0.25% sterile trypsin solution from Irvine Scientific (Santa Ana, Calif.); flasks and dishes were manufactured by Falcon.

Preparation of ^{125}I-Labeled CYP. CYP was iodinated as described by Engel and co-workers (15). [^{125}I]CYP was stored at −20° for at least 6 months, when it still migrated as a single peak on silica gel thin-layer chromatography with pyridine/glacial acetic acid/water (0.33:0.6:9.07, v/v) as solvent. The [^{125}I]CYP was assumed to be iodinated to the maximal theoretical specific activity of 2200 Ci/mmole; correction for decay of [^{125}I]CYP assumed that the products of radioactive decay diluted this theoretical specific activity.[5] Fresh [^{125}I]CYP was prepared every 2–3 months.

Culture of BC3H-1 muscle cells. The BC3H-1 muscle cells used in this study were a gift from Drs. J. P. Mauger and J. Bockaert, Collège de France (Paris, France). The cell line was originally isolated at the Salk Institute (La Jolla, Calif.) from a neoplasm extracted from offspring of pregnant mice which had received injections of nitrosoethylurea (16). From the initial heterogeneous cell population, several clones were isolated, and one of these (Clone 9) was used in further studies. This cell line carries many of the morphological and ultrastructural characteristics of muscle cells, and synthesizes collagen. The initial clones, unlike those used in the present studies, were capable of spontaneous and electrically induced contraction. The cells were routinely grown in Falcon T-flasks in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and maintained at 37° in a humidified atmosphere of 10% CO_2 in air. The cells were subcultured at 3-day intervals, using 0.25% trypsin to dissociate them from the plastic substrate. For experimental purposes, approximately 1.5×10^6 cells were seeded into 150-mm diameter culture dishes containing 25 ml of culture medium. From this starting density, the cells reached confluence within 2–4 days. Subclones of the parent BC3H-1 clone were obtained by growing colonies from single cells.

Preparation of crude membranes from BC3H-1. A crude membrane preparation, free of nuclei, was prepared in the following manner. After reaching confluence (a minimum of 2 days after seeding) the cell sheet was washed twice at 20° with 10 ml isotonic saline buffered with 50 mM Tris-HCl (pH 7.4) and scraped into 5 ml of the same solution with the aid of a rubber policeman. The cells were collected by centrifugation ($400 \times g$ for 5 min at 20°) and resuspended in one-fifth the initial volume of 5 mM Tris-HCl (pH 7.6)/1 mM $MgCl_2$ at 4°. After 5 min, during which time visible swelling had occurred, the cells were homogenized at 0–4° by five strokes of a motor-driven "zero-clearance" Potter-Elvehjem homogenizer (Kontes Model K-886030). The suspension was centrifuged three times for 2 min at $200 \times g$, each time discarding the pelleted material. The final supernatant, which was substantially free of nuclei and intact cells as judged by microscopic examination, was centrifuged at $38{,}500 \times g_{av}$ for 10 min. The pellet was resuspended in 20 ml of 50 mM Tris-HCl (pH 7.6)/10 mM $MgCl_2$. The particulate material was washed one additional time and resuspended in the appropriate volume (usually 20–40 ml for six dishes of cells) of the above buffer plus ascorbic acid (1 mg/ml). This preparation was used immediately in the binding assays.

[^{125}I]CYP and [^{3}H]prazosin binding assays. The BC3H-1 membranes, suspended in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and ascorbate (1 mg/ml), were incubated in a final volume of either 0.25 ml for [^{125}I]CYP binding or 0.5 ml for [^{3}H]prazosin binding studies, at a protein concentration of 0.1–0.3 mg/ml. The binding reaction was carried out for 60 min at 30° in a shaking water bath (60 oscillations/min), in the presence of the specified concentrations of radioligand and other drugs. Nonspecific binding was defined as the radioligand bound in the presence of 1 μM (±)-propranolol or 10 μM phentolamine in incubations using [^{125}I]CYP and [^{3}H]prazosin, respectively. Nonspecific binding accounted for less than 20% of the total binding when either radioligand was employed at concentrations which produced occupation of less than one-half of its receptors and was always less than 50% in these experiments. Specific and nonspecific binding of both radioligands increased linearly with membrane protein concentrations up to at least 0.24 mg/ml with [^{125}I]CYP and 0.60 mg/ml with

[4] The abbreviations used are: CYP, cyanopindolol; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; WB4101, 2-[*N*-(2,6-dimethoxyphenoxyethyl)]-aminomethyl-1,4-benzodioxane.

[5] We believe that it is correct to assume that the products of radioactive decay do not interfere with the binding of [^{125}I] CYP because single batches of [^{125}I]CYP yield similar values for B_{max} and K_d over several months, when we correct the initial estimate of specific activity for the rate of decay of ^{125}I.

[^{3}H]prazosin. The binding reaction was terminated by the addition of 10 ml of wash buffer [0.9% (w/v) NaCl, 50 mM Tris-HCl, pH 7.4] at either 20° in the case of experiments using [^{125}I]CYP or at 0° for experiments using [^{3}H]prazosin. The diluted membrane suspension was filtered over Whatman GF/C glass-fiber filters and washed once with 10 ml of wash buffer at the appropriate temperature. The entire filtration procedure was accomplished within 45 sec. [^{3}H]Prazosin samples were counted in a Beckman LS-330 liquid scintillation spectrometer at 40% efficiency, and [^{125}I]CYP samples were counted in a Searle Model 1197 Gamma counter at 85% efficiency. The [^{3}H]prazosin was intermittently checked for purity by thin-layer chromatography and found to be unchanged. Membrane-bound [^{125}I]CYP co-migrated with fresh [^{125}I]CYP on thin-layer chromatography.

Protein determination. Protein was determined by the method of Peterson (17), employing log-log transformation to yield a linear standard curve (18). Bovine serum albumin, containing sodium azide (1 mg/ml) as a noninterfering bacteriocide, was employed for standards.

Measurement of cyclic AMP accumulation. Cells were seeded into multiwell plates, each plate containing 24 12-mm diameter wells, and grown until confluent. The growth medium was aspirated, and each well was washed twice with 1 ml of Hanks' balanced salt solution. A portion (0.38 ml) of this salt solution, also containing 5 mM Hepes (pH 7.4) and 0.5 mM IBMX, was added to each well and the plate was equilibrated at 37° for 30 min. Following this equilibration period, drugs were added to each well in a final volume of 0.40 ml. After a 10-min incubation, 0.1 ml of 40% (w/v) trichloroacetic acid was added to each well and the cyclic AMP was subsequently purified by chromatography on columns of Dowex AG50-X8 resin. Cyclic AMP was determined by a competitive binding protein method.

Measurement of phenylephrine-stimulated $^{45}Ca^{2+}$ unidirectional efflux from BC3H-1 cells. Cells were grown on 25 × 75 mm glass microscope slides held in 100-mm diameter culture dishes, containing 15 ml of growth medium. Five days after plating, the slides were transferred to culture dishes containing fresh medium. The growth medium was replaced at days 8 and 11 and experiments were performed on days 12–14.

$^{45}Ca^{2+}$ efflux experiments were performed by a modification of the procedure described by Mauger and Worcel (12) for measuring ^{86}Rb efflux. Cells were equilibrated with $^{45}Ca^{2+}$ (5–10 μCi/ml) under normal growth conditions for at least 18 hr prior to the experiment. Subsequent manipulations were performed in physiological buffer [140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 5.5 mM glucose, and 25.0 mM Hepes (pH 7.4)] maintained at 37° and gassed with 95% O_2:5% CO_2. Individual slides were washed briefly with three 15-ml aliquots of physiological buffer and transferred to a perfusion chamber similar to that described (12). Input to the chamber was provided by a peristaltic pump from reservoirs of physiological buffer containing appropriate drug additions. Flow proceeded upward at 5.5–6.0 ml/min. The total system volume was 12 ml. The perfused effluent was collected in either 3- or 5-ml fractions. After perfusion, the monolayer was solubilized in 3% (w/v) Triton X-100/10 mM sodium phosphate (pH 7.4). Aliquots of the cell suspension and the effluent fractions were analyzed for $^{45}Ca^{2+}$ by liquid scintillation counting. $^{45}Ca^{2+}$ efflux data were analyzed as described previously (12).

Data analysis. For Scatchard analyses of equilibrium binding data (Figs. 1 and 2), total binding at each concentration of radioligand was determined in triplicate and nonspecific binding in duplicate. Linear regression analyses were carried out for all estimates of nonspecific binding, since these varied linearly over the range of radioligand concentrations tested. Specific binding at each concentration of radioligand was then calculated as the difference between the mean of the values for total binding and the value for nonspecific binding computed from the line given by linear regression. Data were fit to the relationship $B = B_{max}[L]/(K_d + [L])$, which was, in turn, used to simulate the saturation binding isotherms shown in the figures. The number of binding sites (B_{max}) and the dissociation constant (K_d) were obtained from Scatchard analysis of the data; [L] represents the free radioligand concentration.

Dissociation constants for various adrenergic agents were routinely calculated from the concentrations required to inhibit specific radioligand binding by 50% (K_{app}). The K_{app} values were determined from 10- to 20-point curves, each point representing duplicate determinations; the true dissociation constants were calculated according to the method of Cheng and Prusoff (19), i.e., $K_d = [K_{app}]/(1 + [S]/K_s)$, where [$S$] and K_s refer to the radioligand concentration and the dissociation constant for the displaced radioligand, respectively. In order to determine homogeneity of binding sites, a nonlinear least-squares curve-fitting program, developed by Munson and Rodbard (20), was used to analyze some of the competitive binding data. Data treated in this way included displacement of [^{125}I]CYP by agonists and the subtype-selective ligands, zinterol and practolol.

RESULTS

Binding isotherms and kinetics of [^{125}I]CYP binding. [^{125}I]CYP bound to BC3H-1 membranes with a specific component of binding [i.e., capable of being competed by 1 μM (±)-propranolol] which saturated in a concentration-dependent manner (Fig. 1). Scatchard plots of these data appeared linear, indicating the presence of a single class of high-affinity binding sites (K_d = 105 ± 25 pM (n = 6)]. The total number of these sites was 87 ± 22 fmoles/mg of membrane protein, corresponding to approximately 9000 receptors/cell.[6]

The dissociation constant determined for binding of [^{125}I]CYP at equilibrium was compared with that estimated from the kinetics of binding (Fig. 2). From the rate constant of association (1.8 ± 0.7 × 10^8 M^{-1} min^{-1}) and the rate constant of dissociation (0.0030 ± 0.0010

[6] In some experiments, it appeared that equilibrium was not fully achieved within 60 min, the amount of complex formed being 80–100% of that formed at longer times. However, Scatchard analysis of binding data obtained after a 5-hr incubation yielded a K_d of 124 pM and a B_{max} of 51 fmoles/mg of protein, values very similar to those obtained at 60 min. In addition, (−)-isoproterenol competition for [^{125}I]CYP binding sites was identical in terms of K_d of high- low-affinity sites and the proportion of these two classes of sites in 1-, 2-, and 4-hr incubations.

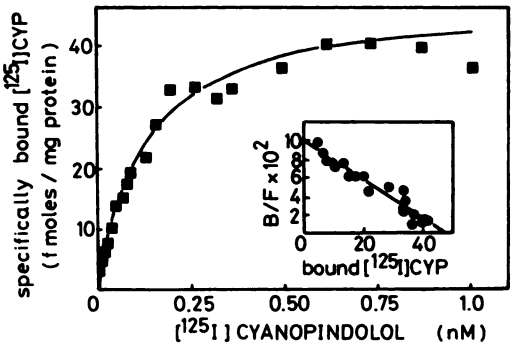

FIG. 1. *Binding isotherm of [^{125}I]CYP to a BC3H-1 membrane preparation*

A crude membrane preparation of BC3H-1 cells was prepared as described and incubated with [^{125}I]CYP in the presence or absence of 1 μM (±)-propranolol. The specifically bound [^{125}I]CYP (■——■) was defined as the difference between the total amount of binding and the nonspecifically bound [^{125}I]CYP at each concentration of radioligand. The *inset* is the Scatchard plot of the data. Data representative of those obtained in six similar experiments are shown.

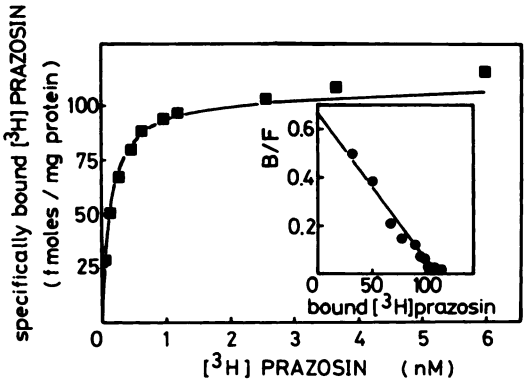

FIG. 3. *Binding isotherm of [^{3}H]prazosin to a BC3H-1 membrane preparation*

A crude membrane preparation of BC3H-1 cells was incubated with [^{3}H]prazosin in the presence or absence of 10 μM (±)-phentolamine. The specifically bound [^{3}H]prazosin (■——■) was defined as the difference between the total amount of binding and the nonspecifically bound [^{3}H]prazosin at each concentration of radioligand. The *inset* is the Scatchard plot of the data. Data representative of those obtained in five similar experiments are shown.

min^{-1}), a K_d of 20 ± 10 pM (n = 3) was determined. In contrast, a kinetic estimate of the K_d, using the method of Brown *et al.* (21), yielded a value of 104 pM, which is closer to the K_d determined by Scatchard analysis of equilibrium binding data.

Binding isotherms and kinetics of [^{3}H]prazosin binding. Several radioligands were investigated as potential probes for *alpha*-adrenergic receptors. Of these, the *alpha*$_2$-selective adrenergic ligands, [^{3}H]yohimbine and [^{3}H]clonidine, at nanomolar concentrations exhibited no binding which was competed by 10 μM phentolamine. In contrast, the nonselective *alpha*-adrenergic ligand [^{3}H] dihydroergocryptine and the *alpha*$_1$-selective adrenergic ligands [^{3}H]prazosin and [^{3}H]WB4101 showed binding which could be competed by phentolamine when tested under the same conditions (data not shown). Of these compounds, [^{3}H]prazosin appeared to possess the highest affinity for the *alpha*-adrenergic receptor on the BC3H-1 cells and was therefore chosen as the compound for more detailed characterization. These preliminary studies suggested that the *alpha*-adrenergic receptors present on BC3H-1 cells were of the *alpha*$_1$ subtype.

[^{3}H]Prazosin bound to a single component of high-affinity binding sites [K_d = 86 ± 38 pM (n = 5)] (Fig. 3). The number of *alpha*-adrenergic receptors (137 ± 32 fmoles/mg of membrane protein, or approximately 14,000 receptors/cell) was approximately 1.5-fold higher than that of the *beta*-adrenergic receptors.

The rates of association and dissociation of [^{3}H]prazosin from its binding sites were determined (Fig. 4). Analysis of these data yielded an association rate constant of 2.4 × 10^{8} M^{-1} min^{-1} and a dissociation rate constant of 0.018 min^{-1}. The K_d determined from the above kinetics was 75 pM, which was in good agreement with that obtained from Scatchard analysis.

Stability of adrenergic receptor expression in subclones. The data presented thus far indicate that BC3H-1, a cloned cell line, possesses both *alpha*$_1$- and *beta*$_2$-adrenergic receptors. To test whether these two classes of receptors might be present on different cell populations, which conceivably might have arisen during extended periods of time in culture, we subcloned the

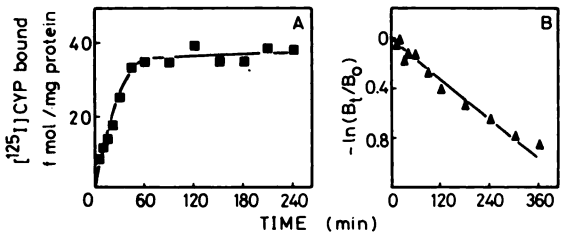

FIG. 2. *Kinetic analysis of [^{125}I]CYP binding to BC3H-1 membranes*

Specific binding (■——■) of 0.14 nM [^{125}I]CYP was followed as a function of time. After 60 min, the rate of dissociation (▲——▲) of [^{125}I] CYP from its receptors was followed subsequent to the addition of propranolol to a final concentration of 1 μM. The dissociation rate constant (k_2) was calculated from the slope of the line (B) as 0.003 ± 0.001 (n = 3) min^{-1}. The rate of association (k_{obs}) was calculated from the data in A transformed to ln ($B_{eq}/B_{eq} - B_t$), and k_1 was determined using the equation $k_{obs} = k_1[L] + k_2$ as 1.8 ± 0.7 × 10^{-8} M^{-1} min^{-1} (n = 3). Thus the kinetically determined K_d was 20 ± 10 pM.

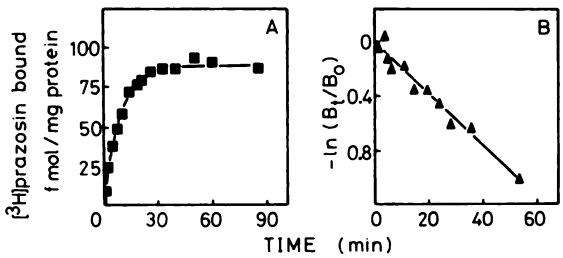

FIG. 4. *Kinetic analysis of [^{3}H]prazosin binding to BC3H-1 membranes*

Specific (■——■) binding of 0.3 nM [^{3}H]prazosin was followed as a function of time. After 45 min, the rate of dissociation (▲——▲) of [^{3}H] prazosin from its receptors was followed subsequent to the addition of phentolamine to a final concentration of 10 μM. The dissociation rate constant (k_2) was calculated from the slope of the line in B as 0.018 min^{-1}. The rate of association (k_{obs}) was calculated from A, and k_1 was determined from the equation $k_{obs} = k_1[L] + k_2$ as 2.4 × 10^{8} M^{-1} min^{-1}. Thus the kinetically determined k_d was 75 pM.

TABLE 1

Expression of adrenergic receptors in BC3H-1 cell subclones

BC3H-1 cells were subcloned, and *alpha*$_1$- and *beta*$_2$-adrenergic receptor properties in three of these subclones were examined. The data presented below were derived from Scatchard analysis of saturation binding isotherms.

	Alpha$_1$ receptor		*Beta*$_2$ receptor	
	B_{max}	K_d	B_{max}	K_d
	fmoles/mg	*pM*	*fmoles/mg*	*pM*
Parent	137 ± 32 (n = 5)	86 ± 38	87 ± 22 (n = 6)	105 ± 25
Subclone 1	151	76	70	201
Subclone 2	173	91	76	80
Subclone 3	268	90	90	73

parent BC3H-1 cells. As shown in Table 1, subclones expressed *alpha*- and *beta*-adrenergic receptors with B_{max} and K_d values similar to those of the parent cells.

Interaction of adrenergic ligands with the [^{125}*I]CYP binding site(s).* The interactions of both *alpha*- and *beta*-selective radioligands with their binding sites were characterized in competitive binding studies with unlabeled adrenergic ligands of known receptor subtype selectivity.

Specific binding of [^{125}I]CYP was competed stereoselectively by the (−)-isomer of the *beta*-adrenergic antagonist propranolol. The two isomers showed a 200-fold difference in affinity for the [^{125}I]CYP binding site (Table 1). Similarly, the (+)- and (−)-isomers of the *beta*-adrenergic agonist isoproterenol showed a 60-fold difference in the concentration required to compete [^{125}I]CYP from 50% of its binding sites (Table 1).

Since CYP is a nonselective *beta*-adrenergic antagonist, binding of [^{125}I]CYP would not be expected to differentiate between the two *beta*-adrenergic subtypes. In order to define further the nature of the [^{125}I]CYP binding site, the *beta*$_1$- and *beta*$_2$-selective ligands practolol and zinterol were employed (22). Zinterol (tested in the presence of 200 μM GTP) was 1000-fold more potent than practolol in competition for [^{125}I]CYP binding sites on BC3H-1 membranes (Table 2; Fig. 5). Furthermore, when these data were analyzed by using a nonlinear, least-squares curve-fitting program (20), there was an insignificant ($p > 0.7$, $n = 7$) improvement in the goodness of fit when a two-site model was compared with a one-site model. This observation is consistent with [^{125}I]CYP's binding to a homogeneous class of *beta*$_2$-adrenergic receptor sites.

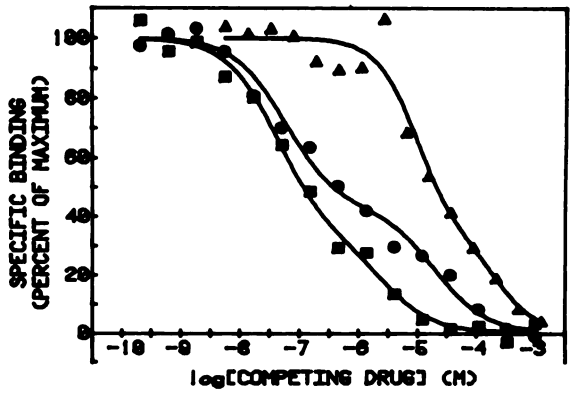

FIG. 5. *Agonist competition for [*^{125}*I]CYP sites on BC3H-1 membranes*

A crude membrane preparation of BC3H-1 cells was prepared as described and incubated with 200 pM [^{125}I]CYP in the absence (0% inhibition) or the presence (100% inhibition) of 1 μM (±)-propranolol. The indicated concentrations of competing ligands were added to the incubation medium in the absence of propranolol, and the percentage inhibition of specific binding was determined. Results are expressed as the mean of duplicate determinations using (−)-isoproterenol (■——■), (−)-epinephrine (●——●), and (−)-norepinephrine (▲——▲); the curves were fitted by the nonlinear least-squares curve-fitting program.

The rank order of potency of adrenergic agonists in inhibiting [^{125}I]CYP binding was also indicative of a *beta*$_2$-adrenergic receptor; (−)-isoproterenol > (−)-epinephrine ≫ (−)-norepinephrine (Fig. 6). However, the curves generated by competition of these agonists and [^{125}I]CYP for binding sites were shallow, having Hill coefficients of 0.5 ± 0.12 (n = 14). When these curves were analyzed by the nonlinear least-squares curve-fitting program, a significantly better fit ($p < 0.02$, $n = 14$) was obtained with a two-site model as opposed to a one-site model (Table 2). All three agonists showed about a 100-fold difference in the affinities of these two binding sites, and 60–80% of the receptors existed in the higher affinity form. The addition of 200 μM GTP, which had no effect on [^{125}I]CYP binding per se, shifted the (−)-isopro-

TABLE 2

Affinities of adrenergic amines for the BC3H-1 beta$_2$*-adrenergic receptor*

The K_d values listed below for these adrenergic amines were calculated from their respective K_{app} values for displacement of [^{125}I]CYP (at 80–200 pM concentrations) from specific binding sites on BC3H-1 particulate fractions or by the nonlinear least-squares curve-fitting program. The K_{app} values were corrected to K_d as described under Experimental Procedures, using a value of 104 pM as the K_d for [^{125}I]CYP. Results are expressed as the mean ± standard deviation of values obtained from 10- to 20-point curves, each point representing duplicate determinations in the number of experiments (n) indicated.

Drug	n	$K_{d,H}$	$K_{d,L}$	$\%R_H$
		M	*M*	
(−)-Propranolol	2	6.4 (±2.9) × 10^{-10}		
(+)-Propranolol	2	1.1 (±0.5) × 10^{-7}		
(±)-Zinterol + 200 μM GTP	2	2.3 (±1.1) × 10^{-8}		
(±)-Practolol	5	3.5 (±2.3) × 10^{-5}		
(−)-Isoproterenol	6	4.2 (±2.1) × 10^{-8}	3.6 (±1.1) × 10^{-6}	79 ± 10
(−)-Isoproterenol + 200 μM GTP	6	1.5 (±1.8) × 10^{-7}	1.4 (±1.1) × 10^{-5}	84 ± 18
(+)-Isoproterenol	2	1.1 (±0.2) × 10^{-6}	4.5 (±1.7) × 10^{-5}	68 ± 2
(−)-Epinephrine	4	9.3 (±5.7) × 10^{-8}	1.0 (±0.8) × 10^{-5}	63 ± 11
(−)-Norepinephrine	4	7.2 (±2.7) × 10^{-7}	2.9 (±1.9) × 10^{-5}	58 ± 12

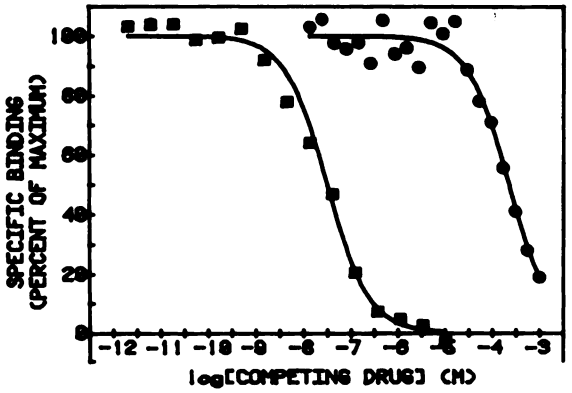

FIG. 6. *Zinterol and practolol competition for [^{125}I]CYP sites on BC3H-1 membranes*

Experimental details are the same as for Fig. 5 except that the competing drugs were (±)-zinterol (■——■) in the presence of 200 μM GTP and (±)-practolol (●——●).

terenol competition curve to the right, with an accompanying increase in the Hill coefficient from 0.49 ± 0.16 (n = 7) to 0.70 ± 0.06 (n = 4), $p < 0.04$. The addition of GTP appeared to decrease the affinity of both classes of sites recognized by isoproterenol (Table 2), and generated a similar proportion of high- and low-affinity sites. Substitution of 5′-guanylyl imidodiphosphate for GTP yielded similar results (data not shown).

Interaction of adrenergic ligands with the [^{3}H]prazosin binding site. Competitive binding studies were used to characterize the interaction of [^{3}H]prazosin with its high-affinity binding site in the BC3H-1 membrane preparation.

Binding of [^{3}H]prazosin was inhibited stereoselectively by the (+)- and (−)-isomers of epinephrine, the latter being 15-fold more potent (Table 3). [^{3}H]Prazosin is highly selective for the *alpha*$_1$-adrenergic receptor (23). The *alpha*$_1$-selective ligands prazosin and WB4101 were far more potent than the *alpha*$_2$-selective ligands yohimbine and clonidine in competing for [^{3}H]prazosin binding sites (Table 3). Furthermore, all of these competition curves showed Hill coefficients near unity [0.88 ± 0.25 (n = 8), p (that n_H = 1) = 0.10], and computer modeling of the binding data indicated the existence of only one class of binding sites.

Adrenergic agonists competed with [^{3}H]prazosin for its receptors with the rank order of potency (−)-epinephrine ≥ (−)-norepinephrine ≫ (−)-isoproterenol (Table 3; Fig. 7). This is compatible with binding to an *alpha*-adrenergic receptor. Again, these competition curves showed Hill coefficients approximating unity [0.91 ± 0.23 (n = 12), p (that n_H = 1) > 0.10], and fitting to binding equations for single or multiple sites indicated the existence of one binding site. GTP (200 μM) had no effect on the ability of (−)-epinephrine to compete for [^{3}H]prazosin binding sites on BC3H-1 membranes (Table 3).

Adrenergic-mediated cyclic AMP accumulation. Cyclic AMP accumulation in BC3H-1 cells was stimulated by *beta*-adrenergic agonists (Fig. 8). The rank order of potency of stimulation by the classical agonists [(−)-isoproterenol > (−)-epinephrine ≫ (−)-norepinephrine] and the high potency of the *beta*$_2$-selective agent zinterol

TABLE 3

Affinities of adrenergic amines for the BC3H-1 alpha$_1$*-adrenergic receptor*

Using K_{app} values for the displacement of [^{3}H]prazosin (at 0.3–1.0 nM concentration) from specific binding sites on BC3H-1 particulate fractions, K_d values were calculated as described under Experimental Procedures, using a value of 86 pM as the K_d for [^{3}H]prazosin. Results are expressed as the mean ± standard deviation of values obtained from 10- to 15-point curves, each point representing duplicate determinations in the number of experiments (n) indicated.

Drug	n	K_d
		M^{-1}
Phentolamine	2	$7.0\ (\pm 0.2) \times 10^{-9}$
Prazosin	4	$4.8\ (\pm 2.3) \times 10^{-11}$
(±)-WB4101	3	$4.0\ (\pm 1.9) \times 10^{-9}$
(±)-Yohimbine	2	$1.2\ (\pm 0.9) \times 10^{-6}$
Clonidine	3	$1.2\ (\pm 0.6) \times 10^{-6}$
(−)-Epinephrine	4	$2.1\ (\pm 1.4) \times 10^{-6}$
(−)-Epinephrine + 200 μM GTP	2	$2.4\ (\pm 0.6) \times 10^{-6}$
(+)-Epinephrine	2	$3.0\ (\pm 0.4) \times 10^{-5}$
(−)-Norepinephrine	3	$4.2\ (\pm 0.6) \times 10^{-6}$
(−)-Isoproterenol	2	$>2 \times 10^{-4}$

(EC_{50} ≃ 100 nM) indicated that this stimulation was mediated by a *beta*$_2$-adrenergic receptor. Moreover, the (−)-isoproterenol-mediated stimulation of cyclic AMP accumulation was blocked by propranolol but not by phentolamine (data not shown). We found no evidence that *alpha*-adrenergic receptors in BC3H-1 cells promote either stimulation or inhibition of cyclic AMP accumulation: phenylephrine had no effect on cyclic AMP accumulation.

Adrenergic-mediated Ca^{2+} efflux. Stimulation of BC3H-1 cells by the *alpha*$_1$-adrenergic agonist phenylephrine increased the rate of unidirectional $^{45}Ca^{2+}$ efflux (Fig. 9). This efflux response was characterized pharmacologically by using antagonists of known adrenergic

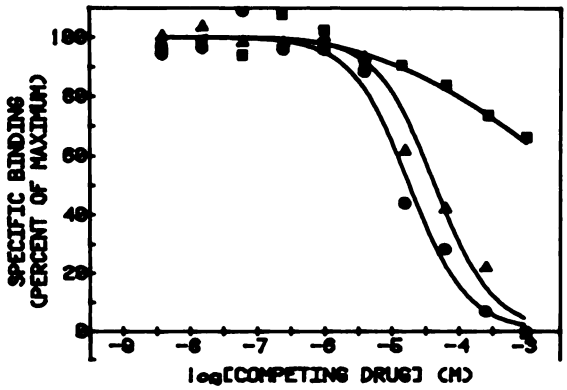

FIG. 7. *Competition by agonists for [^{3}H]prazosin sites on BC3H-1 membranes*

A crude membrane preparation of BC3H-1 cells was prepared as described and incubated with 300 pM [^{3}H]prazosin in the absence (0% inhibition) or presence (100% inhibition) of 10 μM (±)-phentolamine. The indicated concentrations of competing ligands were added to the incubation medium in the absence of phentolamine, and the percentage inhibition of specific binding was determined. Results are expressed as the mean of duplicate determinations using (−)-isoproterenol (■——■), (−)-epinephrine (●——●), and (−)-norepinephrine (▲——▲); the curves were fitted by the nonlinear least-squares curve-fitting program.

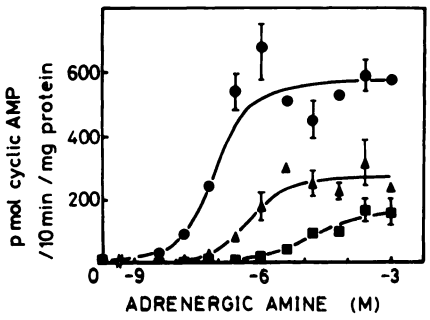

FIG. 8. *Agonist stimulation of cyclic AMP accumulation in BC3H-1 cells*

BC3H-1 cells were grown in multiwell plates. (−)-Isoproterenol (●——●), (−)-epinephrine (▲——▲), and (−)-norepinephrine (■——■) were added to the cell incubation medium [Hanks' balanced salt solution containing 5 mM Hepes (pH 7.4) and 0.5 mM IBMX], and the cyclic AMP which accumulated over 10 min was measured. Results are expressed as the mean of duplicate determinations. The protein content of at least two wells per plate was determined.

receptor subtype selectivity. The phenylephrine-stimulated $^{45}Ca^{2+}$ efflux was blocked by the *alpha*-adrenergic antagonist phentolamine, but not by the *beta*-antagonist propranolol (Fig. 9A). At a concentration of 10^{-7} M, the $alpha_1$-selective antagonist prazosin abolished phenylephrine-stimulated $^{45}Ca^{2+}$ efflux, whereas the $alpha_2$-selective antagonist yohimbine only partially inhibited the response (Fig. 9B). These results are consistent with activation of $^{45}Ca^{2+}$ efflux being mediated by *alpha*-adrenergic receptors on BC3H-1 cells. Furthermore, the $alpha_2$-selective antagonist prazosin proved more effective than the $alpha_2$-selective antagonist yohimbine in inhibiting the efflux response. However, yohimbine produced greater inhibition in intact cells than would be predicted from its dissociation constant determined in isolated membranes. The decrement in functional response on repeated application of phenylephrine may reflect both receptor desensitization and the presence of multiple components of exchangeable cellular Ca^{2+}.

Turnover of $beta_2$- and $alpha_1$ adrenergic receptors. Cycloheximide (1 μg/ml) inhibited ≥95% of protein synthesis by BC3H-1 cells over a period of 3 hr as determined by incorporation of [^{3}H]leucine (data not shown). Cell viability could be maintained for up to 5 days when cells were incubated in growth medium containing cycloheximide (1 μg/ml). The numbers of $alpha_1$- and $beta_2$-adrenergic receptors on BC3H-1 cells were examined in confluent cultures grown in the presence and absence of cycloheximide (1 μg/ml). In the absence of cycloheximide, the density of the two receptors was unchanged. However, in the presence of cycloheximide (1 μg/ml), a dramatic decrease in $alpha_1$-adrenergic receptor density was measured ($t_{1/2}$ = 24 hr), whereas there was only a small decrease in $beta_2$-adrenergic receptor density ($t_{1/2}$ ≥ 96 hr) (Fig. 10).

DISCUSSION

Receptors for adrenergic amines can be divided into at least four classes, namely $alpha_1$, $alpha_2$, $beta_1$, and $beta_2$. This classification not only defines receptors with distinct modes of action but also accounts for the ability of these receptors to recognize various adrenergic amines, both natural and synthetic, with quite distinct relative affinities. On the basis of functional studies using ^{86}Rb efflux, Mauger and colleagues (11) reported that BC3H-1 muscle cells responded to catecholamines with a specificity indicative of the presence of both *alpha*- and *beta*-adrenergic receptors. The data presented in this paper provide a direct quantitation of $alpha_1$- and $beta_2$-adrenergic receptors on BC3H-1 cells. By using [^{3}H]prazosin to label the *alpha*-adrenergic receptors and a newly developed radioligand, [^{125}I]CYP, to label the *beta*-adrenergic receptors, we found that BC3H-1 cells possess 1.5 times as many $alpha_1$- as $beta_2$-adrenergic receptors and that these receptors appear to be distinct as judged by three criteria: (a) the differing abilities of a variety of adrenergic agents to compete for the high-affinity binding sites occupied by the two radioligands, (b) different second messengers ($alpha_1$, Ca^{2+} and $beta_2$, cyclic AMP) for the two receptors, and (c) distinct turnover rates for the two types of receptors.

Lands *et al.* (2) reported that $beta_2$-adrenergic receptors were primarily present on smooth muscle cells as opposed to $beta_1$-adrenergic receptors, which were lo-

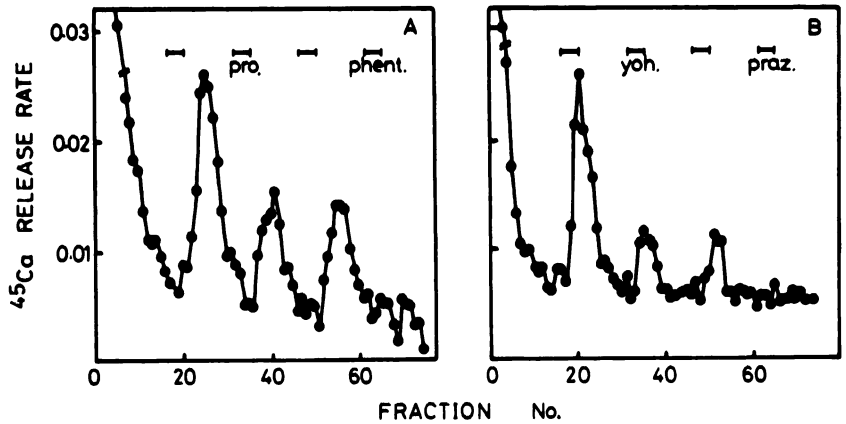

FIG. 9. *Characterization of phenylephrine-stimulated $^{45}Ca^{2+}$ efflux from BC3H-1 cells*

$^{45}Ca^{2+}$ efflux experiments were performed as described under Experimental Procedures. Drug perfusions were applied as indicated, each bar indicating 10^{-5} M phenylephrine plus other drugs, as appropriate. A, Propranolol (*pro.*) = 10^{-5} M; phentolamine (*phent.*) = 10^{-5} M. B, Yohimbine (*yoh.*) = 10^{-7} M; prazosin (*praz.*) = 10^{-7} M.

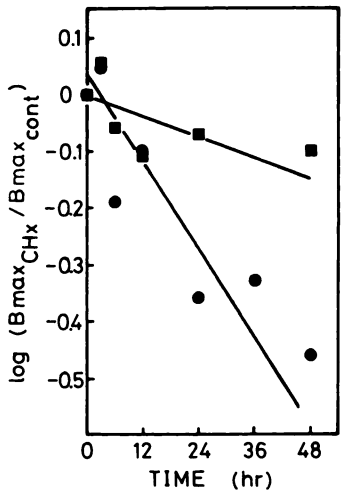

FIG. 10. *Cycloheximide-induced decrease in adrenergic receptor number*

BC3H-1 cells were grown to confluence in 150-mm diameter dishes, the growth medium was renewed, and cycloheximide 1 μg/ml was added to one-half of the plates. The numbers of $alpha_1$ (●——●) and $beta_2$ (■——■) adrenergic receptors were measured over the ensuing 48 hr. $Alpha_1$-receptor number was estimated by the difference between the femtomoles of [^{3}H]prazosin bound in incubations containing 200 pM [^{3}H]prazosin in the presence and absence of 10 μM phentolamine. $Beta_2$-receptor number was likewise estimated from the femtomoles of [^{125}I]CYP bound in incubations containing 200 pM [^{125}I]CYP in the presence and absence of 1 μM (±)-propranolol. Data are expressed as the fraction of receptors remaining in cells incubated with cycloheximide as compared with simultaneous controls. All determinations were made in quadruplicate, and results shown represent the mean of two experiments. Initial receptor densities were 177 fmoles/mg of protein for $alpha_1$-adrenergic receptors and 94 fmoles/mg of protein for $beta_2$-adrenergic receptors.

cated on cardiac, adipose, and other tissues. Furthermore, postsynaptic $alpha_1$-adrenergic receptors are believed to be responsible for the *alpha*-adrenergic response of target cells to catecholamines, including smooth muscle contraction. Thus, adrenergic receptors on BC3H-1 cells conform to their purported origin of smooth muscle cell, as judged by the nature of the adrenergic receptors which they express. However, it is noteworthy that they also possess nicotinic-cholinergic receptors (9, 10), which are typically not seen in mammalian smooth muscle tissues.

Burns *et al.* (6) recently reported the presence of $alpha_1$-, $alpha_2$-, and *beta*-adrenergic receptors on a human adipocyte preparation for which only morphological criteria were used to demonstrate homogeneity in cell population. Data for the BC3H-1 cells offer much firmer evidence to conclude that a single cell can express multiple types of adrenergic receptors. The BC3H-1 cell line, which expresses $alpha_1$- and $beta_2$-adrenergic receptors, was derived from a single cell and, in addition, demonstrates stable expression of these receptors on subclones of the BC3H-1 parent.

Several lines of evidence indicate that BC3H-1 cells possess a single population of $beta_2$-adrenergic receptors: (1) [^{125}I]CYP binds to a single class of sites, as determined by both kinetic and equilibrium binding studies; (b) subtype-selective antagonists compete for these sites with Hill coefficients close to unity, and computer analysis indicates that the data are satisfactorily described by a one-site model; (c) the rank order of potency for competition by nonselective agonists is typical of $beta_2$-adrenergic receptors; (d) binding of receptor subtype-selective compounds (i.e., zinterol, $beta_2$ and practolol, $beta_1$) (22) demonstrates that [^{125}I]CYP shows marked selectivity for competition by the $beta_2$-adrenergic agent, zinterol. Agonist binding to the $beta_2$-adrenergic receptor, assessed through displacement of [^{125}I]CYP, is described by shallow competitive binding curves which are consistent with the presence of two classes of binding sites possessing distinct affinities. The presence of GTP shifted the affinity of both classes of sites observed with (−)-isoproterenol to lower concentrations and revealed a larger Hill coefficient. Such a GTP-dependent decrease in agonist affinity is usually seen with *beta*-adrenergic receptors when studied in membrane preparations and appears dependent on the coupling of the receptor to a functional guanyl nucleotide-binding regulatory component (23). Our inability to detect an elimination of high-affinity sites in the presence of GTP is in apparent contrast to data in other systems (23). Mauger and coworkers (12) recently reported a GTP-induced decrease in affinity of agonists in competition with [^{3}H]dihydroalprenolol for binding sites on BC3H-1 membranes.

In contrast to the situation seen with the *beta*-adrenergic receptors, agonist binding to the *alpha*-adrenergic receptors on BC3H-1 could be described by one binding site, and the addition of GTP had no effect on the (−)-epinephrine competition curve (Table 2). The $alpha_1$-selective compounds prazosin and WB4101 were far more potent than the $alpha_2$-selective ligands clonidine and yohimbine (23) in competing for [^{3}H]prazosin binding sites. In addition, all of the curves generated from competition of these compounds for [^{3}H]prazosin sites could be fitted satisfactorily with a one-site model. Further evidence that BC3H-1 cells possess $alpha_1$- but not $alpha_2$-adrenergic receptors is provided by the observation that Scatchard analysis of [^{3}H]prazosin binding is consistent with a single class of binding sites and that the tritiated $alpha_2$-selective ligands do not display the high-affinity binding characteristic of $alpha_2$-adrenergic receptors in other cell types.

Measurement of the rate of receptor loss following blockade of protein synthesis by cycloheximide indicated that the two classes of adrenergic receptor expressed by BC3H-1 cells were cleared from the plasma membrane at different rates. A similar protocol was previously employed to study the turnover of nicotinic cholinergic receptors on the BC3H-1 cells, and these receptors were cleared from the plasma membrane faster ($t_{1/2}$ ~ 8 hr) than the adrenergic receptors were in our study (9). Thus, it appears that these three classes of receptors are independently regulated in these cells. Thus, receptor turnover in muscle cells does not appear to represent simply bulk membrane protein degradation.

There has been some debate, over the past few years, as to whether *alpha*- and *beta*-adrenergic receptors are interconvertible (24). Surprisingly, all of the data arguing for interconversion accrue from impure cell populations in which the two classes of receptor may not even coexist on the same cell. The BC3H-1 cell should offer a system

in which this interconversion hypothesis can be rigorously tested. In addition, this system should prove useful as a paradigm for the study of the interaction between *alpha*$_1$-adrenergic, *beta*$_2$-adrenergic, and nicotinic cholinergic receptors on the same cell.

ACKNOWLEDGMENTS

We express our thanks to L. Mahan for performing the iodination of CYP; S. Camp, K. Berger, and M. Mullen for their help with the tissue culture; and S. Dutky for preparation of the manuscript.

Note added in proof. We would like to note that an independent estimate of the BC3H-1 *alpha*-adrenergic receptor half-life, 23 hr, was recently reported (25).

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