Proc. Natl. Acad. Sci. USA
Vol. 74, No. 3, pp. 873–877, March 1977
Biochemistry

Induction of functional β-adrenergic receptors in HeLa cells

(*l*-[^{3}H]dihydroalprenolol/3′:5′-cyclic AMP/sodium butyrate/*l*-isoproterenol-stimulated 3′:5′-cyclic AMP production/catecholamines)

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Communicated by Roscoe O. Brady, December 6, 1976

ABSTRACT **HeLa cells contain β-adrenergic receptors that are characterized by specific binding of *l*-[^{3}H]dihydroalprenolol, increased 3′:5′-cyclic AMP production in intact cells after incubation with *l*-isoproterenol, and increased adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity in the presence of *l*-isoproterenol. After cells were cultured with butyrate, the number of β-adrenergic receptors, cyclic AMP production in intact cells, and adenylate cyclase activation by *l*-isoproterenol were increased severalfold over those of untreated cells. The increase involved the induction of synthesis of new receptor molecules with identical affinities for *l*-[^{3}H]-dihydroalprenolol; all three processes were blocked by cycloheximide and actinomycin D. This induction was relatively specific for butyric acid and only the closely related short-chain fatty acids, propionic and valeric acids, were capable of partially inducing the same effect. In contrast to induction of β-adrenergic binding sites, there was no increase in basal or fluoride-activated adenylate cyclase activity, indicating that the β-adrenergic receptor and adenylate cyclase are different molecules that may be controlled separately.**

There is strong experimental evidence for the concept of specific cell surface receptors for polypeptide hormones and neurotransmitters. Much progress has been made in their identification through the use of binding studies with radioactive ligands of high specific activity and through the study of events subsequent to receptor occupancy by agonists (1–5). In spite of this progress, the biochemical mechanisms regulating the number of receptors per cell and their response in a given cell have not been studied in detail. Elucidation of these mechanisms should hasten understanding of such phenomena as desensitization of receptors after stimulation by agonists (6, 7) and supersensitivity after denervation and other manipulations (8, 9) and the role of modulators in the production of intracellular "second messengers."

Because of the widespread distribution of β-adrenergic receptors, which are important in catecholamine-mediated physiological events, and the apparent tight coupling between these receptors and adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], this system is a valuable model for the study of biochemical factors regulating receptors. Through the use of radioactive β-adrenergic antagonists it has been possible to characterize unambiguously a binding site of the β-adrenergic receptor in various cell types and to correlate its binding characteristics with studies of β-receptor-dependent activation of adenylate cyclase (7, 8, 10–12). Because it is possible to study the binding site of the β-adrenergic receptor separately from the ability of β-adrenergic agonists to activate adenylate cyclase, independent evaluation of the number of receptors and their ability to function can be made (12).

To further elucidate the regulatory mechanisms controlling β-adrenergic receptor biosynthesis and events subsequent to receptor stimulation, a cultured cell line in which receptor synthesis can be manipulated by the investigator would be valuable. In recent years, sodium butyrate has been found to induce various changes in mammalian cells in culture (13–20).

Because of the large number of butyrate-induced changes in the membranes of HeLa cells, we asked if this cell line contained receptors whose function might be altered by exposure to butyrate. In this report we demonstrate functional β-adrenergic receptors in HeLa cells and report a system for the induction of synthesis of functional receptors by butyrate.

EXPERIMENTAL PROCEDURES

Materials. ATP, butyric acid, and other fatty acids were purchased from Sigma Chemical Co.; cycloheximide and actinomycin D from P-L Laboratories; *l*-[^{3}H]dihydroalprenolol (specific activity 32.6 Ci/mmol) was purchased from New England Nuclear Co.; and other drugs were obtained from either Regis Chemical Co. or Sigma Chemical Co. *l*-Alprenolol was a gift from Dr. M. Zatz, National Institute of Mental Health. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co.

Cell Culture. HeLa cells were obtained from the Grand Island Biological Co. (GIBCO) and grown in minimal essential medium containing nonessential amino acids and 10% fetal calf serum as described (21); this complete medium is referred to as "MEM". Cells were subcultured by exposing them to a minimal volume of 0.05% trypsin at 37° for 3 min and suspending them in MEM. Cell numbers were determined in an Electrozone/Celloscope (Particle Data Corp.).

For the experiments described below, 150-mm plastic tissue culture dishes (Falcon Plastics) were seeded at a density of 30,000 to 40,000 cells per cm^2 and cultured for 24 hr before experimentation. For harvesting, monolayers were washed with 10 ml of phosphate-buffered saline (pH 7.4) containing 0.2% gelatin; cells were detached from the substratum by scraping with a rubber policeman, suspended in 10 ml of the same buffer, and collected by centrifugation (3000 rpm for 4 min).

The cells were resuspended for assay in 0.9% NaCl buffered with 50 mM Tris·HCl (pH 7.4) containing 5 mM theophylline and 9 mM 3-isobutyl-1-methylxanthine by agitating on a Vortex mixer.

Receptor Binding Studies. The receptor binding study utilized 100 μl of either untreated or sodium-butyrate-treated HeLa cells containing approximately 4×10^5 untreated cells (approximately 250 μg of protein) and 1×10^5 sodium-butyrate-treated cells (approximately 130 μg of protein) in a total incubation volume of 500 μl. The incubations were done in quadruplicate at 37° and were initiated by the addition of [^{3}H]dihydroalprenolol (75,000 cpm, 5.0 nM final concentra-

Abbreviation: cAMP, adenosine 3′:5′-cyclic monophosphate.

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Table 1. Effects of inhibitors and activators on cAMP production in control and butyrate-treated HeLa cells

Contents (concentration 10 μM)	cAMP production (pmol/mg protein·min)	
	Untreated	Butyrate-treated
No additions	2	3.6
l-Isoproterenol	4	77
l-Epinephrine	2.2	81
l-Norepinephrine	2.4	31
l-Isoproterenol + *l*-alprenolol	n.d.*	4
l-Isoproterenol + *dl*-propranolol	n.d.*	6

Cells were treated with 5 mM butyrate for 24 hr. Incubations were carried out as described in the *text* for each agent with intact cells for 5 min at 37°. Each assay was done in triplicate, with no difference greater than ± 10% of the mean.
* n.d. = not determined.

tion). Duplicate tubes containing 10^{-5} M *dl*-propranolol were incubated to determine nonspecific binding. Five-minute incubations were halted by the addition of 7.0 ml of 0.85% saline at 4°. The solution was immediately filtered under low vacuum through a Whatman GFB filter and the filter was washed twice with additional 7-ml aliquots of cold saline. The filters were dried, and bound tritium was measured by liquid scintillation counting techniques. Nonspecific binding is defined as those counts not displaced by 10^{-5} M *dl*-propranolol.

Measurement of Adenylate Cyclase and Production of 3':5'-Cyclic AMP (cAMP) in Intact HeLa Cells. The reaction was started by the addition (10 μl) of the compound under study to 100 μl of the cell suspension. The reactions were carried out in triplicate for 5 min at 37° and halted by placement of the tubes in boiling water. The incubation mixture was diluted to 500 μl with distilled water and an aliquot was assayed for cAMP by a protein binding technique (22, 23).

HeLa cells were disrupted by homogenization for 15 sec in 2 mM Tris·HCl buffer (pH 7.4) using a Polytron tissue disrupter. Aliquots of homogenate (approximately 100 μg of protein) were incubated for 15 min at 37° and contained, in addition to any drug under study, 80 mM Tris·HCl (pH 7.4), 6 mM $MgSO_4$, 1.5 mM ATP, and 10 mM theophylline in a total volume of 100 μl. Incubations were done in triplicate, being initiated by the addition of ATP and terminated by boiling the incubation vessels for 3 min. An aliquot of the incubation mixture was assayed for cAMP as described above.

RESULTS

Properties of the β-Adrenergic Receptor. Both untreated and butyrate-treated HeLa cells contain receptors that fit the criteria for classification as β-adrenergic receptors. These receptors were activated by *l*-isoproterenol > *l*-epinephrine > *l*-norepinephrine to stimulate adenylate cyclase and cAMP production. This effect is blocked by *dl*-propranolol and *l*-alprenolol, two potent β-adrenergic antagonists, but is not blocked by phentolamine or phenoxybenzamine, α-adrenergic antagonists (Table 1).

The β-adrenergic receptors were measured through binding studies utilizing the radioactive ligand, *l*-[^{3}H]dihydroalprenolol. Binding was rapid, reversible, stereospecific, and saturable. The concentration of *l*-[^{3}H]dihydroalprenolol required for half-maximal saturation of the stereospecific saturable binding sites was 12–14 nM for either untreated or sodium-butyrate-treated cells (Fig. 1). The maximal binding for sodium-butyrate-treated cells was 3.5 pmol/10^6 cells and for untreated cells, 0.8 pmol/10^6 cells; if a uniform distribution of receptors in the cell population is assumed, sodium-butyrate-treated cells have about 2×10^6 receptors per cell and untreated cells have 5×10^5 receptors per cell.

Time Course of Induction of β-Receptors. Butyrate induction of *l*-[^{3}H]dihydroalprenolol binding and *l*-isoproterenol-stimulated cAMP production occurred in parallel and reached a maximum by 10 hr (Fig. 2). The levels of both re-

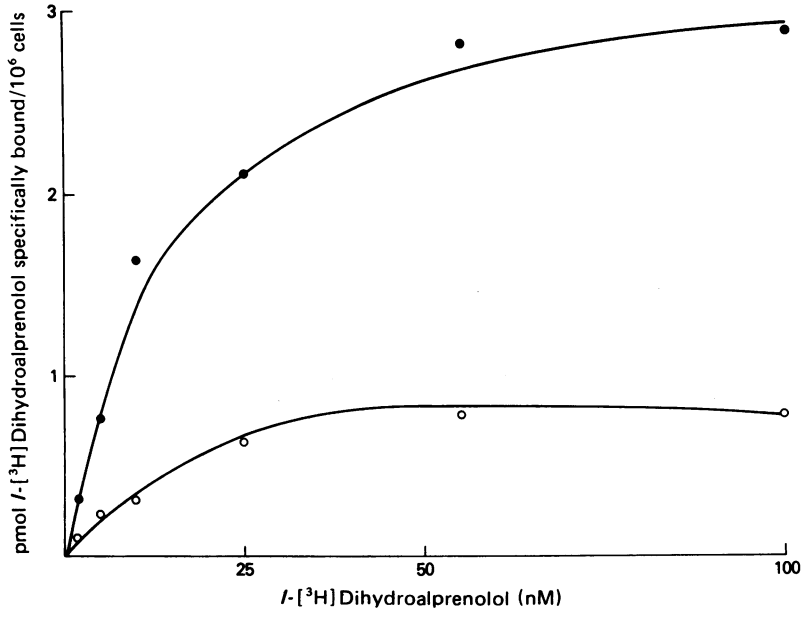

FIG. 1. Effects of varying concentrations of *l*-[^{3}H]dihydroalprenolol on binding to untreated (○) and butyrate-treated (●) HeLa cells. Cells were incubated with or without butyrate (5 mM) for 24 hr and harvested. Binding was determined as described in *Experimental Procedures*. In some cases at the higher concentrations incubation mixtures contained *l*-alprenolol to dilute the specific activity of the *l*-[^{3}H]dihydroalprenolol. The affinity of both these compounds for the β-adrenergic receptor is said to be equivalent in earlier studies (5).

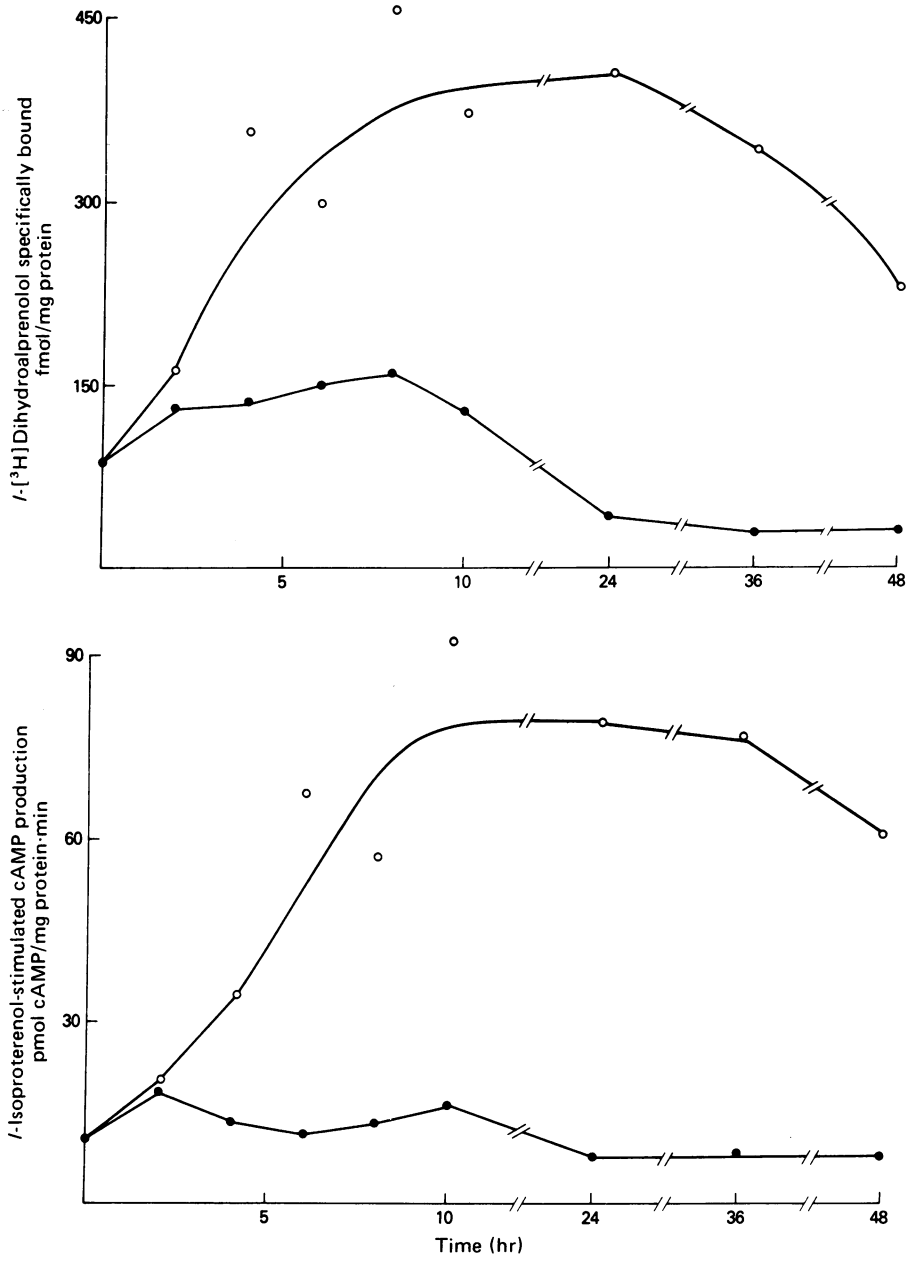

FIG. 2. Time course of induction of β-adrenergic receptors (*upper*) and *l*-isoproterenol-stimulated cAMP accumulation (*lower*) in untreated (●) and butyrate-treated (○) HeLa cells. Cells were incubated for varying times with or without 5 mM butyrate, harvested, and assayed as described in *Experimental Procedures*. After 24 hr the protein content of individual HeLa cells increased and was about 20–30% higher at 48 hr.

ceptor and *l*-isoproterenol-stimulated cAMP production remained at this high level on a cell basis for up to 72 hr, the longest time studied. In contrast, the levels of β-receptor and receptor-dependent cAMP production remained about 4- to 7-fold lower in untreated cells. The decrease in activity expressed on a protein basis may be entirely accounted for by the known increase in protein content of butyrate-treated cells (13).

Effect of Inhibitors of Protein Synthesis on Induction of β-Receptors. Cycloheximide (20 μg/ml) and actinomycin D (1 μg/ml) were tested for their ability to inhibit the appearance of functional β-receptors upon treatment with butyrate (Table 2). Cycloheximide abolished and actinomycin D (1 μg/ml) partly blocked the appearance of both increased *l*-[^{3}H]dihydroalprenolol binding and β-receptor-dependent cAMP production after butyrate treatment. At 0.5 μg/ml, actinomycin D enhanced induction of functional β-receptors, as measured by cAMP production, while it inhibited the increase in binding.

Effect of Related Fatty Acids on Induction of β-Adrenergic Receptors. To investigate the specificity of this induction of binding of [^{3}H]dihydroalprenolol and appearance of *l*-isoproterenol-stimulated cAMP production, we examined a series of short-chain saturated fatty acids for their ability to cause these inductions (Fig. 3). Propionic and valeric acids were somewhat less potent than butyric acid in inducing *l*-isoproterenol-stimulated cAMP production in HeLa cells. Interestingly, although there seems to be a correlation between binding and receptor stimulation for propionic and butyric acids, in the case of valeric acid, the binding of *l*-[^{3}H]dihydroalprenolol remained high while *l*-isoproterenol-stimulated cAMP production failed to increase in proportion to the increase in binding.

Table 2. Effect of inhibitors of protein synthesis on induction of β-adrenergic receptors

Condition	*l*-[^{3}H]Dihydroalprenolol specifically bound (fmol/mg protein)	*l*-Isoproterenol-stimulated cAMP production (pmol/mg protein·min)
Untreated	140	17.7
Butyrate-treated	370	94.0
Butyrate-treated + Cycloheximide (20 μg/ml)	170	18.2
Butyrate-treated + Actinomycin D (0.5 μg/ml)	270	180.0
Butyrate-treated + Actinomycin D (1.0 μg/ml)	220	62.6

HeLa cells were incubated for 10 hr in MEM with no additions, with 5 mM butyrate, or with butyrate plus the inhibitor. Cells were morphologically examined for cell damage, then harvested and assayed as described in *Experimental Procedures*. In all experiments in this table the appearance of the cells was normal and there was no indication of gross cell damage due to the protein synthesis inhibitors.

Effect of Butyrate on Adenylate Cyclase. The effects of butyrate on adenylate cyclase activity were studied after disruption of the cells. There was no difference between untreated and butyrate-treated cells in basal or fluoride-stimulated adenylate cyclase activity (Table 3). Butyrate-treated cells had greater response to *l*-isoproterenol than control cells.

Table 3. Adenylate cyclase activity of untreated and butyrate-treated HeLa cells

Condition	Adenylate cyclase activity (pmol cAMP formed/mg protein·min)	
	Untreated	Butyrate
Basal activity	13	15
+ 10^{-2} M NaF	75	83
+ 10^{-5} M *l*-Isoproterenol	18	34

Butyrate-treated and untreated cells were incubated as described in the *text* and harvested. Cells were homogenized by brief (15 sec) disruption using a Brinkmann Polytron and assayed in triplicate for adenylate cyclase as described in *Experimental Procedures*.

DISCUSSION

Much recent research interest has centered on the relationship between the β-adrenergic receptor and adenylate cyclase through studies of cell lines that contain β-receptors and adenylate cyclase and in somatic cell hybrids between such lines (12, 24, 25). A pharmacological approach has also been used to study the response of various model systems after specific desensitization of β-adrenergic receptors with *l*-isoproterenol (6–8). This desensitization involved a decrease in the number of binding sites, but not in their affinity for *l*-[^{3}H]dihydroalprenolol (7, 8), and could be reversed with β-adrenergic antagonists (7); desensitization and resensitization of these receptors apparently did not involve new protein synthesis (7).

A more direct approach to these problems is suggested by the experiments reported in this communication. Through the use of a specific agent, sodium butyrate, it has been possible to induce the synthesis of new binding sites for *l*-[^{3}H]dihydroalprenolol as well as *l*-isoproterenol-stimulated cAMP production. This process may be differentiated from the desensitization/resensitization process described above because new protein synthesis is required for the increase in β-adrenergic receptors after butyrate treatment (Table 2). We have not yet definitively shown a requirement for RNA synthesis; however, similar ambiguities in the interpretation of actinomycin D effects have been seen for tryptophan pyrrolase and tyrosine aminotransferase induction (26, 27). As noted above, the *l*-[^{3}H]dihydroalprenolol binding site has the same affinity for its ligand in untreated and butyrate-treated cells and would appear to be the same receptor (Fig. 1). The number of sites per cell is higher than other reported numbers of sites in different cell lines (10, 12, 28) and higher than we see in mixed leukocyte experiments (unpublished data); at least in the case of the leukocytes, some of the differences are accounted for by the larger surface area of the HeLa cell.

As in other butyrate phenomena (13, 17, 20), the relative specificity of the butyrate effect is apparent because only the

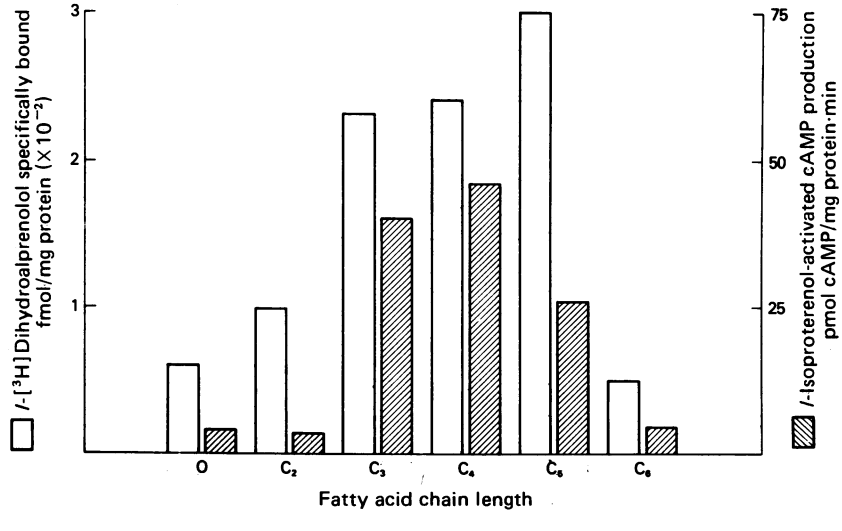

FIG. 3. Effect of saturated fatty acids on induction of β-adrenergic receptors and *l*-isoproterenol-stimulated cAMP production. Fatty acids of different chain lengths were incubated with HeLa cells at a final concentration of 5 mM for 24 hr. The cells were then harvested and assayed as described in *Experimental Procedures* for *l*-isoproterenol-stimulated cAMP formation and *l*-[^{3}H]dihydroalprenolol binding.

two most closely related short-chain fatty acids show any significant stimulation of induction for either *l*-[^{3}H]dihydroalprenolol binding sites or *l*-isoproterenol-stimulated cAMP production. It is interesting to note that valeric acid, although capable of inducing *l*-[^{3}H]dihydroalprenolol binding maximally, does not increase *l*-isoproterenol-stimulated cAMP production in proportion to the rise in receptor number. This suggests that it may be possible to separate the induction of β-adrenergic receptors from induction of stimulatability of adenylate cyclase by β-adrenergic agonists.

In contrast to the number of receptors, the basal and fluoride-stimulated activity of adenylate cyclase is not increased in butyrate-treated cells, which indicates that adenylate cyclase is a separate molecule from the β-adrenergic receptor and presumably under independent regulatory control (24, 25). At present, the adenylate cyclase from untreated and butyrate-treated cells is being studied to examine other factors that might control the activity of the adenylate cyclase in HeLa cells or the efficiency of its coupling to the β-adrenergic receptor. The ability to manipulate this responsiveness using butyrate as a tool should prove a valuable model for elucidating the regulatory phenomena associated with β-adrenergic receptors and adenylate cyclase's responsiveness to catecholamines.

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