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# Catecholamine hormone receptor differences identified on 3T3 and simian virus-transformed 3T3 cells

(β-adrenergic/$\beta_1$, $\beta_2$ receptors/cyclic AMP/cell division)

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ABSTRACT **Identification and characterization of hormone receptors on the cell surface is an effective tool for studying the plasma membrane. Using the direct binding of a radiolabeled antagonist, (−)[$^3$H]alprenolol, to crude membrane preparations, and a physiological response (cellular cyclic AMP levels), I demonstrated a catecholamine (β-adrenergic) hormone receptor site coupled to a catecholamine responsive adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] on 3T3 and simian virus 40 (SV40)-transformed 3T3 cells. At a concentration of 1 μM, epinephrine and isoproterenol elevate cellular cyclic AMP levels 8- and 12-fold, respectively, in both cell lines. Norepinephrine was also a potent agonist on 3T3 cells (8-fold stimulation), but SV3T3 cells showed a lesser (2-fold) response to this hormone. The specificity of the physiological response (as well as the direct binding studies using the alprenolol radiolabel) is indicated by the increased effectiveness of (−) compared to (+) stereoisomers, rapid and reversible kinetics (steady state within 2 min), high affinity ($K_d$ approximately 30 nM) and saturability (indicating a finite number of hormone receptors). These hormone receptor studies indicate the 3T3 cells have a $\beta_1$ adrenergic receptor while the SV3T3 cells have a receptor with $\beta_2$ qualities. In addition, the number of β-adrenergic hormone receptors appear to be increased in the normal 3T3 cells by approximately 2-fold over the SV3T3 cells (300 versus 120 femtomol/mg of protein).**

The plasma membranes of normal and transformed cells differ in chemical composition (1, 2) and biological function (3, 4). These differences may influence cellular proliferation via the membrane-associated hormone-sensitive adenylate cyclase (ATP pyrophosphate-lyase(cyclizing), EC 4.6.1.1.) which catalyzes the production of adenosine 3′:5′-cyclic monophosphate (cAMP) from ATP. cAMP is known to inhibit cell division (5, 6).

Basal specific activities for adenylate cyclase have been determined in several control and tumor tissues (7) as well as in normal and transformed cultured cell lines (8). Clear and consistent differences are not evident when the basal specific activities of the normal and transformed cell enzymes are compared, e.g., increased activity (9–11), decreased activity (3, 12, 13), and no difference (11, 14) have been observed when the basal activity of adenylate cyclase of normal or control cells was compared to the transformed or tumor cells.

A difference that does prevail in the characterization of adenylate cyclase is a decreased hormonal responsiveness of the enzyme in transformed cells compared to the growth regulated cells (7, 15–17). Cell cultures, such as the 3T3 cells, are often useful for studying the regulation of cell division. From this growth regulated line, were generated cell lines that have lost contact inhibition or density dependent inhibition of growth. This transformed (unregulated growth) phenotype occurs spontaneously but is generally elicited by virus infection or exposure to chemical carcinogens. The purpose of the present study was to determine if the adenylate cyclase of normal 3T3 and simian virus 40 (SV40)-transformed 3T3 cells was responsive to catecholamine hormones, and if so, to determine whether any differences in the hormone response or receptors might exist.

Catecholamine hormone receptors are classically distinguished as α or β receptors depending on the potency of epinephrine, norepinephrine, or isoproterenol to elicit a physiological response. These three adrenergic agonists react differently with α and β receptors. Hormonal antagonists can also distinguish α from β receptors (e.g., propanolol and alprenolol are specific β-adrenergic antagonists). Of the three catecholamines, epinephrine is the most potent α-adrenergic agonist while isoproterenol is the most potent β-adrenergic agonist. More recently, the β-adrenergic receptors were further subdivided into two categories, $\beta_1$ and $\beta_2$, depending on the potency of norepinephrine to act as an agonist. For $\beta_1$ receptors, the efficacy of isoproterenol is greater than either epinephrine or norepinephrine and the latter two are equally potent; for the $\beta_2$ receptor, the potency of isoproterenol is greater than epinephrine and much greater than norepinephrine. Using these characteristics, we can classify cells by their hormonal responsiveness to catecholamines—e.g., myocardial muscle cells have $\beta_1$ receptors, whereas smooth muscle cells have $\beta_2$ receptors. See ref. 18 for further information.

Heretofore, hormone responsiveness has not been demonstrated on 3T3 cells or its various transformants. Only prostaglandins had been shown to elevate cellular cAMP levels in 3T3 cells (6). This study describes the β-adrenergic hormone sensitivity of 3T3 and SV3T3 by demonstrating elevated cAMP levels after exposure to the catecholamines epinephrine, norepinephrine, and isoproterenol. Direct binding studies using the radiolabeled β-adrenergic antagonist (−)[$^3$H]alprenolol were also performed and some chemical properties of the catecholamine hormone receptor site were characterized using crude membrane preparations from these two cell lines.

## METHODS

Cell lines designated 3T3 and SV3T3 originated from the Balb $A_{31}$ and $SVT_2$ lines established by G. Todaro (National Institutes of Health, Bethesda, Md.). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. No antibiotics were used on stock cultures but gentamycin (20 μg/ml) was added to experimental plates and bottles upon plating. Temperature (36.5°) was controlled as well as humidity and $CO_2$ (5%) in Napco incubators.

Membranes from cells grown in glass roller bottles, were prepared by scraping off the cells, then washing with a buffer of 6 mM Tris·HCl and 4 mM $MgCl_2$ adjusted to pH 8.0. The cells were then ruptured by brief sonication or homogenization and the 5000 × *g* pellet was resuspended in the Tris·HCl/$Mg^{2+}$ buffer as a crude membrane preparation. This membrane

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; SV40, simian virus 40.

preparation could be stored at −20° for a month without losing hormone binding activity.

**Cyclic AMP Assay.** Using confluent, but not overgrown, cells plated on plastic 60 mm dishes, I measured the cAMP by a method of Brown *et al.* (19). The solutions of drugs tested for effect on the cellular cAMP levels were freshly prepared each time. The plates were removed from the incubator (six at a time), the medium was removed, and the cells were washed with 3 ml of phosphate-buffered saline with $Ca^{2+}$ and $Mg^{2+}$, another 1.8 ml of phosphate-buffered saline was added, the agent to be tested was added in 0.2 ml, and the cells were incubated for 10 min at room temperature (24°). After incubation, the test solution was removed and 1.5 ml of 5% trichloroacetic acid at 0° was added. The procedure that followed is described in ref. 6. Protein was determined by the method of Lowry *et al.* (20).

**Direct Binding of (−)[$^3$H]Alprenolol.** To tubes containing (−)[$^3$H]dihydroalprenolol (final concentration 1–80 nM) with and without (±)propanolol (final concentration) was added 160 μl of crude membranes 0.2 mM/μl (40–130 μg of protein) to give a total volume of 200 μl. Tubes were incubated 13 min at 37°. Incubation was terminated by adding 4 ml of ice-cold incubation buffer to the tube and rapidly filtering contents through a Gelman type A/E glass-fiber filter under low vacuum. The filter was rinsed (ice-cold incubation buffer) with a 4-ml wash of the tube and a final 8-ml rinse. Filters were placed in 10 ml of 10% BioSolv. (Beckman)/90% toluene/0.4% omnifluor scintillation cocktail and allowed to stand overnight.

In each experiment, "nonspecific" binding was determined by parallel assay tubes that contained a large excess (200 μM) of (±)propanolol. "Specific" binding was defined as the difference between the total and the nonspecific binding.

The (−)[$^3$H]alprenolol was purchased from New England Nuclear who prepared the radiolabeled product by catalytic reduction of (−)alprenolol using tritium gas with paladium as a catalyst. The molecular structure, determined by mass spectroscopic methods, is dihydroalprenolol and the tritium is probably found at the unsaturated bond in the aliphatic chain on position two of the aromatic ring.

Other compounds used were (−)propanolol and (+)propanolol from Ayerst laboratories New York, (−)alprenolol from the Hassle Co., Sweden, (−)isoproterenol, (−)epinephrine, and dopamine from Sigma Chemical Co., St. Louis, (+)isoproterenol and (+)epinephrine from Winthrop Laboratories, New York.

## RESULTS

### Physiological response

Evidence that 3T3 and SV3T3 cells respond physiologically (i.e., activate adenylate cyclase resulting in an elevation of the cellular cAMP level) to β-adrenergic hormones is presented in Fig. 1. The cellular level of cAMP is normally 25 and 12 pmol/mg of protein for confluent 3T3 and SV3T3 cells, respectively. After exposure to μM isoproterenol and epinephrine, the cAMP levels are increased by approximately 10- and 8-fold for both the 3T3 and SV3T3 cells. These numbers represent the minimum stimulation observed; on several occasions, up to a 20-fold stimulation was seen in the two cell lines, but the response at μM was always proportionate to the values cited above (i.e., isoproterenol > epinephrine).

While both normal and transformed cell lines responded similarly to isoproterenol and epinephrine, a major difference was noted in their response to norepinephrine. With this agonist, the 3T3 cells responded with an 8-fold elevation of their cAMP level. The SV3T3 cAMP level, however, was not stimulated by norepinephrine. Fig. 1 illustrates the similarities and the differences of normal and transformed 3T3 cells in response to varying doses of the β-adrenergic agonists.

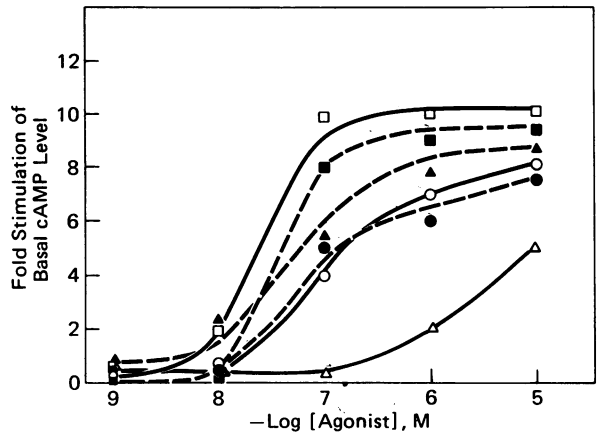


FIG. 1. β-Adrenergic agonists were tested for their effect on the cAMP levels of Balb 3T3 and SV3T3 cells. The conditions of the test and the cAMP assay are described in the *text*. The points are averages of triplicate determinations. This experiment was performed five times with the data presented here consistent with the other experiments except that maximum stimulation of the basal cAMP level varied between 6- and 20-fold. Each experiment, however, showed the same dose response curve for Balb 3T3 and SV3T3 cells. Balb 3T3 cells were studied with isoproterenol (■), epinephrine (●), and norepinephrine (▲). SV3T3 cells were studied with isoproterenol (□), epinephrine (○), and norepinephrine (△).

The kinetics of the cellular response to isoproterenol reaches steady state for both lines within 2 min after exposure of the cells to the hormone. Interference with this response is observed if antagonists such as propanolol are incubated with the agonist being tested. Table 1 is an example of such an effect and also illustrates the stereospecificityof the effect demonstrating that (−) or *l*-stereoisomers are more potent molecular competitors compared to the corresponding (+) or *d*-stereoisomers.

Inhibition of the isoproterenol-stimulated adenylate cyclase by the β-adrenergic antagonist alprenolol, is depicted in Fig. 2. This dose response curve indicates that the apparent $K_d$ of the alprenolol antagonist is the same value for both cell lines and is approximately 30 nM. This value is comparable to that derived by the direct binding studies presented below.

### Direct binding of (−)[$^3$H]alprenolol

Specific binding of (−)[$^3$H]alprenolol to a crude membrane preparation of 3T3 and SV3T3 cells is very rapid at 24° and reaches steady state in less than 2 min. This binding of the radiolabeled ligand was completely reversible by 10 μM propanolol added prior to or after alprenolol binding had reached a steady state (Fig. 3). No kinetic differences were observed between the two cell lines.

The binding exhibited specificity for the (−) stereoisomer of agonists and antagonists. Table 2 is an example of the stereospecificity observed. In addition to the specific reversal of alprenolol binding by the (−)propanolol isomer, the (−) agonist isomers, epinephrine in this case, were also more effective competitors for the hormone receptor site.

Compounds that had no physiological effect such as pyrocatechol, dopamine, or phenotolamine also had no effect on the binding of (−)[$^3$H]alprenolol. These compounds at 0.1 mM had less than a 20% reversal effect on (−)[$^3$H]alprenolol binding with each cell line.

Saturability of the reaction is an indication of a finite number

Table 1. Stereospecificity for propanolol reversal of isoproterenol stimulation of cellular cAMP levels

| Treatment | Fold stimulation of cellular cAMP levels: BALB 3T3 | SV 3T3 |
|---|---|---|
| Isoproterenol (1 μM) | 12 | 10 |
| Isoproterenol (1 μM) + (−)propanolol (10 μM) | 1.5 | 1.0 |
| Isoproterenol (1 μM) + (+)propanolol (10 μM) | 13 | 10 |

Reversibility and stereospecificity of the antagonist propanolol upon the β-adrenergic agonist's stimulation of cellular cAMP levels. Conditions of the experiment and the cAMP assay are described in the *text*. Each value is the mean of triplicate determinations. This experiment is representative of five others.

of binding sites and both cell lines exhibited saturation around $5 \times 10^{-8}$ M alprenolol. However, the actual number of hormone receptor sites on the two cell types is different as shown in Fig. 4. The Balb 3T3 cells have approximately 300 fmol of (−)[$^3$H]alprenolol bound per mg of membrane protein which correspond to 17,000 hormone receptor sites per cell, while the SV3T3 cells have about 120 fmol of ligand bound per mg of protein or about 2,000 receptor sites per cell (transformed cells are 33% smaller than the normal cell).

## DISCUSSION

The cornerstone of Sutherland's second messenger hypothesis is the hormonal responsiveness of the membrane-associated adenylate cyclase. Polypeptide and catecholamine hormones are thought to elicit their physiological effects by binding to membrane hormone receptors thereby stimulating the adenylate cyclase to catalyze the synthesis of cAMP. Direct proof of such an event was not available until recently when specific radiolabeled probes were developed for the purpose of identifying and characterizing hormone receptor sites (22–24). Prior to these advances, classical physiological responses and pharmacological specificities were used to describe the hormone sensitivity of the adenylate cyclase from a certain tissue or cell type.

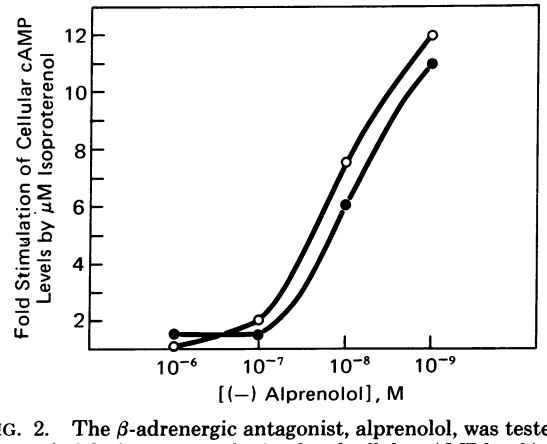


FIG. 2. The β-adrenergic antagonist, alprenolol, was tested for its reversal of the isoproterenol-stimulated cellular cAMP level in Balb 3T3 and SV3T3. The conditions of the test and the assay are described in the *text*. Each point is the mean of triplicate determinations. This figure is representative of four experiments. (○) Balb 3T3; (●) SV3T3.

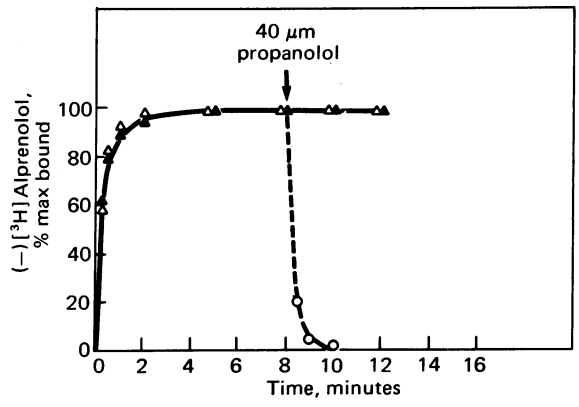


FIG. 3. Binding of (−)[$^3$H]alprenolol to a crude membrane preparation as a function of time. Specific binding was determined after each interval as described in the *text*. Each value shown is the mean of triplicate determinations. This experiment is representative of six experiments. (△) Balb 3T3; (▲) SV3T3; (○) Balb 3T3 or SV3T3 after addition of propanolol.

The catecholamine, β-adrenergic hormone receptor site was especially difficult to study because agents of sufficient specificity, irreversible binding, and radioactivity did not exist. In the past year, Aurbach (24), Gilman (23), and Lefkowitz (22) have described the methodology necessary for directly identifying β-adrenergic hormone receptor sites using radiolabeled antagonists [e.g., (−)[$^3$H]alprenolol].

Our interest in this hormone receptor research stems from the observation that cellular cAMP levels appear to participate in the regulation of cell division (5, 6). Changes in hormone receptors could account for alterations in cyclic nucleotide levels and thereby influence cellular proliferation. Aside from the prostaglandin responsiveness of 3T3 cells, no other receptors have been indicated on 3T3 cells.

In addition to identifying the presence of a β-adrenergic hormone receptor in both normal and virus-transformed 3T3 cells, this paper describes major qualitative and quantitative differences between these two cell types. The growth regulated 3T3 cell is sensitive to norepinephrine while the SV40-transformed cell line is essentially unresponsive, and suggests that the 3T3 line has a $\beta_1$ type of receptor while the SV3T3 line has a $\beta_2$ type adrenergic receptor.

Table 2. Stereospecificity for propanolol reversal of (−)[$^3$H]alprenolol binding to membrane preparations

| Treatment | fmol alprenolol bound per mg protein: BALB 3T3 | SV3T3 |
|---|---|---|
| (−)[$^3$H]alprenolol (10 nM) | 300 | 120 |
| (−)[$^3$H]alprenolol (10 nM) + (−)propanolol (1 μM) | 50 | 20 |
| (−)[$^3$H]alprenolol (10 nM) + (+)propanolol (1 μM) | 280 | 100 |
| (−)[$^3$H]alprenolol (10 nM) + (−)epinephrine (0.1 mM) | 90 | 40 |
| (−)[$^3$H]alprenolol (10 nM) + (+)epinephrine (0.1 mM) | 280 | 110 |

Reversibility and stereospecificity of the specific binding of (−)[$^3$H]alprenolol to a crude membrane preparation from Balb 3T3 and SV3T3 cells. The conditions of the binding assay and competition experiments are described in the *text*. These values are means of triplicate determinations. This experiment is representative of four other experiments.

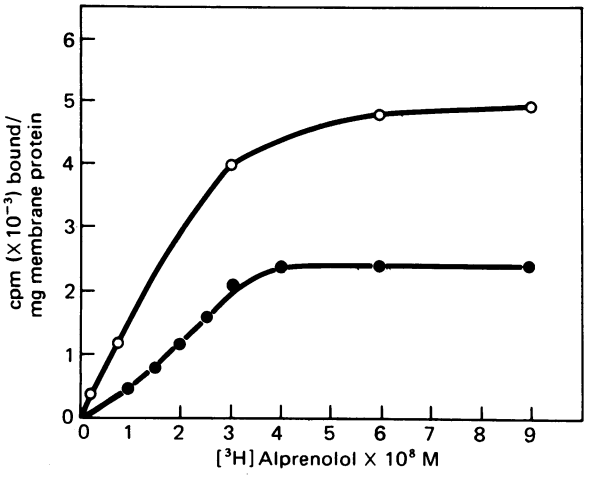


FIG. 4. (−)[³H]Alprenolol-specific binding as a function of the alprenolol concentration in the incubation mixture. The conditions of the binding are described in the *text*. Each point is the mean of triplicate determination. This experiment is representative of six experiments. (○) Balb 3T3; (●) SV3T3.

Quantitative differences also exist between the two cell lines. SV3T3 cells are smaller than the normal 3T3 cells, and should have, therefore, a larger surface area if the cells are compared on a per mg of protein basis. Yet just the opposite is observed. The normal 3T3 membrane preparation has twice as many β-adrenergic receptors as the SV3T3 cells per mg of membrane protein.

The identification and characterization of a β-adrenergic hormone receptor site on 3T3 cells naturally leads to the question of physiological significance. Catecholamines are short-lived hormones and are not found in any appreciable quantity in tissue culture media, thus precluding any direct significance. However, the embryonic mouse cells from which the Balb and Swiss 3T3 lines were derived did have the potential for responding to catecholamines. Recent evidence indicates the Swiss 3T3 cell line has biochemical and biological characteristics similar to adipocytes, a cell type responsive to many different hormones (25–27). Thus, the β-adrenergic hormone receptor site and responsiveness described here for Balb 3T3 cells may not be currently necessary for maintenance of a biological function in culture, but could be an atavism of a former capacity. Other hormone receptor sites, yet to be identified and characterized, may provide further information concerning the biological significance of such receptors on cultured cells, e.g. endothelial cells exhibit various hormonal sensitivities (28).

In extension of these observations concerning hormone receptors of cultured fibroblasts, studies were recently performed comparing normal human lymphocytes and those collected from chronic lymphocytic leukemia patients (16, 29). The leukemia patient's cells were found to have less than 30% of the β-adrenergic sites of the normal lymphocytes. The loss of the hormone receptor could account for the loss of β-adrenergic responsiveness exhibited by these cells (J. R. Sheppard and C. Moldow, submitted for publication) and might also be related to their defective proliferation.

Our studies describe the preliminary identification and characterization of the β-adrenergic hormone responsiveness of normal and virus-transformed 3T3 cells. The data indicate qualitative and quantitative differences exist between the two cell lines. Hormone receptor studies, such as these, provide a powerful tool for studying the plasma membrane structure and its biological functions. Combining cell culture methodology with these hormone receptor techniques promises to yield information on a wide variety of cellular phenomena.

**Note Added in Proof.** Since this manuscript was initially submitted, two other papers (30, 31) have also shown differences in hormone receptors of normal and transformed cultured cells.

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