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Resistance of Human Leukemic CEM-C1 Cells Is Overcome by Synergism between Glucocorticoid and Protein Kinase A Pathways: Correlation with c-Myc Suppression[1]

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ABSTRACT

Glucocorticoids (GCs) induce apoptosis in lymphoid cells that contain functional GC receptors (GRs). However, GC resistance often is seen in cells with demonstrable GRs; one such line is CEM-C1. We have tested the hypothesis that positive interactions between GC and cyclic AMP (cAMP) regulate GC actions in CEM clones. Treatment of both GC-resistant CEM-C1 [resistant to 1 μM dexamethasone (Dex)] and the sensitive sister clone, CEM-C7 (~65% cell death with 20 nM Dex, ~99% death with 1 μM Dex), with a ≤20 μM concentration of the protein kinase A activator, forskolin, had no significant effect on cell viability. Cotreatment with Dex and forskolin resulted in a strong synergistic death response, with only ~10% CEM-C1 cells surviving treatment with 1 μM Dex and 20 μM forskolin. This death was blocked by the GR antagonist RU 38486. However, the extent of apoptosis did not correlate with the amount of GR protein or binding activity in either C7 or C1 cells. As reported previously, Dex-evoked cell death was associated with suppression of c-Myc in C7 cells. In CEM-C1 cells, Dex alone did not affect c-Myc; however, Dex plus forskolin suppressed c-Myc levels. To evaluate mechanisms of Dex-forskolin synergism, fresh subclones of CEM-C7 (clone 14) and CEM-C1 (clone 15) were isolated, to ensure purity of phenotype. In these, forskolin (with or without Dex) caused a similar increase in cAMP (~300-fold) and phospho-cAMP-responsive element binding protein (~4–5-fold) levels, whereas total cAMP-responsive element binding protein expression was not affected. GR transcription function, as tested from a GR-responsive 330-bp mouse mammary tumor virus promoter-luciferase reporter construct, was induced 8- and 4-fold by 1 μM Dex treatment of CEM-C7-14 and CEM-C1-15 cells, respectively. Forskolin (10 μM) significantly potentiated Dex response in CEM-C1-15 cells (13.5-fold) but had only a modest effect (1.5-fold) in CEM-C7-14 cells. These studies suggest that sensitization of CEM-C1 cells by cross-talk between GR and protein kinase A pathways may occur via cooperative effects on GR-mediated gene transcription.

INTRODUCTION

Determining how the interaction of various signal transduction pathways produces cell- and gene-specific events is one of the present challenges facing biologists. Here, we examine the interaction of the PKA[3] and GC/GR pathways as they synergize to cause apoptosis of growing human lymphoid leukemic cells. One fundamental reason for the efficacy of GCs as antileukemic agents is their ability to cause apoptosis in certain lymphoid cells (1–3). The complete molecular and biochemical mechanisms of this cell lysis process are only partially known, but cell and molecular genetic analyses of lymphoid cells selected for resistance to GCs have established beyond doubt the central role of the GR in the GC-mediated apoptotic pathway (4, 5). The predominant characteristic of murine and human cells selected *in vitro* for GC resistance is the loss of functional GR (5–8). In WEHI-7 and S49 mouse lymphoma cells, a direct correlation between the quantity of intracellular GR sites and GC sensitivity has been suggested (9, 10). High GR content in leukemic cells has been shown to favor efficacy of therapy that includes GC (11). In addition, GC-induced increase of GR has been suggested to be an early event in the apoptotic response and a predictor for some instances of GC sensitivity (12, 13).

Although the requirement for functional GR in GC sensitivity has been established, it is also well documented that certain lymphoid cells *in vitro* and *in vivo* resist apoptosis, despite having a GR content similar to or greater than that in other related cells killed by GC (14–18). Clearly, other factors can control GC-induced apoptosis. GC resistance in some GR-positive murine P1798 lymphoma cells has been shown to result from secretion of a protein factor that attenuates the lytic response (19). High-level expression of the *mdr1* gene product, P-glycoprotein, has been described in murine WEHI-7 lymphoma cells that are resistant to GC (20). Alterations in the GC response pathway distal to GR activation also may lead to GC resistance.

GC-induced alterations in the expression of transcription factors and oncogenes, including c-*jun* (21) and c-*myc* (22), have been implicated in apoptosis. Steroid hormones can also modulate the activity of certain cAMP and G-protein-driven pathways (23), and GC-mediated cellular responses can be modulated by other signal transduction pathways, including those involving protein kinase C (24) and PKA (25, 26). In particular, cAMP and GC are known to induce cytolysis synergistically of T cells (27, 28). In WEHI-7 clones, Gruol *et al.* (29) found that loss of cAMP-dependent protein kinase (PKA) activity caused a significant decrease in GC sensitivity. In murine thymocytes, agents that elevate intracellular cAMP levels were shown to enhance the GC-evoked apoptosis (30). Also, cAMP is capable of inducing thymocyte apoptosis without GC stimulation (31). cAMP modulates transcription of target genes bearing cAMP responsive elements via PKA-mediated Ser-133 phosphorylation and activation of CREB (32, 33). CBP, a coactivator, specifically binds to P-CREB to further enhance this process (33). CBP has also been implicated as a component of nuclear receptor coactivator complexes (34, 35).

Although several independent pathways are known to trigger a lytic response in lymphocytes, the precise steps leading to apoptosis are not known. A GC-induced increase or decrease in the expression of key genes involved in cell proliferation/differentiation, such as cyclins, cyclin-dependent kinases, and c-*myc*, has been proposed as one class of mechanisms (22, 36, 37). Down-regulation of c-*myc* is an early, common response in cells arrested and/or killed by GC treatment, documented in S49, P1798, and CEM cells, as well as in Jurkat cells that have been supplied with GR (22, 37–40).

We have established the use of clones from the human acute

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[3] The abbreviations used are: PKA, protein kinase A; GC, glucocorticoid; GR, GC receptor; cAMP, cyclic AMP; CREB, cAMP responsive element binding protein; P-CREB, phospho-CREB; CBP, CREB-binding protein; K_d, dissociation constant; Dex, dexamethasone; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-X-nick end labeling.

lymphoblastic leukemic cell line CCRF-CEM as a valuable model for the investigation of GC-evoked cytolysis. GC-sensitive and -resistant clones of CEM cells have been isolated and well characterized (3, 8). One interesting clone is CEM-C1, cloned originally in 1977 in the absence of any selective pressure and resistant to lysis by GCs (3). Both CEM-C1 and its sister clone, the GC-sensitive CEM-C7, are also resistant to cAMP analogues or to the PKA activator, forskolin. Initial characterization of CEM-C1 cells revealed no significant difference in the number, K_d, or the tested biochemical characteristics of the GR from those of the GR in GC-sensitive clone CEM-C7 (41). Furthermore, the receptor in CEM-C1 cells appears to be functional, in that GCs can induce glutamine synthase and GRs in the cells (41, 42). In recent years, the original CEM-C1 cloned population has been overgrown by a subtype with a level of GR-binding sites that is about one-fourth of that originally observed.[4] Likewise, the CEM-C7 clone has also shown some clonal diversity.

In this derivative CEM-C1 population, we now have discovered that GCs and activators of the cAMP-dependent pathway synergize to restore the apoptotic response. In the CEM-C7 population, activators of PKA potentiate GC-induced apoptosis to cause a greater and more rapid cell death. In both cell populations, the activated GR is critical for this synergism, and suppression of c-Myc correlates with the synergistic effect of PKA and GC pathways in causing apoptosis. This observation has been extended to subsequently recloned CEM-C7 (clone 14) and CEM-C1 (clone 15) cells. Characterization of CEM-C1-15 cells indicates that they contain ~10,000 GR-binding sites per cell,[4] as originally reported for CEM-C1 cells (41). In the recloned cells, we demonstrate that, in either cell type, Dex does not significantly affect forskolin-mediated activation of the PKA pathway, judged by increase in cAMP levels and CREB phosphorylation. Dex-induced transcription of the luciferase gene under the control of a GC responsive element containing 330-bp MMTV-LTR in both cell types. However, transcriptional activity of the promoter-reporter construct was significantly lower in CEM-C1 cells and was strongly enhanced by forskolin. Our studies suggest that activation of the PKA pathway complements and enhances a weak GR-mediated transcriptional response and that a functional change in GR activity may result in restoration of apoptosis in CEM-C1 cells.

MATERIALS AND METHODS

Reagents. Dex and other reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin was purchased from Calbiochem (La Jolla, CA). Reagents for SDS-PAGE, as well as the Bradford protein assay reagent and the PVDF transblot transfer membrane, were obtained from Bio-Rad (Richmond, CA). The Super Signal CL-HP substrate system for ECL was obtained from Pierce (Rockford, IL). RU 38486 was a gift from Roussel-UCLAF (Romainville, France), and [1,2,4,6,7-^{3}H]Dex (86 Ci/mmol) was from Amersham (Arlington Heights, IL). The polyclonal antiserum AhuGR$_{150-175}$, raised against a synthetic peptide corresponding to the amino acids 150–175 within the NH_2-terminal domain of human GR (43), was used for immunodetection of GR. The monoclonal c-Myc antibody Myc1-9E-10.2, raised against a synthetic peptide in the COOH-terminal region of c-Myc (44), was generated as a culture supernatant of the hybridoma cell line CRL1729 purchased from American Type Culture Collection. Rabbit polyclonal antibodies raised against an epitope corresponding to amino acids 295–321 of human CREB (Santa Cruz Biotechnology) and a synthetic phosphopeptide corresponding to amino acid residues 123–136 of rat CREB (Upstate Biotechnology) were used for immunoblot detection of CREB and P-CREB, respectively.

Cell Culture. Tissue culture media and components were purchased from Mediatech (Washington, DC). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). CEM-C7 and CEM-C1 cells used in this study were derived from the parental line CCRF-CEM, obtained from a patient with acute lymphoblastic leukemia (45). These were recloned in semisolid agarose medium in the absence of any selective pressure, and several clones were obtained. Clones CEM-C7-14 and CEM-C1-15, used in some experiments here, were diploid, expressed 10,600 (K_d = 13 nM) and 9,900 (K_d = 12 nM) GR sites per cell, and were Dex sensitive and Dex resistant, respectively, properties that closely correlated with those reported for the original CEM-C7 and CEM-C1 clones (3, 41). The CEM-C1R cells described here are a subpopulation of CEM-C1 cells that were treated continuously with 1 μM Dex plus 10 μM forskolin to select for cells resistant to these agents. All cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum at 37°C in a humidified 5% CO_2 incubator.

Determination of Sensitivity to Dex/Forskolin. Dex stock was prepared at a concentration of 2 mM in ethanol. Forskolin was dissolved in DMSO at a concentration of 40 mM. In all cases, final concentrations of ethanol or DMSO during the treatments did not exceed 0.1%, and appropriate controls with vehicle alone were run in parallel. Cells were treated at a density of 1×10^5 cells/ml with ethanol/DMSO vehicle or Dex and/or forskolin at the appropriate concentrations. Viable cells were counted by trypan blue exclusion method using a hemacytometer. Cell counts were obtained at 24-h intervals for a time course study or between 72 and 96 h for a dose-response study.

Sensitivity of CEM-C1 cells to Dex and/or forskolin was also determined using the MTT assay (46), which measures the conversion of MTT to a colored formazan product by mitochondrial and cellular dehydrogenase enzymes. Appropriately treated cells were incubated for 4 h in the presence of 25 μl of a 5 mg/ml solution of MTT and then lysed and incubated overnight in buffer containing 20% SDS, 0.025 N acetic acid, 0.025 N HCl, and 50% dimethylformamide (pH 4.7) to solubilize formazan crystals. $A_{600\,nm}$ was measured on an ELISA reader to correlate the intensity of the colored product with the number of viable cells.

Analysis of Apoptosis by TUNEL Assay. CEM-C1 cells seeded at a density of 1×10^5 cells/ml were treated appropriately for 48 h. Three million cells were harvested, fixed, and subjected to the TUNEL reaction, according to the protocol provided by the manufacturer (Boehringer Mannheim), with some modifications as described earlier (47). Samples were also stained with propidium iodide to stain DNA and were analyzed for DNA content and strand breaks by flow cytometry using a FACScan (Becton Dickinson, Bedford, MA) and Cell Quest 1.2 (Becton Dickinson) software.

Determination of GR Binding Activity. Whole-cell binding assays were performed in duplicate on appropriately treated cells. Approximately 1×10^7 cells treated for 24 h were washed and resuspended in 250 μl of RPMI 1640 containing 150 nM [^{3}H]Dex (86 Ci/mmol) in the absence (uncompeted) or presence (competed) of 500-fold molar excess of unlabeled Dex. Cells were incubated for 1.5 h in a 37°C humidified CO_2 incubator, then washed three times with PBS and resuspended in 200 μl of PBS. Radioactivity was determined in 100-μl aliquots by liquid scintillation counting. Specific binding was calculated by subtracting nonspecific (competed) from total (uncompeted) binding. Viable cells were determined in an aliquot by trypan blue exclusion to determine specific sites per cell.

SDS-PAGE and Western Blotting. For analysis of GR, ~1.6×10^7 of appropriately treated cells were harvested, washed, and resuspended in 100 μl SDS-PAGE sample buffer [120 mM Tris, 4% SDS, 20% glycerol, and 0.05% bromphenol blue (pH 6.8)], boiled for 3 min, and centrifuged at 16,000 $\times$ g for 20 min to obtain a whole-cell extract as the supernatant fraction. For analysis of c-Myc, cells were washed and resuspended in 500 μl of 50 mM Tris-HCl (pH 7.4) containing 20 μM leupeptin and 400 μM 4-[2-aminoethyl]benzensulfonyl fluoride, lysed by three cycles of freezing and thawing, and centrifuged for 10 min at 8000 $\times$ g to pellet the nuclei. The nuclear pellet was resuspended in SDS-PAGE sample buffer, boiled for 3 min, and centrifuged at 16,000 $\times$ g for 20 min to obtain the nuclear extract. For detection of P-CREB and CREB by Western blotting, cells were treated with the appropriate drugs for 90 min or 18 h, respectively. Harvested cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), containing 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 20 μM leupeptin, 400 μM 4-[2-aminoethyl]benzensulfonyl fluoride, 20 mM sodium molybdate, and 5 mM NaF. In all samples, 2-mercaptoethanol was added to a final concentration of 5%. Protein content in cellular extracts was quantitated by Bradford assay (Bio-Rad), and samples were electrophoresed on a 10% SDS polyacrylamide gel using a mini-slab gel electrophoresis apparatus from Bio-Rad (48). Electroblotting on PVDF membrane was done using the Integrated Separation Systems (Hyde Park, MA) semidry electroblotter. Mem-

[4] B. H. Johnson and E. B. Thompson, unpublished results.

branes were blocked by incubation in 10% nonfat dry milk in PBS at room temperature for 1 h and then incubated overnight at 4°C in the appropriate primary antibody ($AhuGR_{150-175}$ for GR and Myc1–9E-10.2 for c-Myc). Washed membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h. Bands were detected by autoradiography after a chemiluminescent reaction using the Super Signal horseradish peroxidase substrate system from Pierce. Densitometric quantitation of GR or c-Myc protein levels was done on a LYNX 5000 image analyzer.

Assay for cAMP Content. cAMP content was determined using an enzyme immunoassay kit from Amersham (RPN-225). Cells were treated with the appropriate drugs for 60 min, and cAMP was extracted with 65% ethanol. Extracts were dried and reconstituted in assay buffer [50 mM sodium acetate (pH 8.5) containing 0.02% BSA], analyzed for cAMP content as per the protocol provided in the kit, and quantitated based on a standard curve run in parallel.

Evaluation of GR Transcriptional Activity. CEM-C7-14 and CEM-C1-15 cells were cotransfected with 10 μg of pHHLuc (containing sequences from −220 to +110 of the MMTV-LTR driving a luciferase reporter gene; Ref. 49) and 2 μg of pMTneo by electroporation at 300 V and 960 μF. Mass cultures of transfected cells were selected and maintained in the presence of 400 μg/ml Geneticin and treated with appropriate drugs for 18–20 h. Cells were harvested and lysed by three cycles of freezing and thawing, and luciferase activity was determined using a kit from Tropix.

RESULTS

Synergistic Apoptotic Response Mediated by Interaction between the GC and PKA Pathways. When CEM-C7 cells are treated with Dex alone, a concentration of 20 nM causes only a partial kill of the population, which approximately correlates with a partial GR occupancy by the steroid ligand, predicted from affinity. Treatment with cAMP analogues or forskolin, even at fairly high doses, is only weakly inhibitory. An example of these results is shown in Fig. 1. CEM-C7 cells (Fig. 1*A*) treated for 72 h with only 20 nM Dex or 20 μM of forskolin showed modest reductions in growth rates. Examination by light microscopy showed an increase in dead cells and cellular fragments in Dex-treated cultures, whereas the decrease in viable cell numbers seen in forskolin-treated cells appeared to result largely from decreased growth rate rather than cell death. Other activators of the PKA pathway, 8-bromo-cAMP and isobutylmethyl xanthine, also had similar effects as forskolin on cell growth and viability (data not shown). In contrast, CEM-C7 cells treated with 20 nM Dex plus 20 μM forskolin showed a marked decrease in viable cell number as well as extensive cell death by 48 h, and after 72 h, the number of viable cells was <1% of the number of control cells.

In CEM-C1 cells, even 1 μM Dex caused only a slight increase in population doubling time (Fig. 1*B*), without any increase in the apoptotic cell population, as indicated by morphological examination and TUNEL analysis (Figs. 1 and 2 and data not shown). Similarly, 20 μM forskolin caused a small increase in doubling time, without any increase in apoptosis. Isobutylmethyl xanthine had a similar effect as well (data not shown). Cotreatment with 20 μM forskolin and 1 μM Dex produced obvious cell death, and by 72 h, the number of viable cells in doubly treated cells was only 1.4% of control, with a marked increase in dead and fragmented cells. Phase-contrast microscopy of CEM-C1 cells revealed that, after combined treatment, the cells exhibited morphological features characteristic of apoptosis, *e.g.*, cell shrinkage, membrane blebbing, cellular fragmentation, and formation of condensed apoptotic bodies (Fig. 2, *A–D*). Such features were rare in CEM-C1 cells treated with Dex or forskolin alone. The results of flow cytometric analysis using the TUNEL assay and propidium iodide staining were consistent with the apoptotic nature of cell death in CEM-C1 cells treated with Dex plus forskolin. Fig. 2, *E–H*, shows contour plots of cell cycle distributions and terminal deoxynucleotidyl transferase labeling of CEM-C1 cells treated for 48 h with vehicle or 10 μM forskolin and/or 1 μM Dex. Only 1.1% of the control cells were TUNEL positive, and treatment with Dex or forskolin alone resulted in 1.2 and 1.6% TUNEL-positive cells, respectively. However, 12% of cells treated for 48 h with both agents were TUNEL positive, consistent with the data in Fig. 1. There was a time-dependent increase in this terminal deoxynucleotidyl transferase labeling, which preceded cell death (data not shown).

This synergistic response was dose dependent with respect to both Dex and forskolin. Assessing cell viability with the MTT assay, Fig. 3 demonstrates that concentrations of up to 20 μM forskolin alone had little effect on CEM-C1 cells' viability. Synergism with Dex, however, was seen when as little as 2 μM forskolin was added to cultures along with 1 μM Dex. The increasing cell death seen microscopically in the presence of Dex and increasing concentrations of forskolin was paralleled by data from the MTT assay (Fig. 3*A*). Increasing concentrations of Dex, between 2 and 100 nM, in the presence of 20 μM forskolin resulted in cell death with an EC_{50} of ~30 nM (Fig. 3*B*), close to the 19 nM K_d for Dex binding to the GR in these cells (41).

Role of GR in the Synergistic Response. Many studies have suggested that a necessary determinant of the GC response in sensitive cells is the GR. The concordance between the EC_{50} and K_d noted above suggests that one effect of forskolin treatment is to somehow

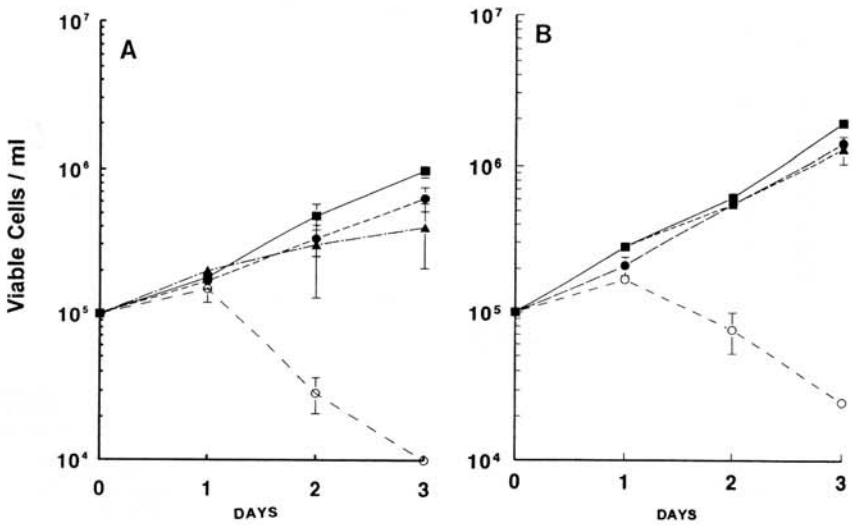


Fig. 1. Synergistic interaction between the GC and the PKA pathways. CEM-C7 (*A*) and CEM-C1 (*B*) cells were seeded at a density of 1×10^5 cells/ml and incubated with Dex (20 nM for C7, 1 μM for C1; ▲); 20 μM forskolin (●); or Dex plus 20 μM forskolin (○) for 72 h. Control cells (■) received ethanol carrier to a final concentration of 0.3%. At intervals of 24 h, growth and viability of cells were determined by trypan blue dye exclusion hemacytometry. *Data points*, means from three independent experiments; *bars*, SD (where no bars are seen, they fell within the size of the symbol).

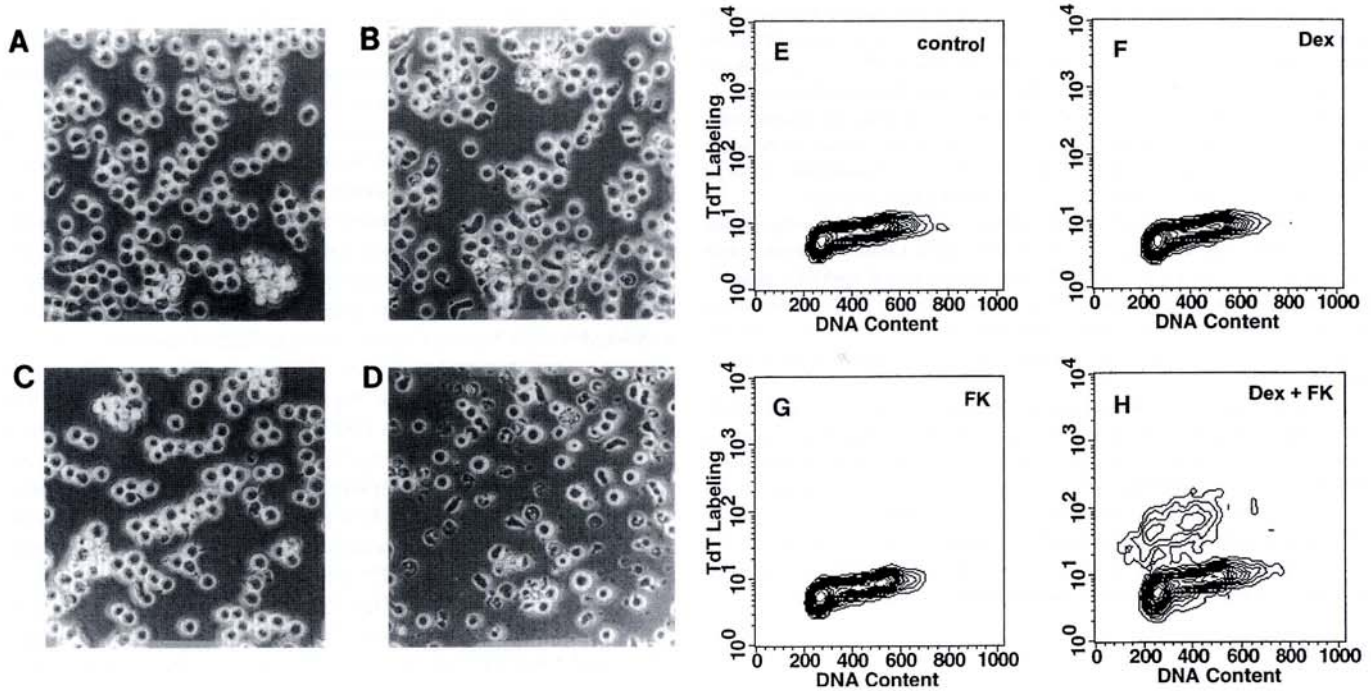


Fig. 2. Synergistic interaction between the GC and the PKA pathways induces apoptosis. CEM-C1 cells were incubated with vehicle (*A* and *E*), 1 μM Dex (*B* and *F*), 20 μM forskolin (*C*), 10 μM forskolin (*G*), 1 μM Dex plus 20 μM forskolin (*D*), or 1 μM Dex plus 10 μM forskolin (*H*), and cultures were subjected to direct phase-contrast microscopy (*A–D*) after 72 h (×600) or TUNEL reaction followed by flow cytometric analysis after 48 h (*E–H*).

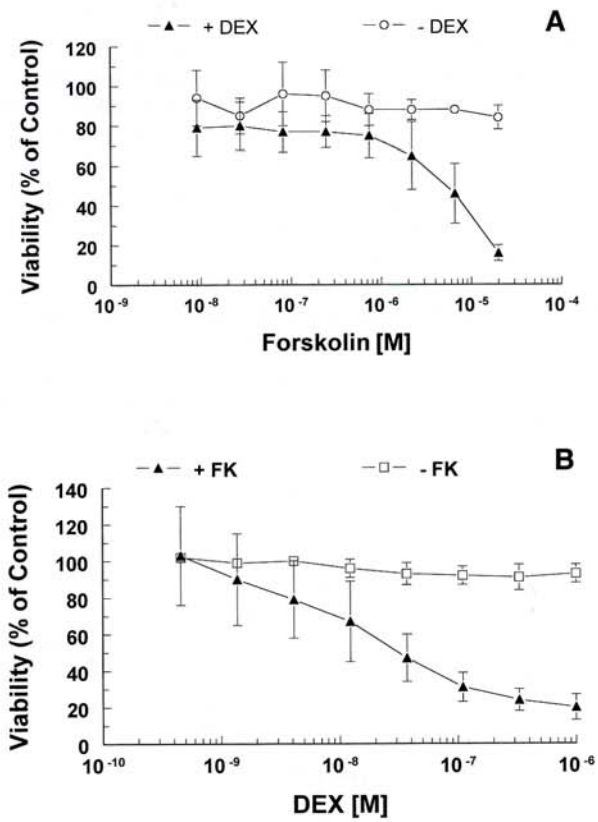


Fig. 3. Dose response of CEM-C1 cells to Dex and/or forskolin. CEM-C1 cells at a density of 3×10^4 cells/ml were treated in sets of six wells with varied concentrations of forskolin with (▲) or without (○) 1 μM Dex (*DEX*; *A*) or with varied concentrations of Dex with (▲) or without (□) 20 μM forskolin (*FK*; *B*). Control cells were treated with ethanol vehicle. After 4 days, each well was assayed for reduction of MTT, as described in "Materials and Methods." The viability of the treated cells was expressed as percentage viability for the control cells. *Data points*, means from three separate experiments; *bars*, SD.

activate/facilitate the GR pathway, leading to cell death. To determine further whether GR plays a critical role in the synergism between the GC and PKA pathways, we used the high-affinity, reversible GR antagonist RU 38486 (50, 51). Previous studies have revealed that equimolar concentrations of RU 38486 prevent Dex-evoked lysis of CEM-C7 cells (52). CEM-C1 cells were treated with 1 μM Dex and/or 20 μM forskolin in the presence or absence of 1 μM RU 38486. RU 38486 alone had no effect on cell growth and viability. Dex and forskolin alone or in the presence of RU 38486 also did not significantly alter cell growth when compared to the control group. The synergistic apoptotic response in cells treated with Dex plus forskolin, however, was completely prevented by RU 38486, implicating GR as a key component of the synergistic response (Fig. 4).

Regulation of GR by Dex and/or Forskolin. The quantity of intracellular functional GRs has been proposed to play a critical role in effects evoked by Dex (9, 53). Recent studies have suggested that GC resistance in CEM-C1 cells is a consequence of low receptor

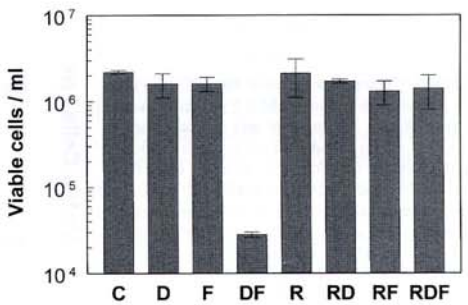


Fig. 4. The competitive GC antagonist RU 38486 reverses synergistic apoptosis by Dex plus forskolin. CEM-C1 cells (1×10^5/ml) were incubated with ethanol (*C*), 1 μM Dex (*D*), 20 μM forskolin (*F*), 1 μM Dex plus 20 μM forskolin (*DF*), 1 μM RU 38486 (*R*), 1 μM RU 38486 plus 1 μM Dex (*RD*), 1 μM RU 38486 plus 20 μM forskolin (*RF*), or 1 μM RU 38486 plus 1 μM Dex plus 20 μM forskolin (*RDF*). After 72 h, viable cell counts were determined by trypan blue dye exclusion hemacytometry. *Columns*, averages from two experiments, each performed in duplicate; *bars*, ranges.

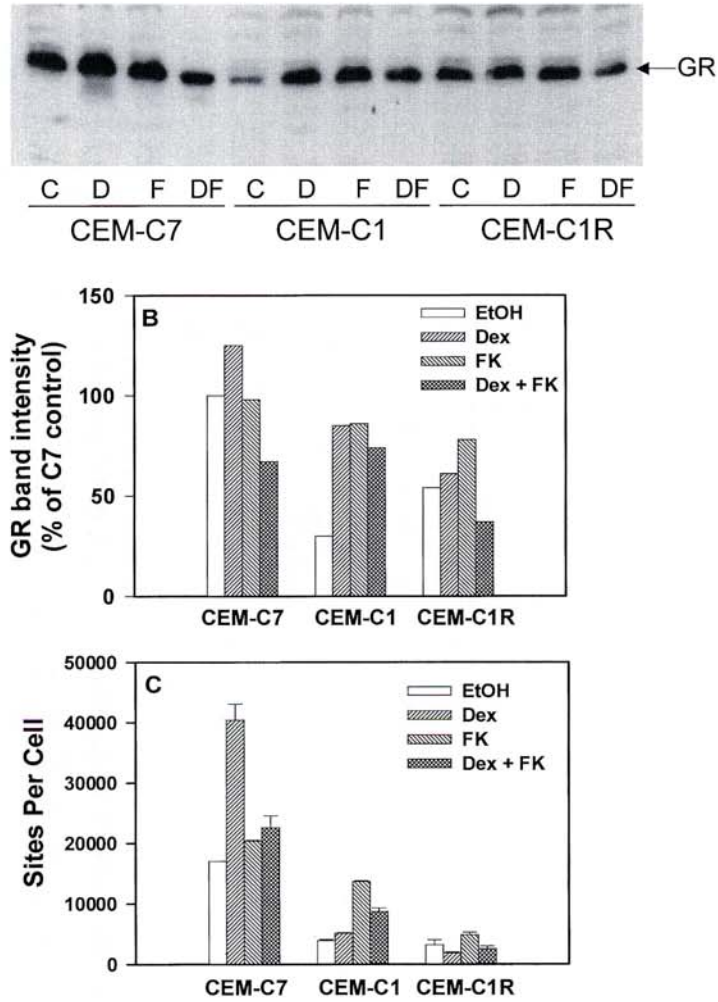
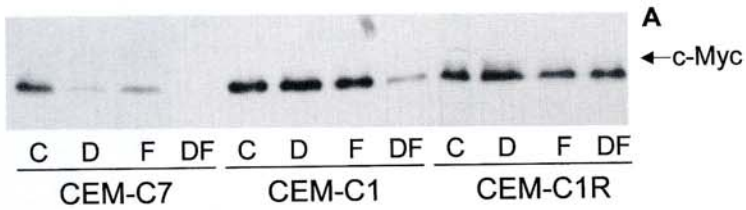


Fig. 5. Regulation of GR protein and binding activity by Dex and/or forskolin. *A*, expression of GR protein in CEM-C7, -C1, and -C1R cells treated with vehicle (*Lanes C*), Dex (*Lanes D*; 20 nM for C7 and 1 μM for C1/C1R), forskolin (*Lanes F*; 10 μM), or Dex plus forskolin (*Lanes DF*; 10 μM forskolin and 20 nM and 1 μM Dex for C7 and C1/C1R, respectively) was determined by Western blotting using the polyclonal antibody $AhuGR_{150-175}$ (35). Sixteen million cells were incubated for 24 h at 37°C in the appropriate agonist at a density of 8×10^5 cells/ml and harvested, and total cellular extracts were prepared as described in "Materials and Methods." Samples (20 μg protein per lane) were resolved on a 10% polyacrylamide gel and electroblotted. The blot was developed using the ECL protocol. *B*, densitometric quantitation of the intensity of bands corresponding to GR. *Columns*, percentages of C7 control. *C*, GR binding activity was determined in CEM-C7, -C1, and -C1R cells treated for 24 h with vehicle (□), Dex (▨; 20 nM for C7, 1 μM for C1/C1R), forskolin (▧; 10 μM), and Dex plus forskolin (▩; 10 μM forskolin and 20 nM and 1 μM Dex for C7 and C1/C1R, respectively). Cell suspensions were incubated for 1.5 h at 37°C with [^{3}H]Dex (150 nM; 86 Ci/mmol) in the absence (total) or presence (nonspecific) of a 500-fold molar excess of unlabeled Dex. After incubation, cells were washed three times with PBS, and radioactivity was determined by liquid scintillation counting. Specific binding was determined by subtraction of nonspecific from total binding. Sites/cell were calculated from hemacytometric determination of cell number after the final wash and specific activity of the radioligand. *Columns*, averages of duplicate assays; *bars*, SD.

number (54). Regulation of GR quantity by GC as well as PKA pathways has been documented previously (26, 55). Hence, we determined the effect of Dex and/or forskolin on GR protein and binding activity in CEM-C7, -C1, and -C1R cells. CEM-C1R cells were derived from CEM-C1 cells by repeated selection in 1 μM Dex plus 10 μM forskolin and are resistant to the combination. GR protein levels were determined by Western blot analysis (Fig. 5, *A* and *B*) using the polyclonal GR antibody $AhuGR_{150-175}$. C7 cells were treated with 20 nM Dex to maintain conditions that cause a submaximal apoptotic response to steroid alone in this steroid-sensitive population. The Dex-resistant C1 and C1R cells were treated with 1 μM Dex. In CEM-C7 cells, treatment with 20 nM Dex alone caused ~25% increase in GR protein, whereas 10 μM forskolin had no significant effect. In contrast, cotreatment with Dex plus forskolin decreased immunoreactive GR protein to ~70% of that in control C7 cells. The unstimulated C1 cells expressed only 30% as much immunoreactive GR as did basal C7 cells. Stimulation of C1 cells with 1 μM Dex, 10 μM forskolin, or 1 μM Dex plus 10 μM forskolin up-regulated GR protein 2.5–3-fold. CEM-C1R cells expressed twice as much basal immunoreactive GR protein as did C1 cells, and this level did not change upon treatment with 1 μM Dex. Ten μM forskolin caused a 45% increase in GR protein, and Dex plus forskolin caused a significant down-regulation (70% of C1R controls) of GR protein levels in C1R cells.

We also determined GR binding activity in cells treated with vehicle, Dex, forskolin, or Dex plus forskolin (Fig. 5*C*). Unstimulated CEM-C7, -C1, and -C1R cells exhibited 17,000, 4,000, and 3,200 sites/cell respectively. Treatment of CEM-C7 cells with 20 nM Dex and 10 μM forskolin caused 2.4- and 1.2-fold increases in GR binding activity, respectively. Simultaneous treatment with both agents resulted in only a 1.3-fold increase in binding activity. GR binding activity in untreated CEM-C1 cells is only one-fourth of that seen in untreated CEM-C7 cells. In CEM-C1 cells, even 1 μM Dex caused only a modest 1.3-fold increase in GR binding activity, whereas 10 μM forskolin elevated GR binding activity to 3.5 times control levels. Cotreatment with both agents resulted in GR binding activity that was ~2-fold higher than untreated CEM-C1 cells. CEM-C1R cells exhibited slightly lower basal binding activity than that of CEM-C1 cells. Treatment with 1 μM Dex resulted in a decrease in binding activity to ~60% of untreated cells. Forskolin treatment caused a 1.5-fold increase; however, cotreatment with Dex plus forskolin resulted in binding activity that was 80% of basal levels in these cells. The GR binding activity in our subcloned Dex-resistant CEM-C1-15 cells is comparable to that in sensitive CEM-C7-14 cells. Each contained ~10,000 sites per cell under basal conditions (data not shown). We conclude that no consistent pattern of GR protein or ligand binding correlated with efficiency of cell kill.



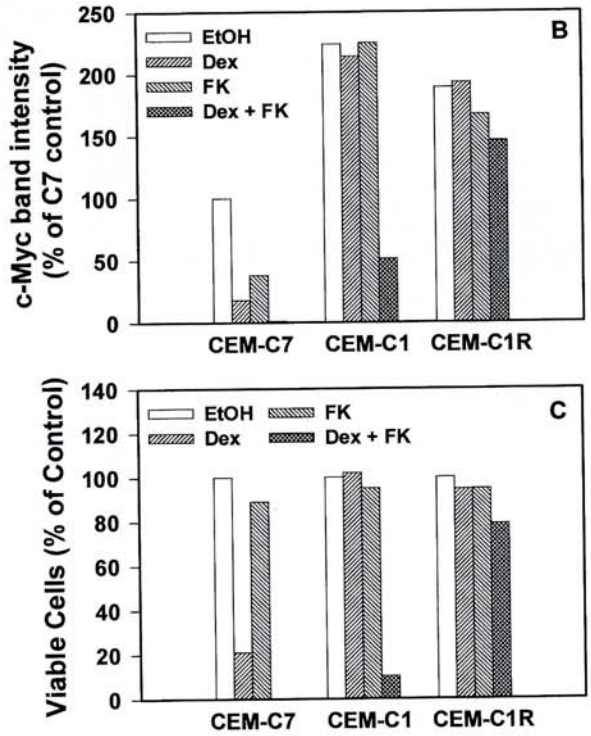


Fig. 6. Dex- plus forskolin-induced cell death is associated with suppression of c-Myc expression. *A*, CEM-C7, -C1, and -C1R cells (16 million at 8×10^5 cells/ml) were treated for 24 h at 37°C with vehicle (*Lanes C*), Dex (*Lanes D*; 20 nM for C7, 1 μM for C1/C1R), 10 μM forskolin (*Lanes F*), or Dex plus forskolin (*Lanes DF*; 10 μM forskolin and 20 nM and 1 μM Dex for C7 and C1/C1R, respectively). After incubation, cells were harvested and lysed by freeze/thaw cycles, and nuclear fractions were separated as described in "Materials and Methods." Nuclei were solubilized in SDS-PAGE sample buffer and centrifuged, and the nuclear extracts (30 μg protein/lane) were resolved on a 10% polyacrylamide gel. Resolved protein was electroblotted on PVDF membrane and visualized by immunostaining with the c-Myc monoclonal antibody Myc1–9E-10.2 and subsequent ECL reaction and autoradiography. A representative experiment is shown here. *B*, immunoblots from two independent experiments were quantitated for band intensity by densitometric analyses. The mean densitometric units for each condition were normalized against a mean C7 control value of 100%. *Columns*, percentages of C7 control. *C*, CEM-C7, -C1, and -C1R cells (1×10^5/ml) were treated for 96 h at 37°C with vehicle (□), Dex (▨; 20 nM for C7, 1 μM for C1/C1R), 10 μM forskolin (▧), or Dex plus forskolin (■; 10 μM forskolin and 20 nM and 1 μM Dex for C7 and C1/C1R, respectively). Viable cells were counted by trypan blue exclusion using a hemacytometer. *Columns*, means of duplicate treatments, presented as percentages of vehicle-treated controls.

Correlation of c-Myc Down-Regulation with Cell Sensitivity. In CEM-C7 cells, suppression of c-*myc* mRNA and c-Myc protein has been previously shown to be critical for GC-induced cell lysis (22). We, therefore, determined whether the cell death in CEM-C1 cells by Dex plus forskolin correlated with down-regulation of c-Myc protein. We also reconfirmed, as reported previously (22), that c-Myc protein is suppressed in CEM-C7 cells in response to Dex. CEM-C7 cells treated with 20 nM Dex for 24 h exhibited 80% down-regulation of c-Myc protein (Fig. 6*A*), and 10 μM forskolin alone caused a 60% down-regulation of c-Myc. When C7 cells were treated with both agents simultaneously, c-Myc became barely detectable. Band intensities in blots from two independent experiments were quantitated and averaged to generate Fig. 6*B*. Untreated C1 or C1R cells expressed approximately twice as much immunoreactive c-Myc protein as did control C7 cells. In various experiments, treatment of C1 or C1R cells with 1 μM Dex had little or no effect on c-Myc protein. Ten μM forskolin caused no significant inhibition of c-Myc expression in each case. Cotreatment with Dex plus forskolin at the same concentrations used for individual treatments significantly down-regulated c-Myc in C1 cells to 20% of C1 control but had only slight effect on c-Myc levels in C1R cells (80% of control). Fig. 6*C* shows the relative numbers of viable cells, compared to time-matched controls, seen in C7, C1, and C1R cells in response to 4 days of treatment with Dex and/or forskolin, and demonstrates that the c-Myc suppression occurring after 24 h treatment precedes and correlates with cell death seen after 96 h. That there was only a minimal apoptotic response (~20%) in C1R cells treated with Dex plus forskolin was confirmed by TUNEL assay (data not shown).

To evaluate how the extent of c-Myc down-regulation correlates with the degree of apoptosis, we determined the effect of very low concentrations of forskolin (in the presence and absence of 1 μM Dex) on c-Myc protein levels and viable cell number in CEM-C1 cells (Fig. 7). Fig. 7, *A* and *B*, shows that treatment with 1 μM Dex causes a 20% decrease in c-Myc immunoreactive protein. Treatment with 0.1, 1, or 5 μM forskolin alone did not cause any significant decrease in c-Myc protein levels. In the presence of 1 μM Dex, 0.1, 1, and 5 μM forskolin caused ~40, 45, and 80% suppression of c-Myc, respectively, which approximately correlated with the 25, 50, and 80% reduction in the number of viable cells at these concentrations, compared to time-matched controls (Fig. 7*C*) seen after 96 h of treatment. Dex (1 μM) alone did not significantly affect viable cell numbers (90% of control) and had little effect on c-Myc levels.

Effect of Dex and/or Forskolin on the cAMP Response Pathway. To begin an evaluation of the mechanism of Dex-forskolin synergism, we performed experiments to determine whether Dex potentiated the cAMP response pathway in either CEM-C7-14 or

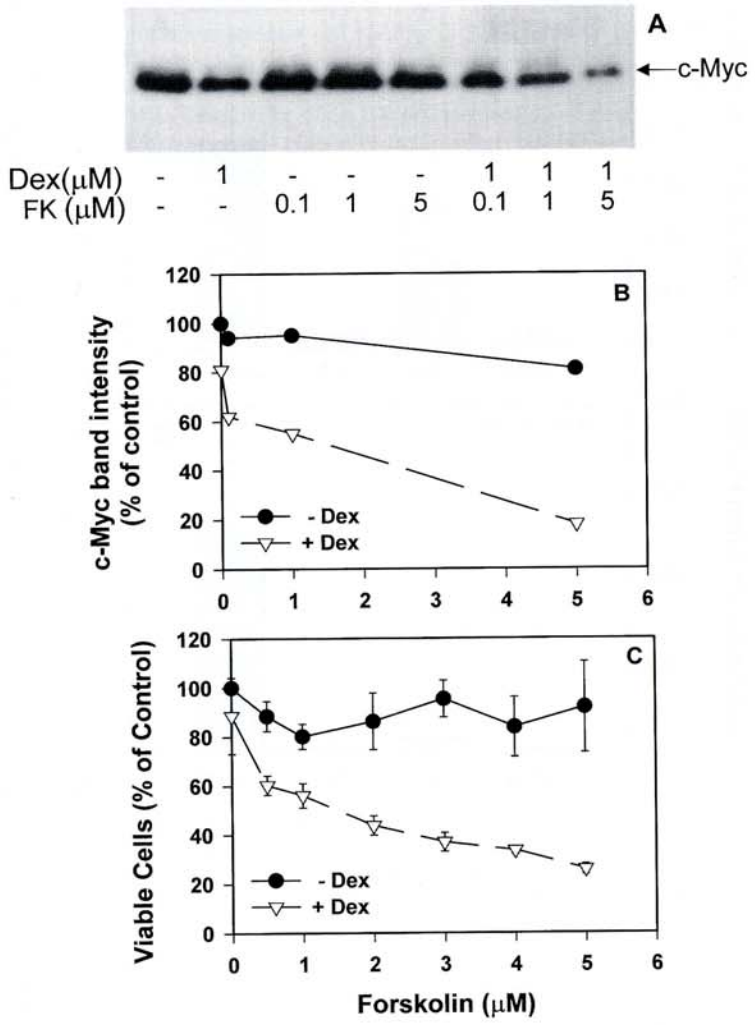


Fig. 7. Down-regulation of c-Myc is dose dependent with respect to forskolin and correlates with extent of cell death. *A*, CEM-C1 cells (16 million at 8 × 10^5/ml) were incubated for 24 h at 37°C with 0.1, 1, or 5 μM forskolin in the absence (*Lanes −*) or presence (*Lanes 1*) of 1 μM Dex (*Dex*). Nuclear extracts of harvested cells were prepared as described in the legend to Fig. 6*A*, and 30 μg of nuclear protein from each sample was resolved on a 10% polyacrylamide gel and subsequently electroblotted on to PVDF membrane. c-Myc expression was determined by immunoblot analysis using the c-Myc monoclonal antibody Myc1–9E-10.2. A representative blot is shown here. *B*, data from densitometric analysis of blots from two independent experiments were averaged and are expressed as the percentage of untreated control. The effects of varying concentrations of forskolin on c-Myc expression in the absence (●) or presence (▽) of 1 μM Dex are shown. *C*, CEM-C1 cells were treated for 96 h at 37°C with sublethal concentrations of forskolin (0.5–5 μM) in the absence (●) or presence (▽) of 1 μM Dex. Trypan blue-excluding cells were counted using a hemacytometer and are expressed as a percentage of untreated controls. *Data points*, means of triplicate treatments; *bars*, SD.

Table 1 *Effect of Dex and/or forskolin on cAMP levels in CEM cells*

Data presented here are averages ± SE; numbers in parentheses indicate numbers of replicates.

	cAMP (fmol/2 × 10^6 cells)	
	CEM-C7-14	CEM-C1-15
Ethanol	155 ± 109 (4)	153 ± 80 (4)
1 μM Dex	190 ± 124 (4)	144 ± 127 (4)
10 μM forskolin	56,250 ± 8,750 (2)	52,500 ± 2,500 (2)
1 μM Dex + 10 μM forskolin	48,750 ± 13,750 (2)	42,500 ± 2,500 (2)
10 μM 1,9-dideoxy forskolin	ND[a]	275 ± 25 (2)
1 μM Dex + 1,9-dideoxy forskolin	ND	190 ± 10 (2)

[a] ND, not determined.

CEM-C1-15 cells. These studies were performed in cells of the fresh subclones C7-14 and C1-15 to avoid complications of potentially mixed responses from any subclonal diversity in the original cultures. As described in "Materials and Methods," these two clones were selected for the study because they most closely resembled, in GR content and ploidy, the originally isolated CEM-C1 and CEM-C7 clones (3, 41). A 1-h treatment with 10 μM forskolin caused about 300-fold increase in intracellular cAMP levels in both C7-14 and C1-15 cells (Table 1). Treatment with 1 μM Dex did not significantly affect basal or forskolin-induced cAMP levels. An analogue of forskolin, 1,9-dideoxy forskolin, that does not activate adenylate cyclase did not affect cAMP levels, nor was it able to restore Dex sensitivity to CEM-C1-15 cells (data not shown). The level and extent of Ser-133 phosphorylation of CREB, the downstream effector of the cAMP response pathway, were evaluated by Western blotting. Expression of immunoreactive CREB protein was similar in both CEM-C7-14 and CEM-C1-15 cells and was not affected by 18 h treatment with either

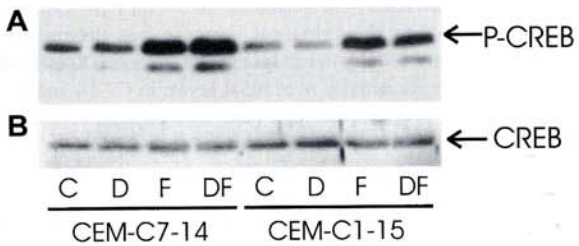


Fig. 8. Regulation of CREB expression and phosphorylation by forskolin and/or Dex. CEM-C7-14 and CEM-C1-15 cells were treated for 1.5 (*A*) or 18 (*B*) h at 37°C with vehicle (*Lanes C*), 1 μM Dex (*Lanes D*), 10 μM forskolin (*Lanes F*), or Dex plus forskolin (*Lanes DF*). Cells were harvested and extracted as described in "Materials and Methods," and a volume corresponding to 50 μg protein from each extract was resolved on a 10% polyacrylamide gel and transferred to PVDF membranes. P-CREB (*A*) and CREB (*B*) levels were determined by probing membranes with specific polyclonal antibodies, as detailed in "Materials and Methods," and by subsequent ECL reaction and autoradiography. A representative of two independent experiments is shown here.

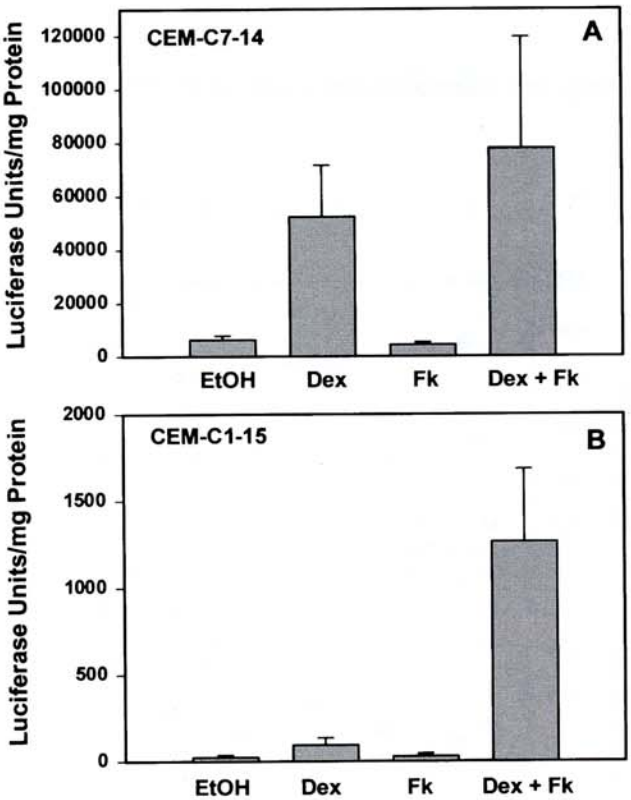


Fig. 9. Potentiation of Dex-induced GR transcriptional activity by forskolin. Mass cultures of CEM-C7-14 (*A*) and CEM-C1-15 (*B*) cells stably transfected with pHHLuc were treated for 18–20 h with ethanol, 1 μM Dex, 10 μM forskolin, or 1 μM Dex plus 10 μM forskolin. The cell extracts were prepared and analyzed for luciferase activity and protein content. Luciferase activity was normalized for protein content. *Columns*, means from three independent experiments; *bars*, SD.

Dex or forskolin, alone or in combination (Fig. 8*B*). Treatment with 10 μM forskolin for 90 min caused a 4–5-fold increase in Ser-133 phosphorylation of CREB in both cell types, whereas treatment with 1 μM Dex did not affect basal or forskolin-induced CREB phosphorylation significantly (Fig. 8*A*). CEM-C1-15 cells appeared to have slightly lower basal and induced levels of P-CREB, in comparison to CEM-C7-14 cells.

GR-mediated Transcriptional Activity from MMTV-LTR. To evaluate the ability of forskolin to modulate the GR transcriptional response, mass cultures of CEM-C7-14 and CEM-C1-15 cells stably transfected with pHHLuc were treated for 20 h with 1 μM Dex and/or 10 μM forskolin. The transcriptional response was determined by measuring luciferase activity in cell lysates. Dex induced 8- and 4-fold increases in luciferase activity over basal levels in C7-14 and C1-15 cells, respectively (Fig. 9), although basal transcription efficiency of CEM-C1-15 mass cultures was much lower than CEM-C7-14 mass cultures. Forskolin did not have any effect of its own, but it potentiated the Dex-induced response. In CEM-C7-14 cells, the combined treatment caused a 12-fold increase in luciferase activity, which was only slightly greater than that seen with Dex alone. Combined treatment of CEM-C1-15 cells sensitized them to a strong GR-mediated transcriptional response, causing a 55-fold induction, which was 13 times more than that seen with Dex alone. These experiments clearly demonstrate that CEM-C1-15 cells evoke a weak transcriptional response to Dex alone, which can be strongly potentiated by coactivation of the PKA pathway.

DISCUSSION

The data presented here demonstrate that, when the GC- and PKA-mediated pathways are simultaneously activated, they complement each other to trigger apoptosis in CEM-C1 cells, which do not show apoptosis following activation of either pathway individually (Fig. 1*B*). We believe ours to be the first report, however, that documents the restoration of apoptosis in an apoptosis-resistant cell line by interaction between the two signal transduction pathways. Microscopic observation and flow cytometric analysis (Fig. 2) of Dex-plus forskolin-treated CEM-C1 cells clearly depicts the apoptotic nature of the cell death, characterized by cell shrinkage, membrane blebbing, formation of apoptotic bodies, and DNA strand breaks. Fig. 1*A* demonstrates that, in the GC-sensitive CEM-C7 cells as well, the PKA activator forskolin significantly potentiates the lytic response induced by suboptimal levels of Dex. Synergism between GC and cAMP analogues has been demonstrated previously in situations in which one or both pathways were strongly functional, *e.g.*, GCs and cAMP synergistically activate transcription of the phosphoenolpyruvate carboxykinase (56) and vasopressin (57) genes. cAMP has also been shown to potentiate GC-induced apoptosis in GC-sensitive cells (23, 29, 30).

Using the GR antagonist RU 38486, we have demonstrated the requirement for functional GR for the synergized apoptotic response (Fig. 4). This is in agreement with previous reports that have shown that cells lacking functional GR are resistant to apoptosis (5, 9). A recent study has demonstrated that a CEM-derived clone, ICR-27, which lacks functional GR, can be sensitized to cAMP by transfection with a GR expression vector (58). In our CEM-C1 cells, activation of PKA did not stimulate the latent agonist activity of RU 38486, as was reported in another system by Nordeen *et al.* (25).

cAMP has been suggested to up-regulate GR protein and binding activity. In a rat hepatoma cell line expressing low levels of functional GR, cAMP restored GC-induced transcriptional activity (59). The authors proposed that a "threshold" level of GR expression is required for GC responsiveness and that cAMP elevates GR levels above this threshold. A number of reports have correlated the level of GR expression to the extent of GC responsiveness (9, 10). In CEM-C1 cells (provided by our laboratory), Geley *et al.* (54) have shown that transfection with a GR expression vector is sufficient to restore GC responsiveness to several subclones. In our laboratory, subclones from the original CEM-C1 line have been derived that express high levels of GR yet remain unresponsive to GC.[4] Thus, it seems that a functional block in GR rather than simply a lack of quantity of GR account for the resistance of at least some CEM-C1 cells.

To determine whether forskolin-induced up-regulation of GR is the mechanism by which apoptosis is triggered in the presence of Dex plus forskolin in these cells, we determined the levels of GR protein and binding activity after stimulation with these agents. Our data demonstrate that there is no direct correlation between the extent of apoptosis and the expression of immunoreactive GR protein or GR-binding activity. Because of the presence of an allele for a functionally defective GR gene (L753F) in both C7 and C1 cells (5), immunoreactive GR protein may not represent functionally active receptor. Hence, the extent of induction of binding activity (Fig. 5*C*) in Dex and/or forskolin treated cells did not always correlate with the extent of increase in immunoreactive protein. In CEM-C1 cells (unlike CEM-C7 cells), forskolin, alone or in combination with Dex, caused a significant increase in GR protein and binding activity compared to basal levels. This up-regulation may raise GR levels above a threshold as proposed by Okret *et al.* (59). However, GR protein and binding activity in doubly treated CEM-C7 cells is significantly lower than that of CEM-C7 cells treated with Dex alone. Yet doubly treated C7 cells exhibit far greater apoptosis than do those treated with Dex alone,

suggesting that forskolin potentiates GC-induced apoptosis by triggering a response distinct from its ability to up-regulate GR protein/binding activity. CEM-C1R cells expressed immunoreactive GR protein at levels comparable to CEM-C1 cells; however, they exhibited very low binding activity, which was not significantly up-regulated by Dex and/or forskolin. Thus these cells may, indeed, be resistant to apoptosis because of subthreshold levels of functional GR expression.

Compared to the sensitive subclone CEM-C7-14, GR-mediated transcriptional activity, based on activation from a 330-bp MMTV promoter fragment, however, was found to be greatly reduced in CEM-C1-15 cells (Fig. 9), a C1 subclone that has a normal number of GR binding sites but is highly resistant to Dex. This may partially be because of poor selection of CEM-C1-15 cells in Geneticin, owing to their inherently greater resistance to the drug. Because these are uncloned mass cultures, it is not possible to accurately estimate/compare transcriptional efficiency of the two cell types. Yet, it is clear that, in CEM-C1-15 cells, coactivation of the PKA pathway complemented the GR-transcriptional response pathway to restore significant transcriptional activity from the MMTV promoter. Along with previous reports, these data suggest that a component of the GR-transcriptional complex is lacking in Dex-only-treated cells and is replenished by coactivation of the PKA pathway. On the other hand, forskolin may down-regulate/inhibit a repressor that blocks basal and Dex-induced transcription in CEM-C1-15 cells. Our experiments do not rule out the possibility that this is a promoter-specific effect. In other systems, a similar potentiation of Dex-induced transcriptional activation by forskolin from the MMTV promoter has been reported (25, 60).

CREB/P-CREB, the downstream mediators of cAMP response, are known to contribute to the transcriptional activity of the GR. Studies shown here (Fig. 8) demonstrate that CREB expression or phosphorylation are not significantly different in CEM-C7-14 and CEM-C1-15 cells. Moreover, Dex and forskolin did not evoke a synergistic response on CREB phosphorylation, as has been reported in WEHI-7.2 cells (61).

A striking response in CEM cells that correlates with Dex-induced lysis is the suppression of c-*myc*. Only those clones that are sensitive to Dex-induced lysis exhibit c-*myc* down-regulation in response to Dex. In other cell culture models of apoptosis, like S49 and B-precursor Reh cells, cAMP has been shown to cause a decline in c-*myc* mRNA levels, with associated G_1 arrest (62, 63). However, constitutive overexpression of c-Myc alone did not relieve Reh cells from cAMP-mediated growth arrest (64), suggesting that another gene product may be required as well. Rescue of P1798 cells from Dex-evoked growth arrest required coexpression of c-Myc and cyclin D_3 (36). Our results (Fig. 6) indicate that C1 and C1R cells express higher levels of immunoreactive c-Myc than do C7 cells. Dex or forskolin administered singly down-regulated c-Myc in C7 cells but strongly synergized to lower c-Myc to virtually undetectable levels. In correlation with their sensitivity to apoptosis, C1 cells exhibit a dramatic down-regulation of c-Myc after treatment with Dex plus forskolin, whereas C1R cells show a minimal change in c-Myc levels. Because c-Myc down-regulation precedes cell death, these data are consistent with our hypothesis that such suppression is an important event in the CEM cell lytic pathway. CEM-C1 cells seem to lack the GR-mediated transcriptional repressive function in addition to its transactivation function. On the other hand, GR-mediated transcription of a repressive protein may be required for c-Myc down-regulation.

In CEM-C7 cells treated with forskolin only, c-Myc is down-regulated to ~40% of control levels without concomitant apoptosis (Fig. 6). Hence, the level of c-Myc expression in each individual cell rather than the process of suppression seems to be important, consistent with our hypothesis that sustained depression of c-Myc below a certain threshold level is involved in the apoptosis of these cells. The reduced level of c-Myc expression in forskolin-treated C7 cells but not in Dex-treated C7 cells or doubly treated C7 and C1 cells seems sufficient to maintain cell viability. It is also possible that forskolin stimulates an "apoptosis-preventive" factor, which protects forskolin-treated CEM-C7 cells from apoptosis despite subthreshold levels of c-Myc. Coactivation of the GR pathway would then be postulated to inhibit/repress this preventive factor, triggering cell death.

We determined whether the average extent of c-Myc suppression in response to Dex plus forskolin correlated with the degree of cell death in C1 cells, using low concentrations of forskolin (Fig. 7). These data show a correlation between the two responses, with increasing extent of cell death being associated with greater average down-regulation of c-Myc. In the presence of Dex, at a concentration of forskolin (1 μM) that caused a 45% decrease in average c-Myc levels, a significant (50%) reduction in viable cell number occurred. This is interesting, because the level of c-Myc attained with this drug combination in CEM-C1 cells is higher than the average level of c-Myc expression in CEM-C7 cells treated with forskolin only (Fig. 6). Those cells are viable. This seems therefore to contradict the "minimal level of c-Myc for viability" hypothesis. There are at least three possible explanations that may reconcile the data within the hypothesis. First, the threshold in CEM-C1 and CEM-C7 cells may differ. Although C1 and C7 cells are "sister" clones, they came as parallel clones from the original uncloned CEM line. Therefore, they probably do not share the same immediate parent and may differ somewhat genotypically and phenotypically. Second, c-Myc levels on immunoblots are averages from millions of pooled cells. The c-Myc protein is expressed throughout the cell cycle in growing cells, but in varying amounts. The hormonally induced down-regulation we observed must be superimposed on this pattern. The hallmark of apoptosis is its random appearance in individual cells. It may well be that when the c-Myc levels in a specific cell drops below the required threshold, that cell dies. Only cell-by-cell analysis can determine whether this is so, and such experiments are underway. Third, the cAMP pathway may not only be permissive for the GC mechanism but may also have independent effects, so that, in addition to the c-Myc regulation, other events that facilitate apoptosis are occurring in the cells treated with two compounds. We note that very high levels of forskolin are lethal in C1 cells, given a background of constant Dex. Our data suggests that C1 cells have lost the ability to down-regulate c-*myc* in response to either Dex or forskolin. This may be because of the loss of an essential factor along the c-*myc* regulatory pathway. Coactivation of both pathways seems to complement this defect.

In conclusion, our studies demonstrate, for the first time, that an interaction between two distinct signal transduction pathways restores sensitivity to apoptosis in cells that are resistant to both pathways individually. This observation may have significant implications for the treatment of cancers that are refractory to conventional therapy.

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