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INDUCTION OF FUNCTIONAL β-ADRENERGIC RECEPTORS IN RAT AORTIC SMOOTH MUSCLE CELLS BY SODIUM BUTYRATE

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Abstract—Rat aortic smooth muscle cells in culture (A-10; ATCC CRL 1476) exhibited low levels of β-adrenergic receptors as determined by specific binding of [^{125}I]cyanopindolol ([^{125}I]CYP) and marginal stimulation of adenylate cyclase in plasma membranes by (−)isoproterenol. When these cells were exposed to 5 mM sodium butyrate, the number of β-adrenergic receptors and the β-agonist-stimulated adenylate cyclase activity increased markedly. However, basal, GTP, Gpp(NH)p, and fluoride-stimulated activities did not change. The induction of β-adrenergic receptors and β-agonist stimulated adenylate cyclase activity was time- and dose-dependent, and was relatively specific for sodium butyrate. Propionate and valerate were less effective than butyrate, while isobutyrate, succinate, and malonate were ineffective. The induction involved RNA and protein synthesis because induction was prevented by treatment with cycloheximide, puromycin, and actinomycin D. Butyrate did not cause a general increase in cell surface receptors, because the number of vasopressin receptors did not change. The sustained presence of butyrate appeared to be necessary for the maintenance of the induced β-receptors. When butyrate was removed, receptor number and β-agonist-stimulated adenylate cyclase activity were decreased by 90% over 24 hr. We conclude that the poor response of rat aortic smooth muscle cell plasma membranes to β-adrenergic agonists is due to the presence of a low number of β-adrenergic receptors. Butyrate markedly increased the number of β-receptors which resulted in a proportional increase in β-agonist-stimulated adenylate cyclase activity. The increase in receptor number was dependent on RNA and protein synthesis. Butyrate treatment did not affect the activity of the cyclase unit and the efficiency of coupling between the receptors and the guanine nucleotide regulatory protein, N_s.

Beta-adrenergic receptors are coupled via the guanine nucleotide binding protein (N_s) to adenylate cyclase [1–3]. Agonist-induced activation of β-adrenergic receptors of smooth muscles induces an increase in adenylate cyclase activity and, consequently, in the concentration of cyclic adenosine monophosphate (cAMP) [4] resulting, in turn, in relaxation of smooth muscle [5].

Beta-adrenergic receptors are subject to a variety of regulatory processes. Chronic exposure to agonist results in decreases in induction of cAMP production by the agonist. This process is known as desensitization [6]. In some cells, this desensitization process is mediated by a reduction in receptor number [7–9]. In other cells, processes such as phosphorylation are involved [10, 11].

Beta-adrenergic receptor number and the efficiency of coupling to cyclase are affected by a variety of agents. Thyroid hormone has been reported to increase β-adrenergic receptor number *in vitro* and *in vivo* [12, 13]. Treatment of 3T3-L1 cells with dexamethasone results in conversion from expression of β_1 to β_2 receptor subtype and an increase in total β-receptor number [14].

Butyrate has been reported to induce a variety of effects on β-adrenergic receptor density and coupling to cyclase. Henneberry *et al.* [15] reported that in HeLa cells, low concentrations of butyrate increase the density of β-adrenergic receptors. However, the receptors are not coupled to cyclase. At higher concentrations, butyrate treatment increases receptor density and cyclase coupling. In contrast, in another strain of HeLa cells, Lin *et al.* [16] reported that all concentrations of butyrate increase the cyclase activation by β-receptors and decrease GTP-induced cyclase stimulation. Employing fusion of stimulatory guanine nucleotide regulatory protein (N_s) from control and butyrate-treated HeLa cells with cyclase from other cells, Kassis *et al.* [17] concluded that butyrate does not increase the number of N_s molecules, but increases their effectiveness along with the number of β-receptors. Thus, a number of seemingly controversial observations have been reported as to the effects of butyrate on β-adrenergic receptors and coupling to cyclase. Furthermore, relatively little is understood about the mechanisms by which butyrate induces these effects.

Less is known about the mechanisms of receptor regulation and signal transduction of other vasoactive hormones, such as vasopressin. Vasopressin is a potent vasoconstrictive agent. Two types of vasopressin receptors (V_1 and V_2) have been reported [18]. V_2 receptors are coupled to adenylate cyclase

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and induce an increase in cAMP and an increase in water permeability of renal collecting duct epithelium [19–21]. V_1 receptors mediate vasoconstriction [22].

We have found that the rat aortic smooth muscle cells in culture (A-10) contain vasopressin receptors of the V_1 subtype [23]. However, they display a minimal number of β-adrenergic receptors. Thus, these cells present an opportunity to study the effects of butyrate on β-adrenergic receptor number and coupling to adenylate cyclase in a relatively simple cell culture system and eventually the opportunity to study the interactions between the vasoconstricting V_1 and the vasorelaxing β-receptors.

The objectives of this study were, therefore, to evaluate the effects of butyrate treatment on β-adrenergic receptor density and coupling and to study the mechanisms of action of butyrate. Additionally, we wished to determine if butyrate affected vasopressin receptors in a similar fashion and to study the effects of butyrate on adenylate cyclase in these cells.

METHODS

Materials. [^{125}I]Cyanopindolol ([^{125}I]CYP), [α-^{32}P]ATP, [^{3}H]AVP (arginine vasopressin), and [^{3}H]cAMP were purchased from New England Nuclear. ATP, GTP, pyruvate kinase, phosphoenolpyruvate, imidazole, and (−)isoproterenol were from Sigma.

Cell culture. Rat aortic smooth muscle cells (A-10) were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 20% fetal calf serum (FCS) in 95% air/5% CO_2 at 37°. Before initiating the experiments, the cells were subcloned twice by limiting dilution. All experiments were carried out with cells passaged for no more than 4 months. Culture flasks (Falcon T-150) were inoculated with 2 ml containing 1.5×10^6 cells, and the cells were cultured in 20 ml medium. Experiments were carried out after 3 days unless otherwise indicated.

Sodium butyrate was dissolved in medium at eleven times more concentrated than the final concentration and 2 ml was added to the culture flask containing the cells in 20 ml medium. Control flasks received 2 ml medium without butyrate. After 24 hr, or as indicated otherwise, the culture medium was removed, and the cells were washed three times with 20 ml of Dulbecco's phosphate-buffered saline (DPBS). Then the cells were scraped in DPBS and collected by centrifugation at 600 *g* at 4° for 10 min. The cell pellet was frozen and thawed, and resuspended in 5 mM Tris, 2 mM $MgCl_2$, 1 mM EDTA (sodium salt), pH 7.5, and homogenized using a Dounce homogenizer (25 strokes). The homogenate was centrifuged at 300 *g* for 10 min. The supernatant fraction was centrifuged at 40,000 *g* for 10 min at 4° and the pellet was resuspended either in buffer A (75 mM Tris, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5) for [^{125}I]CYP binding or in buffer B (75 mM Tris · HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 250 mM sucrose, 0.5 mM dithiothreitol (DTT), pH 7.5) for the adenylate cyclase assay.

[^{125}I]CYP binding assay. Binding studies were done at 37° for 30 min using [^{125}I]CYP as described [3]. The total incubation volume was 250 μl and the amount of membrane protein was 40–100 μg/tube. Non-specific binding was determined by including 100 μM (−)isoproterenol or 10 μM (±)propranolol in the incubation. Separation of bound from free radioligand was achieved using Whatman GF/C filters followed by 3 × 5 ml rapid washes with buffer A at 37°. Radioactivity retained on the filters was quantitated by gamma counting at 75% efficiency.

[^{3}H]AVP binding assay. Vasopressin receptors were measured in [^{3}H]AVP binding experiments using cells grown in monolayer. The culture medium was removed. The cells were washed with DPBS plus 10 mM $MgCl_2$, 0.2% bovine serum albumin (BSA), and 0.1% glucose (DPBS$^+$), and specific binding of vasopressin was determined after incubating the cells with 15 nM [^{3}H]AVP in the presence (non-specific binding) or absence (total binding) of 10 μM AVP for 2 hr at 0°. Bound and free [^{3}H]AVP were separated by rapidly removing the incubation medium, washing the cells with ice-cold DPBS$^+$, scraping the cells, and washing the scraped cells on Amicon filter cups (0.45 μm; mixed ester). Cell-associated [^{3}H]AVP was measured by counting the filters in a liquid scintillation counter.

Adenylate cyclase assay. Assays were performed as described [24]. The final incubation mixture (50 μl) contained 30 mM Tris · HCl, pH 7.5, at 25°, 5 mM $MgCl_2$, 0.6 mM EDTA, 100 mM sucrose, 0.2 mM DTT, 0.12 mM ATP, 1 μCi [α-^{32}P]ATP, 0.1 mM cAMP, 0.06 mM GTP, 2.8 mM phosphoenolpyruvate, 5.2 μg/ml pyruvate kinase, and 10 μg/ml myokinase. The reaction was initiated by the addition of 20 μl of membrane suspension (30–60 μg protein) and continued for 10 min at 37°. The incubation was terminated by the addition of 1.0 ml of ice-cold stop solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (15,000–20,000 cpm). Cyclic AMP was isolated as described by Salomon *et al.* [25]. Each experiment was done in triplicate and repeated at least twice.

Reconstitution experiments. Reconstitution experiments were performed following the procedure of Sternweis *et al.* [26]. Briefly, the guanine nucleotide binding protein (N_s) in the plasma membranes (~5 mg protein/ml) from control and 5 mM sodium butyrate-treated A-10 cells were solubilized using a buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA and 25 mM sodium cholate. After stirring for 1 hr at 4°, the suspension was centrifuged at 100,000 *g* for 30 min. The supernatant fractions (15 μl containing 15 μg protein) were incubated with (10 μl containing 20 μg protein) cyc membranes for 20 min on ice. To this mixture was added 20 μl buffer containing 150 mM Hepes, pH 8.0, 2 mM ATP, 15 mM $MgCl_2$, 150 μM GTP, 30 μg/ml pyruvate kinase, 9 mM phosphoenolpyruvate and 0.3 mg/ml BSA. At this step, the activators such as isoproterenol (100 μM), and sodium fluoride (10 mM) were added and the mixture was then incubated for 10 min at 30°. The adenylate cyclase assay was started by adding 40 μl of 0.25 μg/ml pyruvate kinase, 7.5 mM phosphoenolpyruvate, and 0.5 mM iso-

butylmethylxanthine (IBMX) and the incubation was continued for another 30 min at 30°. Termination of the incubation and isolation of cAMP were done as described above.

RESULTS

Plasma membranes of cultured rat aortic smooth muscle cells (A-10) exhibited low levels of β-adrenergic receptors (Fig. 1A). In addition, the stimulation of adenylate cyclase activity of these membranes by β-adrenergic agonists was poor, although this enzyme was greatly stimulated by Gpp(NH)p, sodium fluoride, and forskolin (Fig. 1B). Attempts to kinetically analyze the β-adrenergic receptors either in intact cells or in plasma membranes were not successful due to high levels of non-specific binding and low number of receptor sites. However,

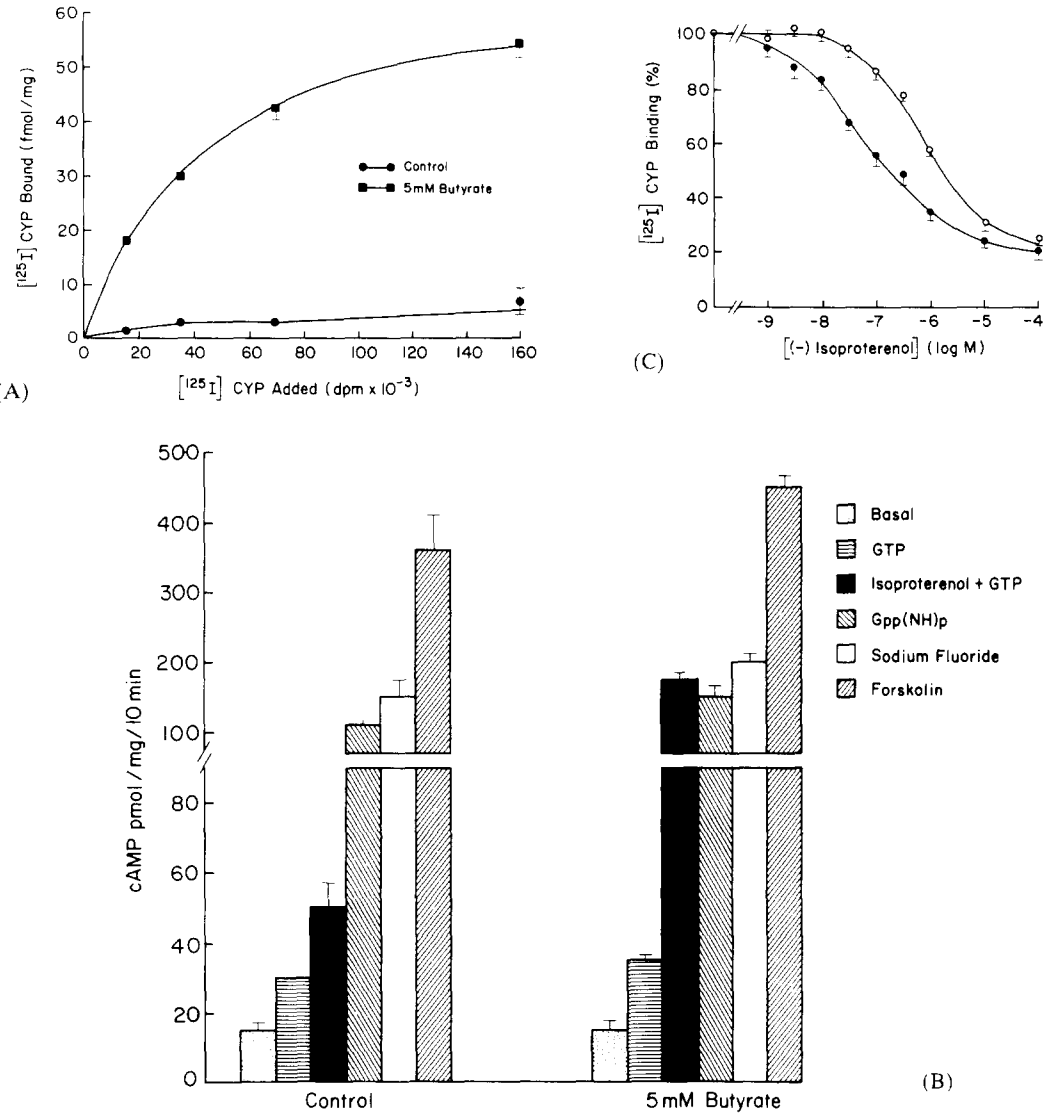

Fig. 1. (A) Effects of different concentrations of [^{125}I]cyanopindolol (CYP) on binding to membranes of control and butyrate-treated smooth muscle cells. Cells were treated with or without 5 mM butyrate for 24 hr and assayed for [^{125}I]CYP binding. Values represent the mean ± S.E.M. of triplicate determinations. The experiment was repeated twice with similar results. The absence of error bars is due to S.E.M. being smaller than the symbols. (B) Adenylate cyclase activity of membranes from control and butyrate-treated smooth muscle cells. Cells were treated with or without 5 mM butyrate for 24 hr, and adenylate cyclase assays were performed. The final concentration of (−)isoproterenol, GTP, Gpp(NH)p and forskolin was 100 μM; the concentration of NaF was 10 mM. Values represent the mean ± S.E.M. of triplicate determinations. The experiment was repeated five times with similar results. (C) Isoproterenol competition of [^{125}I]CYP binding to membranes from cells of control and butyrate-treated smooth muscle cells. Cells were treated with and without 5 mM butyrate for 24 hr. Isoproterenol competition experiments of [^{125}I]CYP binding were performed in the absence (●) and presence (○) of 100 μM Gpp(NH)p. Values represent mean ± S.E.M. of triplicate determinations from a single experiment. The experiment was performed three times with similar results.

when these cells were treated with 5 mM butyrate for 24 hr, the number of β-adrenergic receptors increased 10- to 15-fold with a parallel increase in β-agonist-stimulated adenylate cyclase activity (Fig. 1, A and B). Basal, GTP, Gpp(NH)p, forskolin, and fluoride-stimulated adenylate cyclase activities were not affected significantly by butyrate treatment. The [^{125}I]CYP binding was saturable; B_{max} was 55 fmoles/mg protein, and the K_D was 10–15 pM.

In addition, the butyrate-induced receptors were sensitive to guanine nucleotides as shown in Fig. 1C. In the presence of 100 μM Gpp(NH)p, the agonist competition curve of [^{125}I]CYP binding to the receptor was shifted to about a 10-fold higher agonist concentration; Gpp(NH)p also increased the steepness of the slope.

Induction of β-adrenergic receptors (Fig. 2A) and isoproterenol-stimulated adenylate cyclase activity (Fig. 2B) occurred in parallel and was dependent on the time of incubation with butyrate. Both processes were slow and reached a maximum by 18 hr. The receptor density and hormonally-stimulated adenylate cyclase activity remained at maximal levels for up to 48 hr of incubation with butyrate, the longest time studied.

Figure 3 presents the [^{125}I]CYP binding to β-adrenergic receptors and isoproterenol-stimulated adenylate cyclase activity in membranes derived from cells treated with increasing concentrations of butyrate for 24 hr. We determined the β receptor density of control and butyrate-treated membranes with 50 pM [^{125}I]CYP, assuming that butyrate treatment did not affect the K_D. Beta-adrenergic receptor density and isoproterenol-stimulated adenylate cyclase activity were dependent on the butyrate concentration in a similar fashion. Both levels were maximal at 10 mM butyrate.

The slow process of β-adrenergic receptor induction by butyrate suggested that RNA and protein syntheses might be involved. This was tested by incubating the cells with 5 mM butyrate in the presence of cycloheximide (20 μg/ml), actinomycin D (1 μg/ml) or puromycin (40 μg/ml). At these concentrations, RNA and protein synthesis was inhibited 84, 57 and 87% by these inhibitors respectively. The cell viability as measured by trypan blue exclusion was greater than 85%. All inhibitors blocked the appearance of both β-adrenergic receptors and isoproterenol-stimulated adenylate cyclase activity (Fig. 4, A and B).

To determine if butyrate caused a general increase in synthesis of cell surface receptors, vasopressin receptor number was measured. Although butyrate markedly increased the number of β-receptors, the number of vasopressin receptors was not increased. Cells treated with and without 5 mM butyrate for 24 hr exhibited 90.0 ± 4.6 and 95.1 ± 4.2 fmoles vasopressin receptors per 10^6 cells (mean ± S.E.M.; N = 3) respectively. Furthermore, during butyrate treatment for 24 hr, cell number and total cell protein did not increase significantly, whereas the cell number and protein content of control cultures doubled (data not shown).

The specificity of the induction of β-adrenergic

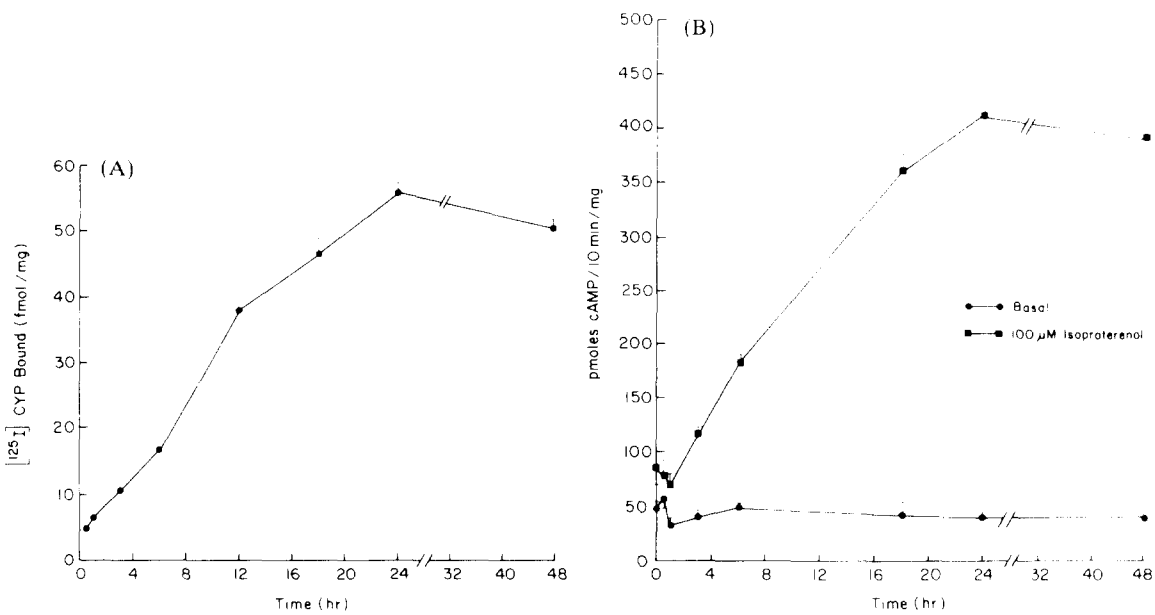

Fig. 2. (A) Time course of beta-adrenergic receptor induction by butyrate in smooth muscle cells. Cells were treated with or without 5 mM butyrate for different time periods. Membranes were prepared and assayed for [^{125}I]CYP binding. Values represent the mean ± S.E.M. of triplicate determinations. The experiment was performed twice with similar results. The absence of error bars is due to S.E.M. being smaller than the symbols. (B) Time course of induction of beta-adrenergic agonist-stimulated adenylate cyclase activity by butyrate in smooth muscle cells. Cells were treated with or without 5 mM butyrate, and adenylate cyclase activity of membranes was measured with (■) and without (●) 10^{-4} M isoproterenol. Values represent the mean ± S.E.M. of triplicate determinations. The experiments was repeated twice with similar results.

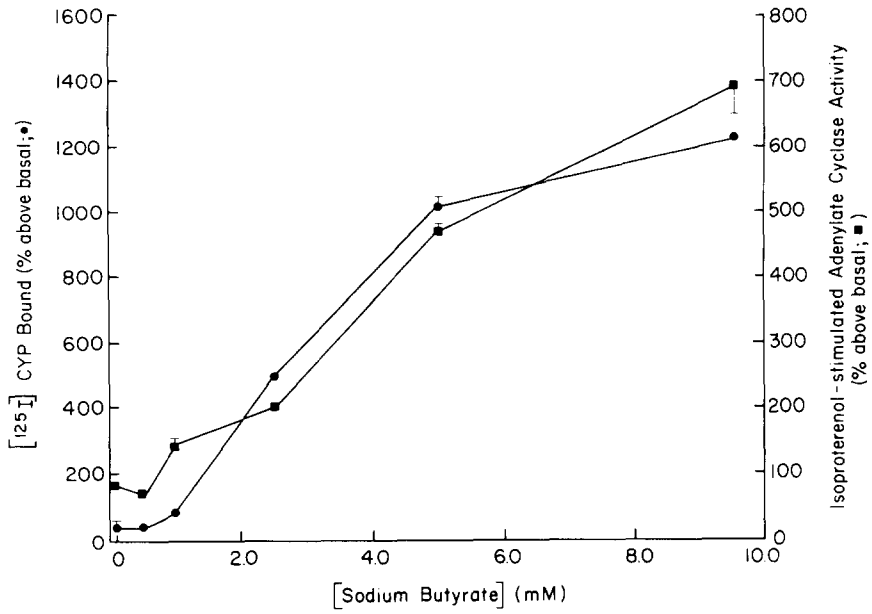

Fig. 3. Effects of treatment of smooth muscle cells with various concentrations of butyrate on [^{125}I]CYP binding and adenylate cyclase activity. Cells were treated with indicated concentrations of butyrate for 24 hr, and membranes were prepared and assayed for [^{125}I]CYP binding (●) and 100 μM (−)isoproterenol-stimulated adenylate cyclase activity (■). Values represent the mean ± S.E.M. of triplicate determinations. The experiment was repeated twice with similar results. The absence of error bars is due to S.E.M. being smaller than the symbols.

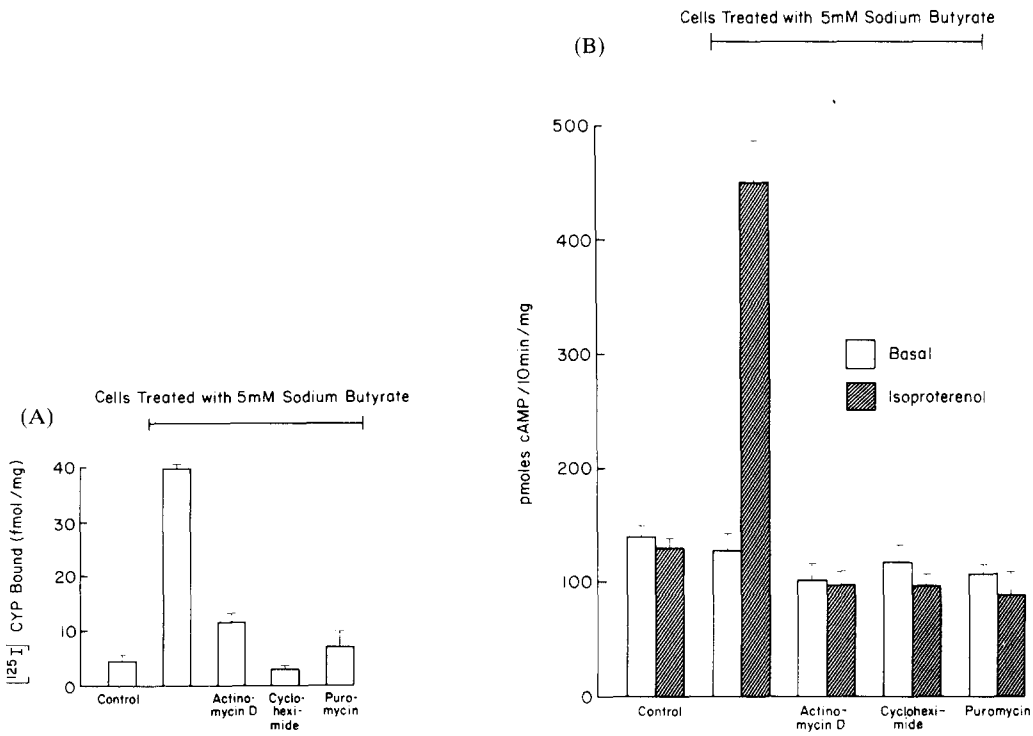

Fig. 4. Effect of RNA and protein synthesis inhibitors on the induction of beta-adrenergic receptors (A) and isoproterenol-stimulated adenylate cyclase activity (B) by butyrate in smooth muscle cells. Cells were incubated with or without 5 mM butyrate for 24 hr in the absence and presence of 20 μg/ml cycloheximide, 1 μg/ml actinomycin D, or 40 μg/ml puromycin. At the end of preincubation, cells were washed, membranes were prepared and assayed for [^{125}I]CYP binding and isoproterenol-stimulated adenylate cyclase activity. Values represent the mean ± S.E.M. of triplicate determinations. The experiment was repeated with similar results.

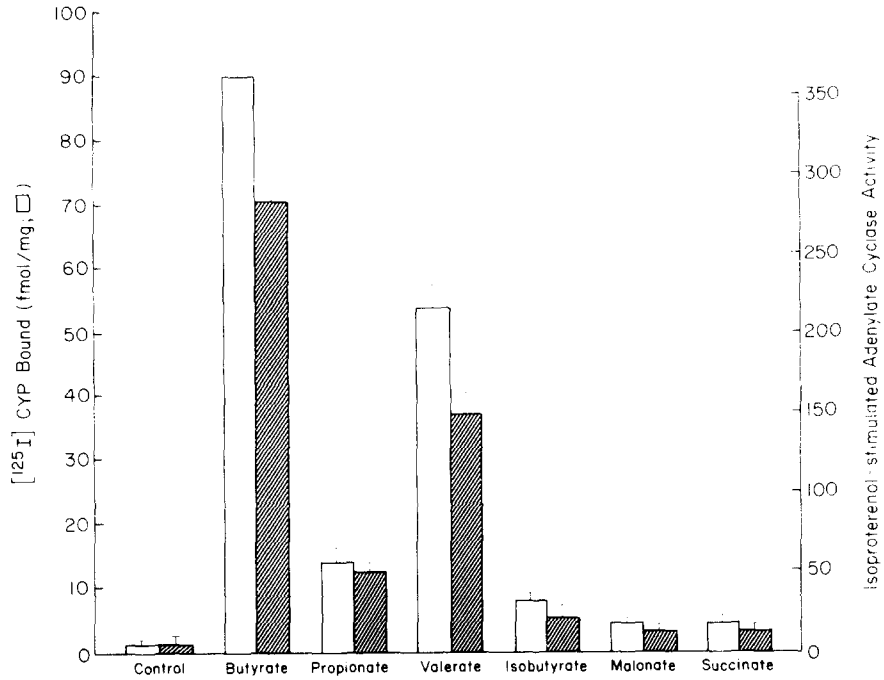

Fig. 5. Induction of beta-adrenergic receptor and isoproterenol-stimulated adenylate cyclase activity in smooth muscle cells by various short chain fatty acids. Cells were incubated for 24 hr with the indicated short chain fatty acids at 5 mM and the membranes were assayed for [^{125}I]CYP binding (□) and adenylate cyclase activities (▨). Values represent the mean ± S.E.M. of triplicate determinations. The experiment was repeated with similar results.

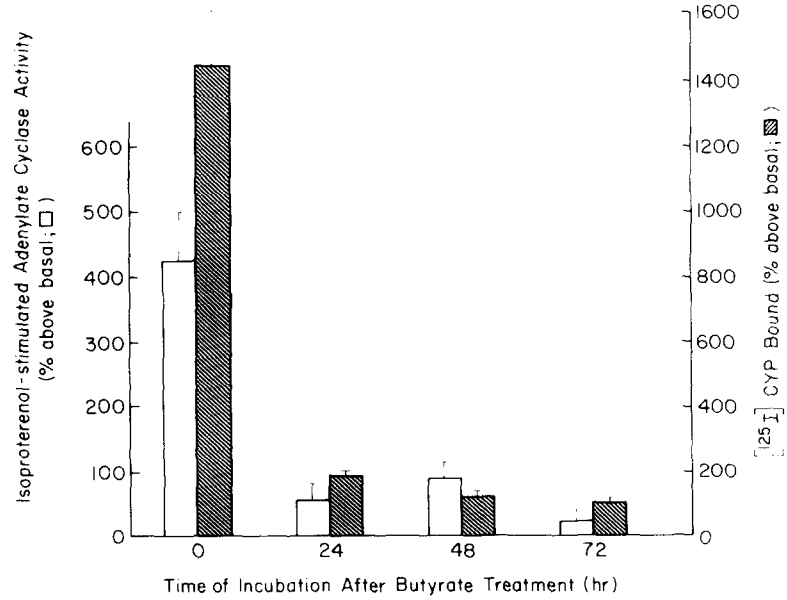

Fig. 6. Reversal of the induction by butyrate of beta-adrenergic receptors and isoproterenol-stimulated adenylate cyclase activity in smooth muscle cells. Cells were treated with or without 5 mM butyrate for 24 hr. Subsequently, the cells were washed and the incubation was continued for 0, 24, 48, and 72 hr in the absence of butyrate. At the end of incubation period, membranes were prepared and assayed for [^{125}I]CYP binding (▧) and adenylate cyclase activity (□). Values represent the mean ± S.E.M. of triplicate determinations. The experiment was repeated with similar results.

Table 1. Reconstitution of cyc⁻ adenylate cyclase with N_s extracted from A-10 cells

Source of N_s	Adenylate cyclase activity (pmoles cAMP/30 min)		
	GTP	GTP + Iso	NaF
Control	0.17 ± 0.06	0.44 ± 0.07	2.24 ± 0.72
Butyrate	0.26 ± 0.08	0.58 ± 0.09	2.74 ± 0.21

Cholate extracts from control and 5 mM sodium butyrate-treated A-10 cells (15 μg protein) were mixed with plasma membranes from S49 cyc⁻ cells (20 μg protein), and the reconstituted membranes were assayed for adenylate cyclase activity as described under Methods. The data represents mean ± S.E.M. of triplicate determinations. The experiment was repeated with similar results.

receptors by short chain fatty acids was tested by incubating the cells with 5 mM butyrate, propionate, valerate, isobutyrate, malonate, or succinate. As shown in Fig. 5, valerate was less effective than butyrate, and propionate was less effective than valerate in inducing β-adrenergic receptors. Malonate, succinate, and isobutyrate showed little or no effect. The density of the β-adrenergic receptor and the level of β-agonist-stimulated adenylate cyclase activity were affected similarly by the different carboxylic acids.

To investigate the reversibility of butyrate induction of β-adrenergic receptors, cells were preincubated with 5 mM butyrate for 24 hr and then butyrate was removed and the incubation continued for up to 72 hr. As shown in Fig. 6, when butyrate was removed, the receptor number and isoproterenol-stimulated adenylate cyclase activity declined sharply over 24 hr and then remained constant over the next 48 hr.

To study whether butyrate treatment also increased the efficiency of coupling between the β-adrenergic receptors and guanine nucleotide regulatory protein (N_s), reconstitution experiments were performed using cyc⁻ membranes and N_s from control and butyrate-treated A-10 cells. As shown in Table 1, butyrate treatment did not increase the efficiency of N_s coupling in these cells.

DISCUSSION

Plasma membranes prepared from cultured rat aortic smooth muscle cells (A-10) exhibited low levels of β-adrenergic receptors and little or no β-agonist stimulation of adenylate cyclase. At the same time, this enzyme was fully responsive to receptor-independent activators such as fluoride, forskolin, GTP, and Gpp(NH)p. This would indicate that the interaction between the nucleotide regulatory protein and the catalytic subunit was not impaired. Treatment of these cells with butyrate resulted in a 10- to 15-fold increase in β-adrenergic receptor number with a parallel increase in isoproterenol-stimulated adenylate cyclase activity. Both increases were time- and concentration-dependent. Maximum induction occurred after an 18-hr incubation with 10 mM butyrate. The induction by butyrate of β-adrenergic receptors required RNA and protein synthesis. To sustain a high density of β-adrenergic receptors, the continued presence of butyrate was required; once butyrate was removed, β-receptor density and hormone responsiveness were reduced by as much as 90% within 24 hr. The induction of β-adrenergic receptors was not due to a general increase in RNA and protein synthesis, because the number of vasopressin receptors, cell number, and total cellular protein did not change during butyrate treatment.

Similar to the findings in hepatocytes by Tallman *et al.* [27], the induction was relatively specific for sodium butyrate in that other short chain fatty acids were less effective (valerate, propionate) or ineffective (isobutyrate, malonate, succinate).

Henneberry *et al.* [15] have reported that, in HeLa cells at lower concentrations of butyrate, the number of β-adrenergic binding sites increased, but there was no concomitant increase in adenylate cyclase activation. At higher butyrate concentrations, these investigators found that the number of β-adrenergic receptors as well as the adenylate cyclase stimulation by β-agonists increased. In our studies with smooth muscle cells, we could not differentiate between the concentrations at which an increase in β-receptor number or hormone responsiveness was observed. The increase in receptor number paralleled the increase in hormonal stimulation of adenylate cyclase activity in these cells. Moreover, after removal of butyrate, the number of β-receptors and the hormone responsiveness declined to a similar extent.

Lin *et al.* [16] suggested that the butyrate-induced increase in responsiveness to β-agonists of HeLa cells and fetal hepatocytes was accompanied by changes in the interaction between β-receptor and guanine nucleotide regulatory proteins. These authors found that butyrate treatment reduces the degree of activation of adenylate cyclase by GTP. We did not find a reduction in activation by GTP. The butyrate-induced β-adrenergic receptors of the smooth muscle cells were coupled to guanine nucleotide regulatory protein, because the agonist competition curves of antagonist ([^{125}I]CYP) binding was shifted as much as 10-fold by Gpp(NH)p. Studying HeLa cells, Kassis *et al.* [17] obtained evidence to indicate that butyrate treatment enhanced the effectiveness of the stimulatory guanine regulatory protein (N_s). This enhancement appeared to be due to a qualitative rather than a quantitative change of N_s. In our studies, the effectiveness of N_s did not appear to be altered by butyrate treatment, because the activation of adenylate cyclase by GTP, Gpp(NH)p, or NaF was not significantly different with or without butyrate treatment. Moreover, reconstitution experiments using cyc⁻ membranes and N_s from control and butyrate-treated A-10 cells suggested that the efficiency of coupling between receptor and N_s is not altered by butyrate treatment. Also, the cyclase units appeared not to be changed, since the basal and forskolin-stimulated adenylate cyclase activities were not affected by butyrate.

We conclude that the poor β agonist stimulation of adenylate cyclase activity of membranes of rat vascular smooth muscle cells in culture is due to low density of β-adrenergic receptors. Treatment of these cells with butyrate caused a marked increase in β-

agonist-stimulated adenylate cyclase activity. This increase appeared to be due to a concomitant rise in β-adrenergic receptor density, and not to increases in the N_s and cyclase components. The increase in β-adrenergic receptor density required RNA and protein synthesis and the continued presence of butyrate. Removal of butyrate resulted in a slow reversal of the receptor density to control levels. Butyrate did not cause a rise in all cell surface receptors, since the number of vasopressin receptors was unaffected.

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