The pathogenesis of neoplastic cellular proliferation may involve loss of responsiveness to normal inhibitory regulatory signals, as well as excessive response to stimulatory signals. Adenylate cyclase catalyzes the generation of intracellular cyclic AMP (cAMP), a second messenger molecule that appears to participate in the regulation of growth and proliferation in a variety of cell populations. The adenylate cyclase (AC) signaling system has therefore received attention for its possible role in neoplastic processes.

Transmission of extracellular hormonal stimuli (such as those from catecholamine and prostaglandin E) to the adenylate cyclase catalyst involves both the agonist-specific membrane receptor and distinct guanine nucleotide-binding proteins (G-proteins), which may either stimulate (Gs) or inhibit (Gi) AC catalytic activity (for review, see ref 5). Disruption of this transmembrane signaling system results from either absence or functional “uncoupling” of any of these membrane-associated components. This mechanism has been elegantly delineated in the murine S49 lymphoma cell line: Wild type cells with an intact transduction mechanism respond to exogenous AC agonists with increased intracellular cAMP, followed by cessation of proliferation and cell death. This response is absent in derivative cell lines lacking either the α subunit of Gs (cyc-variant) or coupling of Gs to Gi (UNC variant).

The mediation of growth factor stimuli in factor-dependent lymphohematopoietic cells may also bear upon the transmembrane activation of adenylate cyclase. Both interleukins 2 and 3 (IL 2 and IL 3) effect stimulatory changes in target cells through a transmembrane signaling system ultimately activating protein kinase C.

Murine myeloid cell proliferation has recently been closely linked to the action of the growth factor IL 3. The present study was performed to investigate the consequences of excessive activation of the IL 3 signaling system on the capacity for proliferative inhibition of immortal IL 3-dependent myeloid cell lines by adenylate cyclase agonists or soluble cAMP. In addition, the capacity of these agents to inhibit the proliferation of a transformed IL 3-independent subline of FDC-P1 (Fl), was studied. In particular, the independent subline, Fl, were resistant to these antiproliferative agents. The in vitro ability of the “differentiation” agent, sodium butyrate, to reverse their resistance to adenylate cyclase agonists was studied. The antiproliferative action of butyrate involved augmentation of transmembrane adenylate cyclase activity. Increased adenylate cyclase catalyst activity was the primary alteration of this transmembrane signaling group leading to the functional inhibitory effects on leukemia cells, although alterations in regulatory G-proteins appear to play a secondary role.

The results of the present investigation demonstrate a mechanistic pathway by which sodium butyrate causes augmented activation of adenylate cyclase in leukemia cells by signals transmitted through GTP-binding proteins. This restoration of transducer function in leukemia cells may have important consequences for altering the capacity for inhibition of leukemia cell proliferation by natural hormones or pharmacologic agents.

Materials and Methods

Murine myeloid cell lines. The immortal myeloid cell line FDC-P1 was originally derived by Dexter et al from nonadherent cells in long-term culture of normal DBA/2 marrow cells that were not exogenously infected by any murine leukemia virus.

Survival...
and proliferation of FDC-P1 cells is dependent on stimulation by exogenous growth factor, either IL 3 or granulocyte-macrophage colony-stimulating factor.\textsuperscript{14} FDC-P1 was maintained as previously described\textsuperscript{15} in RPMI 1640 with 10% fetal calf serum (FCS) with conditioned medium (CM) prepared from the WEHI-3 leukemia cell line (a constitutive producer of IL 3).\textsuperscript{16} FDC-P1 cells were washed three times and incubated for four to 12 hours in WEHI-3-free medium prior to assay; such withdrawal of WEHI-3 CM renders FDC-P1 cells quiescent as assessed by $^{3}$H-thymidine uptake.\textsuperscript{17}

A growth factor-independent cell line (FI) was derived from FDC-P1 by long-term culture of FDC-P1 on bone marrow stromal cells as previously described.\textsuperscript{18} FI cells (subclone 11.2) and the parent FDC-P1 cells have been shown to differ not only in growth factor requirement but also in tumorigenicity: FI cells form tumors upon injection into syngeneic mice.\textsuperscript{19}

**Proliferation assays.** Cells were incubated overnight at 37°C, 5% CO$_{2}$ in triplicate microwells of 96-well flat-bottomed plates (Corning, New York), 5 x 10$^{4}$ cells/well, in final volume of 100 μL, followed by pulsing with 1 μCi of $^{3}$H-thymidine (New England Nuclear, Boston) and further incubation for six to eight hours. Well contents were transferred to glass fiber discs (MASH II, MA Bioproducts, Walkersville, MD) for scintillation counting.

The incubation media consisted of RPMI 1640, 10% FCS, with or without (a) Na butyrate (Sigma Chemical, St Louis) 0.9 to 1.0 mmol/L/PH 7.3, (b) chola toxin (List Biologics, Campbell, CA) 20 to 100 ng/mL, (c) prostaglandin E$_{2}$ (PGE$_{2}$) (Sigma) 10$^{-4}$ mol/L, (d) 8-bromo-cAMP (8-Br-cAMP) (Sigma) 1 mmol/L, and (e) forskolin, 10$^{-4}$ mol/L (Calbiochem). PGE$_{2}$ and forskolin were solubilized in ethanol prior to serial dilution in RPMI 1640. The final ethanol concentration (0.01% to 0.1% for PGE$_{2}$/or 0.1% to 1% for forskolin) was shown in control experiments to have no effect on $^{3}$H-thymidine uptake. Purified homogenous IL 3, provided by Dr James N. Ihle, Frederick Cancer Research Facility, NC!, was added at 0.8 mmol/L cAMP, containing of membranes were isolated by ultracentrifugation (200,000 g for 20 minutes) of the supernatant. The membrane pellets were resuspended in 20 mmol/L Tris pH 7.5 for protein assay by the method of Lowry.\textsuperscript{17}

**Membrane preparation.** Cells (10$^{6}$) were incubated at a concentration of 0.5 to 1 x 10$^{5}$ cells/mL in RPMI 1640 10% FCS, with or without butyrate (1 mmol/L) at 37°C, 5% CO$_{2}$ for 12 to 16 hours. Viability, as assessed by trypan blue exclusion, was not affected (>85% viable) by this treatment. Cells were washed twice in serum-free RPMI and homogenized in a buffered solution of 15 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 400 g for 10 minutes to remove nuclei, and membranes were isolated by ultracentrifugation (200,000 g for 20 minutes) of the supernatant. The membrane pellets were resuspended in 20 mmol/L Tris pH 7.5 for protein assay by the method of Lowry.\textsuperscript{17}

**Adenylate cyclase activity assays.** Adenylate cyclase reactions were initiated by the addition of 20 μL membrane suspension containing 40 μg protein to a final volume of 100 μL containing 40 mmol/L Tris, 1 mmol/L NaATP, 0.1 mmol/L ascorbate, 0.5 mmol/L cAMP, 2.5 U/mL adenosine deaminase, 0.4 mg/mL bovine serum albumin (BSA), 80 mmol/L NaCl, 10 mmol/L MgCl$_{2}$, and an ATP-regenerating system consisting of 5 mmol/L creatine phosphate and 5 U/mL creatine phosphokinase. Reactions were performed to generation of $^{32}$P-cAMP from 2 μCi $^{32}$P-ATP at 30°C for 10 minutes and were terminated by the addition of 0.8 mL of a solution of 0.25% sodium dodecyl sulfate (SDS), 5 mmol/L ATP, 0.175 mmol/L cAMP (pH 7.5). The nonhydrolyzable GTP analogue, guanosine 5' (β, γ-imido) triphospate Gpp(NH)$_{p}$, was used as the stimulus in some reactions in concentrations from 10$^{-4}$ to 10$^{-6}$ mol/L, to assess stimulatory GTP-binding protein-mediated AC activity specifically. Forskolin 10$^{-4}$ mol/L was used as the stimulus in other reactions performed with various concentrations of the divalent cations Mg$^{2+}$ or Mn$^{2+}$ in the presence of guanine nucleotides to evaluate inhibitory influences (Gi) on direct activation of the adenylate cyclase catalyist.\textsuperscript{18} Stimulation of adenylate cyclase by PGE$_{2}$, in concentrations between 10$^{-4}$ and 10$^{-6}$ mmol/L, was assessed in the presence of 10 μmol/L GTP, 3.3 mmol/L MgCl$_{2}$. In other reactions, MnCl$_{2}$ 4 mmol/L, with or without forskolin 10$^{-4}$ mol/L, was substituted for MgCl$_{2}$ to assay adenylate cyclase activity by direct activation of the catalyist (bypassing Gs).\textsuperscript{19}

After termination of each reaction, $^{3}$H-cAMP (10$^{5}$ cpmp) was added to judge recovery of cAMP after chromatographic isolation by the method of Salomon and colleagues.\textsuperscript{20} Each data point represents the mean ± SEM of duplicate or triplicate reactions.

**ADP-ribosylation for SDS polyacrylamide gel electrophoresis (PAGE) assay of Gs or Gi.** The [32P] ADP-ribosylation reactions were performed by a modification of the procedure described previously.\textsuperscript{21} Sixty micrograms of membranes were incubated in a 1.44 mL-vol containing 60 mmol/L potassium phosphate, pH 8.0, 54 mmol/L NaCl, 2.7 mmol/L dithiothreitol, 5 mmol/L MgCl$_{2}$, 10 mmol/L thymidine, 0.1 mmol/L GTP, 1 mmol/L/ATP, 3.3 mmol/L phosphocreatine, 0.3 U/mL creatine phosphokinase, 10 μmol/L [32P] NAD, 10$^{6}$ mol/L/PMol final concentration. Either cholera toxin or pertussis toxin (both are preactivated in 20 mmol/L dithiothreitol: cholera: 30 minutes at 30°C; pertussis: 15 minutes at 37°C) was added at 84 or 20 μg/mL, respectively. The reactions were allowed to proceed for 30 minutes at 30°C, and then were terminated by the addition of 4 mL ice-cold 0.25 mol/L sucrose/10 mmol/L L-histidine. The membranes were then sedimented at 40,000 rpm for 20 minutes in a Beckman 40 rotor. For SDS-PAGE, the membranes were suspended in a final concentration of 0.6 mg/mL and boiled for 5 minutes in gel dissociation medium (62.5 mmol/L Tris, pH 6.8, 1.25% SDS, 0.25% β-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue). Samples were loaded onto 8% resolving gels (SDS-PAGE) and electrophoresed at 20 mA. They were then fixed, stained with Coomassie blue, destained, dryed, and subjected to autoradiography. Cholera toxin catalyzed ADP-ribosylation reactions were performed with the addition of ADP-ribosylation factor (ARF) (80 μL) prepared from canine heart, or also with NADP, with equivalent results.\textsuperscript{22} The major substrate of pertussis toxin-catalyzed ADP-ribosylation has been previously demonstrated to be Gi, the adenylate cyclase inhibitory GTP-binding protein (41 kd α subunit).\textsuperscript{23} In contrast, cholera toxin catalyzes specific ADP-ribosylation of the adenylate cyclase stimulatory GTP-binding protein Gs, (45 kd α subunit).\textsuperscript{24}

**Preparation of pertussis toxin.** Bordetella pertussis strain 165 was grown in modified Stainer-Sholte medium, and pertussis toxin was purified from the culture supernatants using hydroxylapatite and fetuin-agarose chromatography using a modification of procedures described elsewhere as communicated by Dr Erik Hewlett, University of Virginia. Briefly, 7L culture supernatant was passed through a 200-mL column of hydroxylapatite that had been preequilibrated with 10 mmol/L potassium phosphate (pH 9.0). The eluate from the pH 9 column was adjusted to pH 6.0 with HCl and applied to a 200-mL column of hydroxylapatite that had been preequilibrated with 10 mmol/L potassium phosphate (pH 6.0). This column was then washed sequentially with 500 mL 100 mmol/L potassium phosphate (pH 6.0) and 500 mL 100 mmol/L potassium phosphate (pH 7.0). Pertussis toxin was eluted from the column with 400 mL 100 mmol/L potassium phosphate (pH 7.0) containing 0.5 mol/L NaCl. The resulting eluate was applied to a 35-mL column of fetuin-Sepharose. The column was washed with 105 mL 100 mmol/L potassium phosphate (pH 7.0)/0.5 mol/L NaCl. Pertussis toxin was eluted with 100 mL 100 mmol/L potassium phosphate (pH 7.0)/0.05 mol/L NaCl containing 3 mol/L KSCN. The purified toxin was dialyzed and concentrated using an Amicon
AC RESPONSIVENESS IN MURINE MYELOID LEUKEMIA

Fig 1. FDC-P1 cells growing exponentially in RPMI 1640–10% FCS with WEHI CM were washed three times and then replated overnight in medium without IL 3. The next day, the cells were washed and plated at 5 × 10^5 cells/well into microtiter wells, with serial dilutions of purified IL 3, or alone, at a final volume of 100 µL. Identical groups were plated with the IL 3 titration plus a constant dose of cholera toxin 20 ng/mL. Tritiated thymidine uptake was assessed, and the difference of uptake between IL 3-stimulated cultures (with or without cholera toxin) and control cultures was plotted. Results are representative of two consecutive experiments for cholera toxin (also shown in Table 2). Similar results were obtained with PGE, in place of cholera toxin.

YM-10 ultrafiltration membrane (43 mm) and 100 mmol/L potassium phosphate (pH 7.0)/0.5 mol/L NaCl. Pertussis toxin was stable for at least 1 year when stored in frozen aliquots at -20°C.

Determination of intracellular 3H-cAMP generation. Determination of intracellular 3H-cAMP was performed according to the methodology of Kienast et al. with modification.25 Following preincubation with butyrate or control medium, cells were suspended at 10^5 cells/mL in serum-free RPMI with 3H-adrenaline, 15 µCi/µL for 20 minutes at 20°C. Cells were centrifuged and resuspended in medium at the same cell density. Cell aliquots of 450 µL were added to 50 µL medium containing 3-isobutyl-1-methyl-xanthine (MIX) (a phosphodiesterase inhibitor, final concentration, 1 mmol/L), with or without prostaglandin E1 (final concentration 10^-4 mol/L) and incubated at 37°C for 16 minutes. (Preliminary experiments demonstrated that plateau generation of intracellular cAMP occurred by 16 minutes.) Reactions were stopped by addition of 50 µL 3 mol/L trichloroacetic acid containing 3H-cAMP (ICN Biochemicals), 0.2 µCi/µL, vortexed, and then centrifuged at 10,000 g for 5 minutes, and cAMP was isolated from the supernatants by the method of Salomon and colleagues.25 Results were expressed as the percentage of control 3H-cAMP generated in reactions without addition of hormones.

RESULTS

Modest antiproliferative effect of adenylate cyclase agonists (PGE1 and cholera toxin) on murine myeloid cell lines is augmented by butyrate. To evaluate whether the IL 3-dependent and IL 3-independent cell lines were resistant to the antiproliferative action of PGE1 and cholera toxin, thymidine incorporation proliferation assays were performed. The proliferation of FDC-P1 was studied as a function of IL 3 concentration between 7.5 and 0.02 U/culture, and the degree of inhibition of thymidine incorporation by cholera toxin was evaluated (Fig 1). Only IL 3 doses of <0.5 U/culture was a window of specific inhibition seen; the cells were resistant at higher IL 3 doses (Fig 1). The same pattern of inhibition by 1 µmol/L PGE1 was seen: no inhibition occurred at IL 3 doses >0.5 U/culture, but significant inhibition of proliferation was observed at lower IL 3 doses as measured by thymidine incorporation and in cell recovery assays (data not shown). In addition, the transformed factor-independent cell line, F1 clone 11.2, was largely resistant to the effects of PGE1 or cholera toxin. Both FDC-P1 and the F1 clone 11.2, however, were consistently inhibited by 8-BrcAMP, a cAMP analogue that effectively bypasses the transmembrane adenylate cyclase mechanism (Table 1).

Further proliferation assays were performed on FDC-P1 and F1 cells with the addition of sodium butyrate. The antiproliferative effect of PGE1, or cholera toxin on FDC-P1 and F1 11.2 cells was synergistically increased by exposure to butyrate (Table 2). Other experiments performed in both factor-dependent and factor-independent cells demonstrated that the augmented antiproliferative effect of PGE1 or cholera toxin by butyrate did not require the continuous presence of butyrate. Cells incubated in butyrate for 8 to 12 hours and then washed twice prior to assay remained sensitive to PGE1 or cholera inhibition (data not shown).

These proliferation data suggested that impaired cAMP generation by the adenylate cyclase transmembrane signaling complex either at the level of the catalyst moiety or the GTP-binding regulatory proteins was responsible for the native refractoriness of both cell lines to PGE1-mediated inhibition. Cellular generation of 3H-cAMP stimulated by PGE1 (after incorporation of radioactively labeled adenine) was examined in control and butyrate-pretreated FDC-P1

---

Table 1. Inhibition of Proliferation of FDC-P1 and Factor-Independent Cells by 8-BrcAMP Is Superior to that Mediated by Hormonal Agonists

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IL 3 Concentration (U/Culture)</th>
<th>Medium</th>
<th>PGE1 (1 µmol/L)</th>
<th>Cholera Toxin (100 ng/mL)</th>
<th>8-BrcAMP (1 mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td></td>
<td>166,010 ± 4,606</td>
<td>138,423 ± 6,558</td>
<td>109,860 ± 2,325</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>CPM ± SEM (%)</td>
<td>(−)</td>
<td>(−15%)</td>
<td>(−32%)</td>
</tr>
<tr>
<td>F1 11.2</td>
<td>0</td>
<td>247,890 ± 585</td>
<td>26,387 ± 7,172</td>
<td>203,977 ± 5,487</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>CPM ± SEM (%)</td>
<td>(−7%)</td>
<td>(0%)</td>
<td>(−32%)</td>
</tr>
</tbody>
</table>

Representative data of three consecutive experiments.
and factor-independent cells (Fig 2). These experiments showed that in butyrate-pretreated cells [H]-cAMP generation by PGE, was increased twofold (Fig 2).

Next, cell membranes were prepared from control and butyrate-pretreated factor-independent cells to examine whether the augmenting effects of butyrate pretreatment could be attributed to the adenylate cyclase membrane network consisting of the PGE, receptor, regulatory G-proteins, and the adenylate cyclase catalyst. Membranes prepared from butyrate-pretreated factor-independent cells displayed significantly increased hormone-stimulated adenylate cyclase activity compared to control membranes at every dose tested (Fig 3).

**Butyrate increases activity of adenylate cyclase system in plasma membranes by alterations in the cyclase catalyst modulated by G-proteins.** Because PGE, and cholera toxin activate adenylate cyclase at different points in the signal transduction pathway (receptor and GS, respectively) and butyrate appeared to enhance the effects of both these agonists, we inferred that butyrate might act at the level of adenylate cyclase catalyst or the guanine nucleotide-binding regulatory proteins, perhaps by altering the balance of stimulatory and inhibitory coupling. We therefore examined adenylate cyclase activity in membranes prepared from butyrate-treated v control cells on (a) stimulation of the catalyst by Gpp(NH)p-activated GS, and (b) more direct stimulation of the AC catalyst by forskolin. Membranes were prepared from FDC-P1 and Fl cells that had been incubated 12 to 15 hours with or without 1 mmol/L butyrate. Both FDC-P1 cell groups had been supplemented with the same

---

**Table 2. Augmentation of PGE, and Cholera Toxin Inhibitory Effects by Butyrate**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IL 3 Concentration (U/culture)</th>
<th>BT (1 mmol/L)</th>
<th>CT 100 ng/mL</th>
<th>PGE, (1 μmol/L)</th>
<th>Proliferation Response (cpm ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDC-P1</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>359,060 ± 11,174</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>1</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>336,373 ± 6,355</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>356,113 ± 20,957</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>321,000 ± 3,205</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>108,063 ± 18,253</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>222,373 ± 18,759</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>279,133 ± 23,483</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>281,400 ± 5,141</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>253,553 ± 20,381</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>291,810 ± 8,273</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>86,749 ± 12,434</td>
</tr>
<tr>
<td>Fl 11.2</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>399,908 ± 16,808</td>
</tr>
<tr>
<td>Fl 11.2</td>
<td>0</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>271,205 ± 1,933</td>
</tr>
<tr>
<td>Fl 11.2</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>334,298 ± 9,012</td>
</tr>
<tr>
<td>Fl 11.2</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>30,012 ± 2,328</td>
</tr>
<tr>
<td>Fl 11.2</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>204,493 ± 7,781</td>
</tr>
</tbody>
</table>

Abbreviations: BT, butyrate; CT, choleratoxin.

*Background proliferation in the absence of IL 3 was 9,443 ± 1,239 cpm. At 0.2 U IL 3/culture, FDC-P1 proliferation was significantly inhibited by PGE, and cholera toxin alone; control, 194,533 ± 17,068 cpm; +PGE, 127,827 ± 1,704 cpm; + cholera toxin, 140,590 ± 1,234 cpm.*
suboptimal IL 3 concentration to maintain a low level of growth stimulation. Gpp(NH)p-stimulated AC activity (through Gs) was significantly enhanced in both FDC-P1 and Fl membranes by butyrate pretreatment (Fig 4). Furthermore, activity of the cyclase catalyst, as assessed by direct Mn2+ and Mn2+, forskolin stimulation, was also increased to a similar degree (Table 3).

To distinguish the influence of individual G-protein (Gs or Gi) on the augmented divalent cation-dependent adenylate cyclase activity exhibited by membranes from butyrate-pretreated cells, forskolin-stimulated reactions were performed without and with Gpp(NH)p and graded concentrations of Mn2+ from 0.1 to 2 mmol/L (Fig 5). Lower concentrations of Mn2+ (1 to 2 mmol/L) have been reported to uncouple Gi from forskolin-stimulated adenylate cyclase selectively, allowing recognition of Gs-dependent costimulation by guanine nucleotides.2627 In the absence of guanine nucleotide, increasing Mn2+ concentration from 100 μmol/L to 2 mmol/L caused similar augmentation of adenylate cyclase activity in membranes from control and butyrate-treated cells (Fig 5), but the addition of Gpp(NH)p to membranes of butyrate-treated cells caused a greater synergistic GTP-dependent and forskolin-dependent adenylate cyclase stimulation (Fig 5).

We also determined whether butyrate pretreatment effected alterations in G-proteins that were manifested by their availability as substrates for bacterial toxins. Pertussis toxin-catalyzed 32P-ADP ribosylation of cell membranes revealed a twofold increase in the 41-kd Gi substrate of the butyrate-treated membranes vs control membranes from factor-independent cells and FDC-P1 cells, respectively (Fl, ratio of 41-kd band optical density: control/butyrate, 0.5 ± 0.11, n = 5; FDC-P1, ratio of 41 kd optical density control/butyrate 0.57 ± 0.18, n = 2). The 45-kd and 55-kd cholera toxin-substrates (Gs isoforms) available for ADP-ribosylation were diminished in membranes from butyrate-treated factor-independent cells as compared with control cells, however (optical density ratio control/butyrate: 45 kd, 2.08 ± 0.66; 55 kd, 1.5 ± 0.12, n = 2) despite use of methodologies that enhance the efficiency of the cholera toxin-mediated reaction.2122 The availability of 55-kd and 45-kd cholera toxin substrates in control v butyrate-treated FDC-P1 membranes was not reproducibly altered (data not shown).

**DISCUSSION**

The present study demonstrated the capacity of a well-described leukemia cell bioresponse modifying agent, Na butyrate, to promote effective inhibition of proliferation by agonists of adenylate cyclase in two related murine myeloid leukemia cell lines: the IL 3-dependent FDC-P1 line and an IL 3-independent subline, Fl. This action can be described as functionally synergistic because it reversed a relative-to-absolute refractoriness of these cell lines to the adenylate cyclase agonists PGE, and cholera toxin. Indeed, butyrate has been studied extensively for its effect on various human and murine leukemia cell lines, but the mechanism by which it mediates differentiation or inhibition of proliferation has not been completely understood.2829 One confusing aspect regarding butyrate’s action on leukemia cell lines has been distinguishing its endpoint of function from that of cAMP. This is because butyrate has most often been delivered bound with cAMP in the soluble form of dibutyryl cAMP for study of model leukemia cell lines. Recently, however, McCachren and colleagues distinguished the action of butyrate from that

---

**Fig 3.** Membranes were isolated from factor-independent clone 11.2 cells that had been preincubated: control medium (○) or 1 mmol/L butyrate (■) and adenylate cyclase reactions were performed. Duplicate adenylate cyclase reactions containing 10 μmol/L GTP and 3.3 mmol/L Mg2+ and equal protein concentration were incubated for 10 minutes. The reactions were stopped, and 32P-cAMP was isolated by sequential ion resin chromatography by the method of Salomon and colleagues. Recovery of the method of Salomon and colleagues. 1007

**Fig 4.** Membranes were isolated from factor-independent clone 11.2 (A) or FDC-P1 (B) after preincubation at 37°C for 12 hours in medium with 1 mmol/L butyrate (■) or control medium (○). Duplicate adenylate cyclase reactions containing equal protein concentration were incubated for 10 minutes at each concentration of Gpp(NH)p as shown on the horizontal axis. After 10 minutes, the reactions were stopped and 32P-cAMP was isolated by sequential ion resin chromatography by the method of Salomon and colleagues. Recovery of the method was judged by the prior addition of 10,000 cpm 3H-cAMP. Data are expressed as the mean ± SEM of duplicate reactions.
of the membrane-soluble cyclic AMP analogue, 8-BrcAMP on the myeloid leukemia cell line HL-60. These investigators found that butyrate acted synergistically with 8-BrcAMP to reverse cellular resistance to 8-BrcAMP-induced differentiation. These experiments suggested that butyrate favorably affected inhibition of proliferation in FDC-PI and the factor-independent clone 11.2 cells (data not shown), we focused on an activity of butyrate at the level of signal transduction by the hormonal activator of adenylate cyclase, prostaglandin E,.

We reasoned that demonstration of butyrate’s effect on the activity of a hormonal agonist in conveying transcellular signals would be additive with any cytoplasmic or nuclear effects and would be most important to physiologic or clinical approaches to assessing whether a significant portion of this impedance to adenylate cyclase activity is still somewhat subject to modulation of the action of butyrate. This approach derived from our observations that butyrate favorably affected inhibition of cell proliferation not only by PGE, but also by chlora toxin, a direct activator of Gs (adenylate cyclase stimulatory GTP-binding protein). First, membranes from control factor-independent cells displayed very poor adenylate cyclase activity on stimulation by a range of prostaglandin E, doses, but this activity was significantly augmented by butyrate pretreatment (Fig 3). Further experiments were performed to assess whether a significant portion of this impedance to signaling might reside at the level of coupling of Gs to the adenylate cyclase catalyst, which was suggested by the poor anti-proliferative action of cholera toxin in control cells (Tables 1 and 2). Adenylate cyclase activity in isolated membrane preparations mediated through G-proteins by the nonhydrolyzable GTP analogue Gpp(NH)p was significantly augmented by butyrate pretreatment of both FDC-PI and Fl cells (Fig 4). This effect could be explained in part by a quantitative increase of the activity of the adenylate cyclase catalyst as measured in the presence of 4 mmol/L Mn⁺⁺/forskolin (Table 3). The inclusion of Mn⁺⁺ at the concentration of 4 mmol/L, along with forskolin, allowed relatively independent assessment (“quantitation”) of cyclase activity, which is still somewhat subject to modula-

### Table 3. Butyrate Pretreatment of Cells Augments Adenylate Cyclase Activity in Cell Membranes of FDC-P1 and Fl

<table>
<thead>
<tr>
<th>Membrane assay condition</th>
<th>Control Preincubation</th>
<th>Butyrate Preincubation (1 mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>11 ± 0</td>
<td>19 ± 0</td>
</tr>
<tr>
<td>Mn⁺⁺ (4 mmol/L)</td>
<td>22 ± 0</td>
<td>31 ± 0</td>
</tr>
<tr>
<td>Forskolin (10⁻⁴ mol/L) + Mn⁺⁺ (4 mmol/L)</td>
<td>144 ± 3</td>
<td>233 ± 5</td>
</tr>
<tr>
<td>FDC-P1 membrane* assay condition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>14 ± 0.4</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Mn⁺⁺ (4 mmol/L)</td>
<td>18 ± 0.8</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Forskolin (10⁻⁴ mol/L) + Mn⁺⁺ (4 mmol/L)</td>
<td>128 ± 2</td>
<td>176 ± 8</td>
</tr>
</tbody>
</table>

*After factor deprivation, FDC-P1 cells were incubated overnight in a submaximal concentration of WEHI-3 CM in RPMI 10% FCS, with or without 1 mmol/L Na butyrate prior to homogenization.
tion by subunit components of the inhibitory G protein of adenylate cyclase. To examine possible G-protein modulation of adenylate cyclase activity in our system further, forskolin-stimulated adenylate cyclase activity in membrane preparations was studied at Mn\(^{2+}\) concentrations lower than those that fully uncouple G-proteins from adenylate cyclase. These concentrations of Mn\(^{2+}\), which preferentially uncouple Gi, allowed observation of a significant augmentation of forskolin-stimulated adenylate cyclase activity by Gpp(NH)p (Fig. 5). Thus, guanine nucleotide regulatory proteins appeared to play a role in the augmented adenylate cyclase activity observed with butyrate treatment. These observations are concordant with previous studies of the epithelial HeLa cell line in which low concentrations of butyrate (<1 mmol/L) caused an increase in uncoupled \(\beta\)-receptors and higher concentrations (5 mmol/L) appeared to couple receptors to Gs by a functional alteration of the stimulatory G-protein.

Enhancement by butyrate of GTP-dependent adenylate cyclase stimulation led us to examine the G-proteins by SDS-PAGE analysis after covalent modification (\(^{32}\)P-ADP-ribosylation) by cholera toxin (45-kd and 55-kd isoforms of Gs-\(\alpha\) are substrates) or pertussis toxin (41-kd Gi-\(\alpha\) substrate). Several factors, including guanine nucleotide concentration and type (eg, GDP or GTP) and Mg\(^{2+}\) concentration in the ribosylation reaction, as well as the availability of ADP-ribosylation factor (ARF: cholera toxin cofactor) and endogenous membrane NAD glycohydrolase activity may affect the extent of covalent modification of Gi and Gs by pertussis toxin and cholera toxin, respectively, and thus limit their usefulness in G-protein quantitation.

Despite this problem, the observation of consistent changes in the amount of ribosylation product assayed under controlled conditions has been useful in elucidating qualitative aspects of G-protein cellular organization. For example, Woolkalis and colleagues recently observed that phosphorylation of chicken embryo fibroblasts by Rous sarcoma virus resulted in reduction of forskolin-stimulated adenylate cyclase activity. This functional result was accompanied by threefold increase in the amount of the 41-kd cholera toxin substrate in the transformed cells, which was accounted for by a species of lower isoelectric point. A cholera substrate with acidic isoelectric point has previously been identified in the "uncoupled" S49 lymphoma variant. Posttranslational modification by phosphorylation was postulated to have altered function of Gs-\(\alpha\) in transmitting hormonal signals. In the present experiments, the diminution of cholera substrate upon butyrate treatment was a consistent finding in factor-independent cells (FDC-P1 was variable) that correlated with increased function of Gi in adenylate cyclase assays. These autoradiographic observations did not appear to result from problems in assay conditions because of the inclusion of ARF and NADP in our assay. In addition, independent assessment of the presence of 45-kd G-protein by photoaffinity labeling with \(^{32}\)P-8-azido-GTP did not suggest major reduction of 45-kd GTP-binding species (data not shown). Therefore, the present data may reflect the induction by butyrate of an activity modifying Gs (such as a phosphatase). In addition, the observation of a consistent increase in the relative labeling of the 41-kd pertussis toxin substrate, Gi, following exposure of FDC-P1 and Fl 11.2 to butyrate suggests changes in the equilibrium of \(\beta\)-\(y\) subunit exchange between Gs-\(\alpha\) and Gi-\(\alpha\), favoring the inactive Gi-\(\alpha\) \(\beta\)-heterotrimer, which is the preferred substrate for ADP-ribosylation by pertussis toxin. Such an equilibrium shift upon butyrate exposure would be expected to yield higher adenylate cyclase activities in butyrate membranes and control upon Mg\(^{2+}\)-dependent ligand activation. These attractive mechanistic explanations for our functional observations are as yet unsubstantiated and will require further investigations involving the use of purified G-protein subunits in reconstitution assays or antibodies against individual subunits for quantitation by Western blotting. In addition, the present studies do not clarify the possible intrinsic modification(s) of the cyclase enzyme leading to augmented activity. Yoshihara et al. recently demonstrated that phosphorylation may affect enzymatic capacity of the adenylate cyclase catalyst.

An important aspect of the current investigation was the examination of uncoupling of the adenylate cyclase system in both IL 3-dependent and IL 3-independent forms of a murine myeloid leukemia. Most prior studies of the mechanistic or functional effects of differentiating agents have used IL 3-independent myeloid cell lines such as the human HL-60 line. Examination of butyrate's activity in IL 3-dependent cells is particularly relevant, however, since all primary human myeloid leukemias examined so far share this characteristic whereas only in murine leukemias have factor-independent stages been described. In the present study, the inhibitory activities of PGE, and cholera toxin on proliferation of FDC-P1 were inversely related to IL 3 concentration. The factor-independent cell line was constitutively resistant to their inhibitory effects, however. The observation of comparably impaired levels of adenylate cyclase activity mediated by forskolin or Gpp(NH)p in both cell types argues against the possibility that the transformation event (to factor-independence) resulted from impaired adenylate cyclase activity per se. Butyrate appeared to be equally effective, however, in augmenting intracellular cAMP generation in both leukemic stages.

In summary, butyrate augmented the antiproliferative activity of PGE, and cholera toxin in IL 3-dependent and IL 3-independent murine myeloid cell lines. This effect resulted, at least in part, from increased cAMP generated through GTP-binding proteins by an adenylate cyclase enzyme with increased catalytic activity. Further analysis of this signal transduction system may lead to improved understanding of human leukemias. The data suggest the usefulness of biore- sponse modifiers that affect adenylate cyclase enzyme activity in combination with hormonal adenylate cyclase agonists in mediating differentiation or inhibition of proliferation in myeloid leukemias that otherwise may have an adaptive mechanism promoting clonal excess over normal hematopoietic progenitors.

ACKNOWLEDGMENT

We gratefully acknowledge the expert technical assistance of Gem Burgess and Stephanie Moore for typing the manuscript.
REFERENCES


Restoration of adenylate cyclase responsiveness in murine myeloid leukemia permits inhibition of proliferation by hormone. Butyrate augments catalytic activity of adenylate cyclase

L Inhorn, JW Fleming, D Klingberg, TG Gabig and HS Boswell