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Forskolin induces U937 cell line differentiation as a result of a sustained cAMP elevation

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Abstract

The present study examines the effects of forskolin on U937 cell differentiation. We recently reported that dibutyryl cAMP (dbcAMP), but not cAMP-elevating agents such as histamine, promotes U937 cell differentiation. cAMP production elicited by stimulation of histamine H_2 receptors showed a rapid, homologous desensitization, which might explain the dissimilar responses to histamine and dbcAMP. Forskolin induced an increase in cAMP levels in a concentration-dependent manner ($EC_{50} = 30$ μM) for an extended period of at least 24 h. Forskolin but not histamine (up to 100 μM), also inhibited cell growth in a dose-dependent fashion ($EC_{50} = 22$ μM). After 3 days of incubation, 75 μM forskolin induced U937 cell differentiation as judged by an increased rate of reduction of nitrobluetetrazolium (mean $\pm$ S.E.M.: $21.3 \pm 6.6\%$ in treated cells vs. $3.2 \pm 1.9\%$ in the control group, $P < 0.001$) and an augmented chemotactic response to complement 5a (C5a) ($33.2 \pm 5.9\%$ in forskolin-treated vs. $0.34 \pm 0.12\%$ in control cells, $P < 0.01$). Furthermore, c-Myc levels decreased following forskolin treatment, while the histamine H_2 receptor agonist dimaprit had no effect. We conclude that forskolin induces U937 cell differentiation through a sustained rise in cAMP levels.

Keywords: Forskolin; Histamine; U937 cell; cAMP; Cell differentiation; Desensitization

1. Introduction

Cyclic AMP is implicated in the growth regulation and differentiation of normal and malignant cells (Cho-Chung et al., 1991; Hartwell, 1994). This second messenger is also involved in apoptotic cell death in lymphoid (McConkey et al., 1990) and myeloid cells (Duprez et al., 1993; Vintermyr et al., 1993). In the human promyelocytic cell line HL-60, cAMP-elevating agents, including histamine and histamine H_2 receptor agonists, induce granulocyte differentiation (Nonaka et al., 1992; Kaliniyak et al., 1985; Chaplinski and Niedel, 1982).

In the promonocytic cell line U937, dibutyryl cAMP (dbcAMP) induces monocyte maturation (Gavison et al., 1988; Shayo et al., 1997), whereas histamine, which increases cAMP levels via histamine H_2 receptors with a short desensitization half-time, fails to promote differentiation (Davio et al., 1995a; Shayo et al., 1997).

Forskolin, a direct adenylyl cyclase-stimulating agent, has been reported to increase cAMP levels in U937 cells and to augment the number of surface receptors for complement 5a (C5a) in association with 1,25-dihydroxyvitamin D3 (Rubin et al., 1986). Recently we found that forskolin alone induced U937 cell differentiation (Shayo et al., 1997).

Based on these previous results, we supposed that the rapid desensitization of the histamine H_2 receptor might

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explain the failure of the histamine H_2 receptor agonist to cause differentiation of U937 cells.

The aim of the present work was to investigate the kinetic profile of forskolin-induced cAMP production and the characteristics of forskolin-induced cell differentiation.

2. Materials and methods

2.1. Chemicals

Forskolin and dbcAMP were purchased from Calbiochem (San Diego, CA, USA). Dimaprit, famotidine, pyrilamine, histamine, nitrobluetetrazolium and recombinant human C5a were obtained from Sigma (St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based cell titer 96™ kit, purchased from Promega (Madison, WI, USA), was used as specified by the manufacturer. All other chemicals were of analytical grade.

2.2. Cell culture

The U937 cell line was provided by Dr. Rolande Berthier (INSERM, Grenoble, France). Cells were cultured in suspension at 37°C in RPMI 1640 medium supplemented with 300 mg/l L-glutamine, 10% fetal calf serum and 40 μg/ml gentamicin in a 5% CO_2 humidified incubator. Cells were routinely passaged every 3–4 days and seeded at a density of 0.5×10^5 cells/ml.

For differentiation experiments final drug concentrations were 10 μM dimaprit, 75 μM forskolin and 500 μM dbcAMP. Since kinetic studies showed renewed cell proliferation after 48 h in forskolin-treated cultures, cells were harvested at this time, washed and resuspended in culture medium supplemented with fresh forskolin solution. After 3 days, viability (always > 90%) was assessed by using the Trypan blue exclusion test, and the cells were used for the differentiation assays.

2.3. Proliferation assay

The MTT test was performed as described elsewhere (Hansen et al., 1989). Briefly, 10^3 to 5×10^3 cells per well in a final volume of 100 μl were seeded in a 96-well flat bottom tissue culture plate. Each experiment was performed in quadruplicate. Blanks consisted of 100 μl/well of culture medium. After 3–4 days of culture, 15 μl of MTT solution/well was added and the cultures were incubated for 4 h. The reaction was terminated by the addition of 100 μl of solubilization-stop solution/well and overnight incubation. Optical density at 540 nm was recorded in a vertical ELISA Titertek Multiscan MCC/340 reader.

A linear relationship between optical density at 540 nm and U937 cell numbers between 5×10^4 and 7×10^5/ml in the MTT test has been established previously (data not shown).

2.4. cAMP assay

Cells were stimulated for 9 min with different concentrations of forskolin or histamine H_2 agonist (dimaprit) in Hanks' solution with 1 mM 3-isobutyl-1-methylxanthine (IBMX). The reaction was terminated by centrifugation for 3 min at $3000 \times g$, followed by the addition of 1 ml absolute ethanol. Supernatants were centrifuged for 10 min at $3000 \times g$, and the ethanol phase was dried and resuspended in 50 mM Tris–HCl buffer. cAMP content was determined by means of competition with [^{3}H]cAMP for protein kinase A, as previously described (Davio et al., 1995b). Pretreatment of cells (desensitization experiments) with forskolin was performed in Hanks at 37°C in a 5% CO_2 humidified atmosphere. Cells were exposed to 75 μM forskolin (maximal response) for periods ranging from 1 min to 24 h in the absence of IBMX. Thereafter, cells were washed and resuspended in Hanks with 1 mM IBMX at a cell density of 10^6 cells/ml and exposed for 9 min to 75 μM forskolin or 10 μM dimaprit, to determine whether the adenylyl cyclase could still generate cAMP. In every experiment, each point was assayed in triplicate.

2.5. Surface myeloid antigen study

Treatment and control cells were washed twice with phosphate buffer saline (PBS) and incubated for 15 min at 37°C in RPMI 1640, supplemented with 5% human AB serum to block Fc receptors. Indirect immunofluorescence was performed by exposing 10^6 cells to a saturating amount (2 μg) of murine monoclonal anti-cluster designation 14 (anti-CD14) or anti-CD11b antibodies. Goat monoclonal anti-mouse fluorescein-conjugated antibodies were used for fluorescent labelling. Irrelevant monoclonal murine antibodies of the same isotype were used as negative controls. Incubations with antibodies were performed for 30 min at 4°C, in a light-protected environment. Cells were washed twice with PBS, resuspended and analyzed on a FACStar plus flow cytometer (Beckton Dickinson, San Jose, CA), using Lysys software. At least 5000 events per tube were measured. Green fluorescence, and forward and side scatter were recorded. Percentages of positive cells and mean fluorescence intensity differences between specific CD-immunolabelled cells and their negative controls were established for both untreated and drug-cultured cells.

2.6. Chemotaxis assay

The in vitro locomotion of both control and treated U937 cells was assayed using the micropore filter technique. Briefly, 10^5 cells in 0.1 ml of RPMI 1640 containing 10 mM HEPES and 0.5% human serum albumin were

seeded onto the top compartment of chemotactic chambers (Transwell 3415; Costar, Cambridge, MA) placed in a 24-well tissue culture plate (Corning, USA). The top and bottom compartments were separated by a polyvinylpyrrolidone-free polycarbonate filter with a pore size of 5 μm. The bottom compartment was filled with 0.6 ml of control medium with or without recombinant human C5a. Chambers were incubated 1 to 3 h at 37°C in a 5% CO_2 atmosphere. The assay was stopped when high migration rates were observed with an inverted light microscope (between 1 to 3 h). Migrated cells were collected and counted by flow cytometry (Cytoron; Ortho Diagnosis, NJ, USA)

2.7. Nitrobluetetrazolium reduction

Treated and control cells were mixed with equal volumes of 0.2% nitrobluetetrazolium and 200 nM of 4β-phorbol 12-myristate 13-acetate (PMA) in PBS. The mixture was incubated for 1 h at 37°C and the proportion of cells containing intracellular formazan deposits was determined on May Grunwald-Giemsa-stained cytospin slide preparations. A minimum of 300 cells per treatment were assessed.

2.8. Western blots

Treated and control cells (3×10^6) were lysed in 300 μl of 50 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 100 mM 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue, and sonicated to shear DNA. Samples were then boiled for 5 min, and 20-μl samples were electrophoresed in 12% SDS-polyacrylamide gels and transferred to nitrocellulose paper. The residual binding sites were blocked with 5% nonfat dried milk in TBST (20 mM Tris–HCl pH 7.6, 137 mM NaCl and 0.05% Tween 20) and membranes were incubated with 1 μg/ml of anti-Myc rabbit sera (purchased from Santa Cruz Biotechnology, CA, USA) in 3% nonfat dried milk in TBST. All subsequent washes were performed in TBST. Binding was visualized by using an anti-rabbit polyclonal antibody linked to horseradish peroxidase and enhanced with chemiluminescence reagents, according to the manufacturer's instructions (Amersham Life Science, UK).

2.9. Statistical analysis

Optical densities, cAMP levels and natural logarithms of the number of cells that had migrated were compared by using a one-way analysis of variance (ANOVA). A Bonferroni post-hoc test for multiple pairwise comparisons was then performed if significant differences were detected. An $\alpha = 0.05$ level of statistical significance was adopted.

Mean fluorescence intensity differences between CD-immunolabelled cells and their negative controls, and percentages of positive cells, were compared by using a two-sample *t*-test. An $\alpha = 0.01$ level was considered statistically significant.

In dose–response studies nonlinear regression analyses were performed by using the GraphPad Prism 2.0 software.

The proportions of nitrobluetetrazolium-reducing cells were compared by using the χ^2 test. To perform comparisons between some treatment groups, χ^2 values were calculated by splitting the degrees of freedom (Armitage and Berry, 1992). α levels of 0.01 were adopted as being statistically significant.

3. Results

3.1. Forskolin and dbcAMP inhibit U937 cell proliferation in a dose-dependent fashion

Forskolin inhibited U937 cell-line proliferation in a concentration-dependent fashion with an EC_{50} value of 22 μM (Fig. 1). A maximal response with 83.3% inhibition of control growth was observed at concentrations greater than 50 μM. dbcAMP also induced a dose-dependent decrease in proliferation, with an $EC_{50} = 116$ μM and a maximal inhibition of 82% compared to control values at 1 mM (Fig. 1).

3.2. Histamine agonists do not modify U937 cell proliferation

Cell proliferation was evaluated in U937 cells treated with 1 nM–100 μM histamine. No significant differences between control and treated cells were observed ($P =$

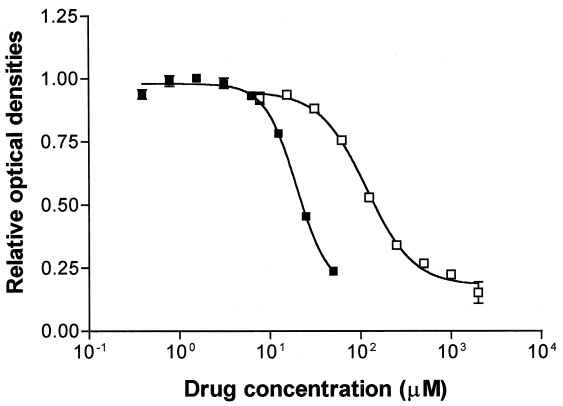

Fig. 1. Forskolin- and dbcAMP-induced inhibition of proliferation. U937 cells were seeded in tissue culture plates and incubated for 3 days with different concentrations of forskolin (filled squares) or dbcAMP (open squares). The MTT test was performed as described in Section 2. Relative optical densities are the recorded optical densities expressed as proportions of the mean control optical density. Results are expressed as means ± S.E.M. of quadruplicate assays. Forskolin $IC_{50} = 22$ μM (CI_{95} = 20.7–23.4 μM). dbcAMP $IC_{50} = 116$ μM (CI_{95} = 102–131 μM)

0.1775 and $P = 0.4027$, one-way ANOVA). Similar results were obtained with specific histamine H_1 and H_2 agonists, in a concentration range between 100 nM and 10 μM (data not shown). Nonspecific inhibition of cell proliferation was produced by higher concentrations of both histamine H_1 and H_2 receptor agonists, as demonstrated by the failure of the specific antagonists pyrilamine and famotidine to reverse this growth inhibition (data not shown).

3.3. Forskolin induces a dose-dependent cAMP elevation in the U937 cell-line

Forskolin induced an increase in cAMP levels in a concentration-dependent fashion, with an $EC_{50} = 30.7$ μM and a maximal response of 45.3 pmol of cAMP/10^6 cells (Fig. 2).

3.4. Forskolin-induced cAMP elevation does not desensitize for at least 24 h.

In order to investigate whether the cAMP response to forskolin in this cell line is susceptible to desensitization, cells were exposed to 75 μM forskolin (maximal response) for periods ranging from 1 min to 24 h. Cells were subsequently washed and exposed to forskolin for 9 min in the presence of 1 mM IBMX, to determine whether the system was still capable of generating cAMP. Furthermore, we examined the effects of pretreatment with forskolin on the response to dimaprit (a histamine H_2 receptor agonist).

As shown in Fig. 3, both dimaprit and forskolin induced similar cAMP levels in non-pretreated cells (inset). A decrease in cAMP production was observed after stimulation with the histamine H_2 receptor agonist in cells previously exposed to forskolin. Dimaprit could no longer induce a response in cells pretreated 4 h with forskolin, eliciting a half-maximal desensitization of 120 ± 13 min (mean $\pm$ S.E.M., $n = 4$). However, there were no changes in cAMP production after incubation with forskolin for at least 24 h.

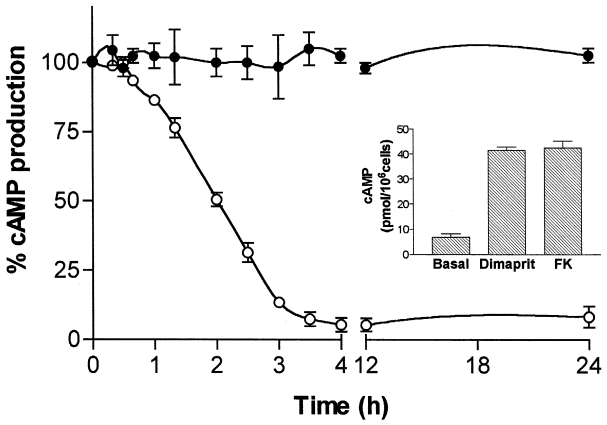

Fig. 3. Kinetics of forskolin-induced cAMP elevation. U937 cells were incubated for different lengths of time with 75 μM forskolin in Hanks' balanced solution. Cells were subsequently washed and incubated for 3 min in Hanks' medium supplemented with 1 mM IBMX, followed by stimulation with 10 μM dimaprit (open circles) or 75 μM forskolin (filled circles). Data are expressed as the percentage of cAMP production in response to forskolin and dimaprit as a function of the basal level in pretreated cells, or as pmol of cAMP/10^6 cells (inset). Data are the means $\pm$ S.E.M of triplicate assays. Similar results were obtained in at least three independent experiments.

3.5. Forskolin and dbcAMP increase the burst oxidative capacity of U937 cells

A significant increase in the proportion of nitrobluetetrazolium-reducing cells was observed following exposure to dbcAMP (10.8 ± 3.1%, mean $\pm$ S.E.M.), especially after forskolin treatment (21.3 ± 6.6%), in three successive assays. Dimaprit did not modify the low basal rate of nitrobluetetrazolium reduction by U937 cells (3.9 ± 1% and 3.2 ± 1.9%, respectively) (Fig. 4).

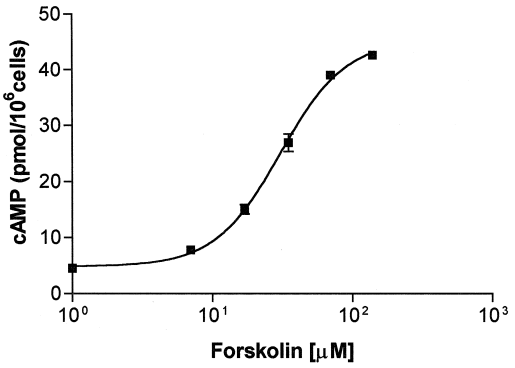

Fig. 2. Concentration–response curves for cAMP production. U937 cells were incubated for 9 min with increasing concentrations of forskolin at 37°C in Hanks' medium supplemented with 1 mM IBMX, and cAMP levels were determined. Data are the means $\pm$ S.E.M of triplicate assays. Similar results were obtained in at least four independent experiments.

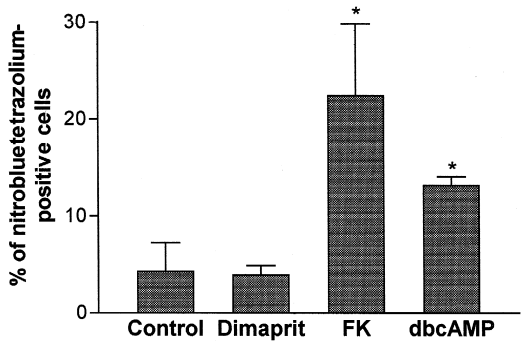

Fig. 4. Nitrobluetetrazolium reduction by U937 cells. Control and treated cells were incubated for 1 h with 1‰ nitrobluetetrazolium, cytocentrifuged and stained, as described in Section 2. Results are expressed as means $\pm$ S.E.M. and were obtained from three independent experiments. $* P < 0.001$ respect to control (χ^2 test).

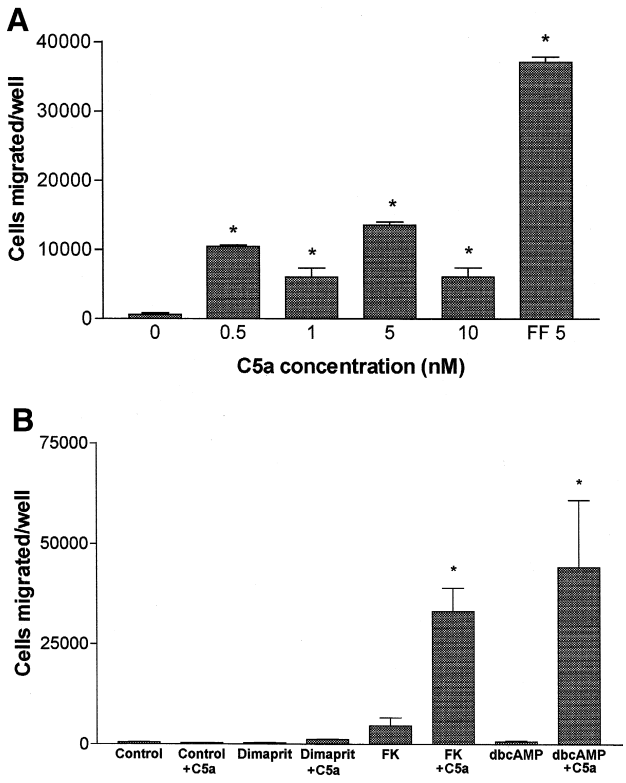

Fig. 5. (A) Recombinant human C5a concentration-dependent chemotactic responses of forskolin-treated U937 cells. U937 cells were incubated with 75 μM forskolin for 3 days before being washed and exposed to the recombinant human C5a concentrations indicated. Chemotactic assays were performed as stated in Section 2. After 48 h of culture a group of cells was harvested, washed and resuspended in fresh culture medium containing 75 μM forskolin (represented by the FF5 bar). Results are expressed as means ± S.E.M. $*P < 0.01$ with respect to migration of untreated cells and of forskolin-treated cells unexposed to recombinant human C5a. (B) Treatment-dependent chemotactic response to recombinant human C5a. Chemotactic assays were performed with control and treated cells, with and without 5×10^{-9} M recombinant human C5a. Results are expressed as means ± S.E.M and were obtained from three independent experiments. $* P < 0.01$, with respect to the other groups.

3.6. Forskolin induces a chemotactic, dose-dependent response to rhC5a

U937 cells treated for 3 days with 75 μM forskolin showed a significant chemotactic response to recombinant human C5a in a range of 0.5 to 10 nM. Maximal cell migration was detected at 5 nM recombinant human C5a (Fig. 5A), and especially when forskolin and culture medium were replaced after 48 h of incubation.

Forskolin- and dbcAMP-treated cells showed a large chemotactic response to 5 nM recombinant human C5a (44.3 ± 16.8% and 33.2 ± 5.9%, respectively, mean ± S.E.M.). However, no significant differences were observed between dimaprit-treated cells (1.18 ± 0.15%) and controls (0.34 ± 0.12%) (Fig. 5B).

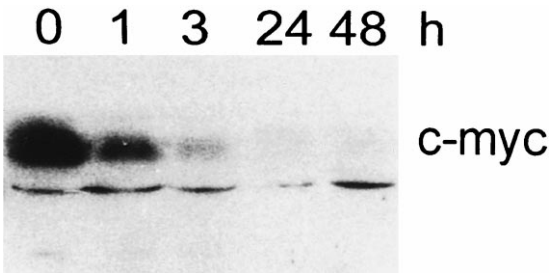

Fig. 6. Time-dependent effects of forskolin on Myc protein level. U937 cells were incubated with 75 μM forskolin for the indicated periods of time before being harvested and lysed as described in Section 2. Samples were electrophoresed in 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with polyclonal purified anti-Myc rabbit serum.

3.7. Modulation of surface myeloid antigen expression

dbcAMP induced a significant increase in the levels of two myeloid markers of differentiation in U937 cells, CD11b (α subunit of the β_2 integrin Mac-1) and CD14 (a putative receptor lipopolysaccharide-binding protein complex). Forskolin induced increases in the levels of both antigens, but these increases did not reach statistical significance (Table 1).

3.8. Myc proteins levels in forskolin-treated U937 cells

Kinetic analysis of Myc protein content was carried out on cells treated with forskolin. Myc protein levels decreased markedly after treatment with 75 μM forskolin

Table 1
Treatment-induced changes in U937 cell expression of differentiation antigens

	CD11b		CD14	
	M.F.I. difference (mean ± S.E.M.)	% of positive cells (mean ± S.D.)	M.F.I. difference (mean ± S.E.M.)	% of positive cells (mean ± S.D.)
Control	16.54 ± 2.73	34.9 ± 5.05	0.61 ± 1.24	6.3 ± 3.35
H_2 Agonists	19.95 ± 5.02	37.3 ± 3.42	2.2 ± 3.57	8.5 ± 7.47
Forskolin	25.69 ± 3.01	60.39 ± 12.58	7.65 ± 2.27[c]	18.43 ± 13.03
dbcAMP	39.32 ± 3.01[a]	68.7 ± 1.74[b]	14.71 ± 3.98[d]	25.5 ± 4.51[e]

Cells were treated 72 h with dimaprit, forskolin, or dbcAMP, immunolabelled and analyzed in a flow cytometer as described in Section 2. Results were obtained from three to five independent experiments.

M.F.I. difference: difference between the mean fluorescence intensities of specific CD-immunolabelled cells and their corresponding negative controls. [a]$P < 0.002$, [b]$P < 0.001$, [c]$P < 0.04$, [d]$P < 0.006$, [e]$P < 0.02$, with respect to control (two-sample t-test).

(Fig. 6). Similar results were obtained previously after treatment with dbcAMP (Shayo et al., 1997).

4. Discussion

Several chemicals induce monocyte differentiation in the human monoblastic cell line U937: phorbol esters (Nilsson et al., 1980), transretinoic acid (Olsson and Breitman, 1982), 1,25-dihydroxy vitamin D_3 (Olsson et al., 1983; Rigby et al., 1984), and dbcAMP (Gavison et al., 1988), but not cAMP-elevating agents such as histamine (Shayo et al., 1997). There is as yet no satisfactory explanation as to why dbcAMP causes differentiation in these cells while cAMP-elevating agents do not.

Dibutyryl cAMP is a membrane-permeable cAMP analogue that is resistant to phosphodiesterase hydrolysis (Posternak et al., 1962). Protein kinase A activation is thought to be the main cellular target for the multiple pharmacological effects of dbcAMP. Although the dbcAMP molecule has two butyrate residues and butyric acid causes differentiation in the HL-60 cell line (Collins et al., 1978; Fischkoff et al., 1985; Hutt-Taylor et al., 1988), the same is not true for U937 cells, where only cell proliferation is inhibited (Laskin et al., 1990). The differentiating effect of dbcAMP on the U937 cell line, thus, seems to involve activation of protein kinase A. The natural diterpene, forskolin, binds and stimulates adenylyl cyclase by a direct mechanism, increasing cAMP synthesis and thereby activating protein kinase A (Seamon et al., 1981; Daly, 1984). Histamine also increases cAMP levels in this cell line, through the histamine H_2 receptor–G protein–adenylyl cyclase pathway (Shayo et al., 1997). In the present study, we found that forskolin, unlike histamine and the histamine H_2 receptor agonist dimaprit, inhibited U937 cell proliferation in a dose-dependent manner at the same concentrations required to increase cAMP levels (10–100 μM). Furthermore, forskolin, but not dimaprit, at the concentration that elicited a maximal response also induced U937 cell differentiation, as shown by the increased reduction of nitrobluetetrazolium, the chemotactic response to C5a, and c-Myc down-regulation. In spite of the similar cAMP levels induced by both drugs, forskolin produced a sustained increase in cAMP levels. Thus, in this model, continuous activation of protein kinase A induces changes in the expression of multiple genes: a down-regulation of c-*myc*, expression of the oxidative burst enzymatic machinery, and the appearance of functional C5a receptors.

Desensitization is a phenomenon observed in almost all G protein-coupled receptors. It is characterized by attenuation of the cellular response despite the presence of the agonist. Histamine H_2 receptors in U937 cells show a fast homologous desensitization, with a $T_{1/2}$ of approximately 20 min (Shayo et al., 1997). The present work shows that forskolin was unable to induce down-regulation of its own stimulus but produced a heterologous desensitization to the histamine H_2 response. Heterologous desensitization shows a longer half-time than homologous desensitization, and probably is mediated by a different pathway.

We hypothesize that short-lasting activation of protein kinase A is, in our system, insufficient to induce an antiproliferative and differentiating response, whereas a long-lasting and stable activation of protein kinase A induces U937 cell differentiation. Reinforcing this idea, we showed that forskolin induced a sustained activation of adenylyl cyclase with a subsequent decrease in c-Myc levels and cell differentiation.

Agents that cause cell differentiation may generate dissimilar maturation patterns, not only in leukemic cell lines but also in normal myeloid precursors (Ôberg et al., 1993; Gavison et al., 1988; Nakamura et al., 1996; Hewison et al., 1992). In U937 cells, for example, transretinoic acid induces a strong inhibition of proliferation together with increased expression of CD23 but not CD14, a significant nitrobluetetrazolium reduction rate, a low phagocytic capacity, and no chemotactic response to C5a and *N*-formyl-methionine–leucine–phenylalanine (fMLP) (Ôberg et al., 1993; Gavison et al., 1988). In contrast, 1,25-dihydroxyvitamin D_3 induces a moderate inhibition of cell growth, CD14 but not CD23 expression, a high nitrobluetetrazolium reduction rate, and marked phagocytosis of yeast (Olsson et al., 1983; Ôberg et al., 1993; Testa et al., 1993; Rigby et al., 1984). Phorbol esters completely block cell proliferation, induce adhesion to plastic, phagocytosis, and both CD14 and CD23 synthesis and expression (Ôberg et al., 1993; Nambu et al., 1989). Finally, dbcAMP does not induce adhesion to plastic but markedly inhibits cell growth, induces C5a receptor expression (Rubin et al., 1986), together with a chemotactic response to C5a and fMLP (Gavison et al., 1988), reduction of nitrobluetetrazolium, and CD14 and CD11b expression.

Forskolin-induced cell differentiation showed similar but not identical characteristics to dbcAMP-induced differentiation, including a higher rate of nitrobluetetrazolium reduction, lower levels of CD14 and CD11b expression, and subtle differences in morphological appearance. These discrepancies might be explained by an increased phosphodiesterase activity following cAMP elevation, with subsequent attenuation of differentiation in the presence of forskolin, the activation of other concurrent cell responses (Posternak et al., 1962), or the coexistence of protein kinase A isoforms selectively modulated by cAMP or dbcAMP.

In conclusion, we found that forskolin increases cAMP levels accompanied by a decrease in c-Myc protein levels and subsequent cessation of cell proliferation concomitant with cellular differentiation. This suggests that elevation of cAMP levels exerts an important role in leukemic, and perhaps also in normal, myeloid differentiation. Receptor desensitization may be a relevant physiopathological event in neoplastic biology to avoid progression of differentiation.

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