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Type 4 cAMP phosphodiesterase (PDE4) inhibitors augment glucocorticoid-mediated apoptosis in B cell chronic lymphocytic leukemia (B-CLL) in the absence of exogenous adenylyl cyclase stimulation

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Abstract

cAMP-mediated signaling potentiates glucocorticoid-mediated apoptosis in lymphoid cells, but an effective means by which to take advantage of this observation in the treatment of lymphoid malignancies has not been identified. The primary objective of the current study was to determine whether PDE4 inhibitors, a class of compounds in late clinical development that raise intracellular cAMP levels by inhibiting type 4 cyclic nucleotide phosphodiesterases (PDE4), increase the efficacy of glucocorticoid-mediated apoptosis in leukemic cells from patients with B cell chronic lymphocytic leukemia (B-CLL). Rolipram, a prototypic PDE4 inhibitor, synergized with glucocorticoids in inducing B-CLL but not T cell apoptosis. Rolipram also augmented glucocorticoid receptor element (GRE) transactivation in B-CLL cells. In contrast, inhibition of protein kinase A (PKA) with the cAMP antagonist Rp-8Br-cAMPS reversed both glucocorticoid-induced apoptosis and GRE transactivation. CCRF-CEM cells, a well-studied model of glucocorticoid and cAMP-induced apoptosis, differed from B-CLL cells in that stimulation of adenylyl cyclase with the diterpene forskolin was required to increase both glucocorticoid-mediated apoptosis and GRE activation, while PDE4 inhibition had no effect. Consistent with these results, inhibition of PDE4 induced cAMP elevation in B-CLL but not CCRF-CEM cells, while forskolin augmented cAMP levels in CCRF-CEM but not B-CLL cells. While rolipram treatment up-regulated PDE4B in B-CLL, forskolin treatment up-regulated PDE4D in CCRF-CEM cells. These studies suggest that PKA is required for and enhances glucocorticoid-induced apoptosis in B-CLL by modulating glucocorticoid receptor signal transduction. Clinical trials that examine whether PDE4 inhibitors enhance the efficacy of glucocorticoid-containing chemotherapy regimens in B-CLL are indicated.

Keywords: Glucocorticoid therapy; B-CLL; PDE4 inhibitors; cAMP; PKA; GRE

1. Introduction

Specific subsets of normal and malignant B and T lineage lymphoid cells are unique in their sensitivity to the induction of apoptosis by agents that increase intracellular levels of the second messenger cAMP [1–3]. The same subsets of lymphoid cells are unusually sensitive to the induction of apoptosis by glucocorticoids [4–6]. Several groups have identified similarities in the signaling pathways activated by these two stimuli in such cell types. Early studies demonstrated that certain genes were up-regulated in lymphoid cells by both glucocorticoids and cAMP analogs [7]. Subsequent studies by McConkey and co-workers demonstrated that in CCRF-CEM cells, a human lymphoid cell line derived from a patient with T-ALL, loss of glucocorticoid receptor (GR) led to loss of sensitivity to cAMP-induced apoptosis [8]. Glucocorticoid

Abbreviations: B-CLL, B cell chronic lymphocytic leukemia; PDE4, type 4 cAMP phosphodiesterase

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and protein kinase A (PKA) signaling pathways have also been shown to synergize in inducing apoptosis in glucocorticoid-resistant CCRF-CEM cells [9–11]. Interestingly, cAMP-mediated potentiation of glucocorticoid-induced apoptosis has been reported to be independent of cAMP response element (CRE)-associated transcriptional activation [12]. Most recently, the catalytic subunit of PKA was found to associate with the GR [13].

Although the studies noted above suggest that cAMP-mediated apoptosis in lymphoid cells may be mediated through the GR, the mechanism by which glucocorticoids themselves induce lymphoid apoptosis remains unclear. GR signaling both positively and negatively regulates transcription. While positive regulation of gene transcription is mediated through palindromic GRE elements, several mechanisms for negative regulation of gene transcription by the GR have been described including negative GREs, composite elements and tethering [14–16]. Surprisingly, most of the clinically beneficial activities of glucocorticoids, such as inhibition of lymphoid proliferation and inflammatory cytokine secretion, appear to be mediated by a tethering mechanism, in which GR suppresses NFκB or AP1-mediated transcription in a manner independent of the ability of the GR to bind to DNA itself [17].

The majority of studies examining glucocorticoid and cAMP-mediated apoptosis have utilized leukemic cell lines as the experimental model. Primary leukemic cells differ in numerous ways from such immortalized cell lines, most strikingly in that primary cells fail to proliferate to any significant degree in tissue culture. In this study, we have performed parallel studies of GC and cAMP-induced apoptosis in freshly isolated B-CLL cells and the CCRF-CEM cell line. To stimulate PKA signaling, we have utilized inhibitors of type 4 cAMP phosphodiesterase (PDE4), the predominant cAMP PDE in lymphoid cells [18–20]. Cyclic nucleotide phosphodiesterases (PDEs) are a diverse group of 11 or more enzyme families that catabolize cAMP and/or cGMP [21,22]. PDE4 phosphodiesterases are derived from four genes, PDE4A–PDE4D, each of which generates a variety of PDE4 isoforms as a result of alternative splicing in their amino termini [23,24]. As a result of differential expression and subcellular localization, PDE4 isoforms vary in their signal transduction properties. In some instances, differential localization results from association of distinct splice isoforms of PDE4 with A-kinase-anchoring proteins (AKAPs) which tether PKA holoenzyme along with some PDE4 splice isoforms and other associated proteins into a signaling complex [25].

Our prior studies have demonstrated that rolipram, a prototypic PDE4-specific inhibitor, induces apoptosis in B-CLL cells but not peripheral blood T cells, by a mitochondrial pathway and in a PKA-dependent manner [26–29]. Here, we report that in B-CLL cells, PDE4 inhibitors synergize with glucocorticoids to induce apoptosis and transactivate GRE-containing reporter constructs in the absence of exogenous adenylyl cyclase activation.

2. Materials and methods

2.1. Materials

The following reagents were obtained from commercial sources: cilostamide and rolipram (Calbiochem); forskolin, 1,9-dideoxyforskolin, phenazine methosulfate (PMS) (Sigma); Hoechst 33342 and $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) (Molecular Probes); MTS and St-Ht31 AKAP inhibitor peptide (Promega); RO20-1724 (Biomol), (R_p)-8-Br-cAMPS (Biolog).

2.2. Patient selection

Blood samples were obtained by IRB-approved consent from flow cytometry-confirmed B-CLL patients that were either untreated or for whom at least 1 month had elapsed since chemotherapy. Patients with active infections or other serious medical conditions were not included in this study.

2.3. Cell purification and culture

CCRF-CEM cells were obtained from ATCC [31]. Leukemic or normal mononuclear cells were obtained by centrifugation over Histopaque 1077 (Sigma). For purification of T cells, whole mononuclear cells from normal subjects were incubated with magnetic beads coated with appropriate antibodies, then positively purified using a magnet (Miltenyi). Cells were cultured in RPMI 1640 media (Biowhittaker) supplemented with 10% fetal calf serum, 50 μmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 10 mM Hepes pH 7.4, 100 μg/ml penicillin, and 100 U/ml streptomycin (Sigma). For isolation of glucocorticoid resistant clones, parental CCRF-CEM cells were treated with 1 μM dexamethasone for 10 days, and surviving cells diluted to $\leq$1 cell/well in a 96-well flat-bottom tissue culture plate, grown for 3 weeks in media supplemented with 20% fetal calf serum and 1% insulin–transferrin–selenium (GIBCO), and then transferred to regular growth medium. A glucocorticoid-resistant subclone (CEM-R8) was completely resistant to dexamethasone-induced apoptosis up to at least 10 μM dexamethasone. For isolation of glucocorticoid-sensitive clones, the same procedure was used, except that treatment with dexamethasone was omitted. A glucocorticoid-sensitive subclone (CEM-S2) was inhibited in its survival by dexamethasone with an IC_{50} = 0.007 μM.

2.4. Apoptosis and cell survival assays

Hoechst 33342 and $DiOC_6(3)$ apoptosis assays were performed as previously described [28,32]. For CCRF-CEM cell survival assays, cells were plated at a density of 3×10^4/well in 96-well flat-bottom plates in the presence of test reagents or vehicle in 0.1 mL media. Following

incubation for 72 h, 20 μl of a 20:1 MTS (2 mg/mL)/PMS (0.92 mg/ml) solution was added to each well, and the plates incubated for 2 h at 37°. The absorbance (O.D.) of the formazan product was determined at 492 nm using a plate reader (Titertek Multiscan Plus, Labsystems). Percent cell viability was calculated as follows: (O.D. control sample − O.D. blank)/(O.D. test sample − O.D. blank) ×100. All assays were performed in triplicate.

2.5. Transfection technique

An MMTV GRE luciferase construct, originally created by Dr. R. Evans, was a gift from Dr. Remco Spanjaard (Department of Otolaryngology, Boston Medical Center) [33]. One hundred million B CLL cells in 200 μl of PBS were added to 500 μl of Nucleofector Solution D containing 30 μg of endotoxin-free (Qiagen) MMTV luciferase constructs. One hundred microliters was used per transfection using a Nucleofector electroporation instrument (Amaxa Biosystems) using program U16. Pilot experiments with a GFP expression vector demonstrated 20% transfection efficiency and 50% cell survival at 18 h. Up to six such aliquots of transfected cells were diluted in media and pooled, prior to distribution of 8 million cells/well in 48 well plates. Two hours after transfection, drugs, cytokines or vehicle alone were added to the transfected cells. Fourteen hours after transfection, samples were processed using a luciferase assay kit (Promega). Triplicate samples were analyzed with an MGM Instruments Optocomp I luminometer. Analysis of cell viability (trypan blue exclusion) and total protein (Pierce) verified that equivalent numbers of viable B-CLL cells were analyzed. One hundred million CCRF-CEM cells were similarly transfected, cultured and analyzed except that Nucleofector program O17 was utilized. Pilot experiments with a GFP expression vector demonstrated 50% transfection efficiency and viability. Data were analyzed using a two-sided paired *t*-test for means.

2.6. cAMP assay

Half a million B-CLL or CCRF-CEM cells were incubated for 30 min in 1 mL media alone or with the addition of drugs. Cells were centrifuged and lysed in 80% ethanol. After vortexing and incubation on ice for 10 min, cellular debris was removed by re-centrifugation. The supernatant was dried in a Speedivac and the sample brought up in 250 μL of sample buffer as provided by the makers of a cAMP RIA kit (Amersham). One hundred microliters of this sample was used for each cAMP assay. The RIA kit was used according to the manufacturer's instructions.

2.7. Western analysis

Thirty million B-CLL cells or 10 million CCRF-CEM cells were incubated for 18 h in media alone or with the addition of drugs as indicated in the text. Western analysis was then carried out as previously described [34]. The PDE4A (66C12H), PDE4B (96G7A) and PDE4D (61D10E) antibodies, each of which recognize all splice isoforms derived from that PDE4 gene, were kind gifts from Dr. Sharon Wolda (ICOS) and have been previously described [34,35].

2.8. Statistical analysis

Data are reported as the mean ± S.E. Comparisons between multiple groups were performed using single factor ANOVA and secondary comparisons were performed using Duncan's test.

3. Results

3.1. PDE4 inhibitors synergize with glucocorticoids to induce apoptosis in B-CLL but not T cells

To examine the effect of PDE4 inhibition on glucocorticoid-induced apoptosis in B-CLL, leukemic cells were cultured for 48 h with media alone, the PDE4-specific inhibitor rolipram, the glucocorticoid hydrocortisone, or the two drugs in combination, followed by assessment for apoptosis (Fig. 1A). A steep dose–response curve for hydrocortisone-induced apoptosis was observed between 0.1 and 10 μM, with little additional effect of hydrocortisone concentrations above 10 μM (Fig. 1B). Subsequently, the effect of combining a glucocorticoid with a PDE4 inhibitor was tested at 1 μM hydrocortisone.

Patient to patient variability was observed both for basal and drug-induced apoptotic rates. However, for all of the 11 leukemic cell samples examined, treatment with a combination of rolipram and hydrocortisone induced a higher percentage of apoptotic B-CLL cells than either agent alone. In patients with higher basal levels of apoptosis (patients 7–11) combined treatment with the two agents induced >65% apoptosis, but this was less than additive of the apoptosis induced by each agent alone (Table 1). In contrast, in the six patients with less than 68% apoptosis (patients 1–6) following combined therapy, treatment with both agents induced a supra-additive or ''synergistic'' effect (Table 1). It is likely that the lack of synergy observed in the leukemic cell samples with high levels of apoptosis following combined treatment reflects the plateau at 75–85% maximal apoptosis we have observed in B-CLL cells, regardless of the apoptotic stimulus examined, using these in vitro apoptosis assays. Of note, basal rates of apoptosis in the leukemic cells did not correlate significantly with a prior history of chemotherapy in the six untreated and five previously treated B-CLL patients in this study.

To verify that addition of a PDE4 inhibitor augmented killing of B-CLL cells even when maximally effective

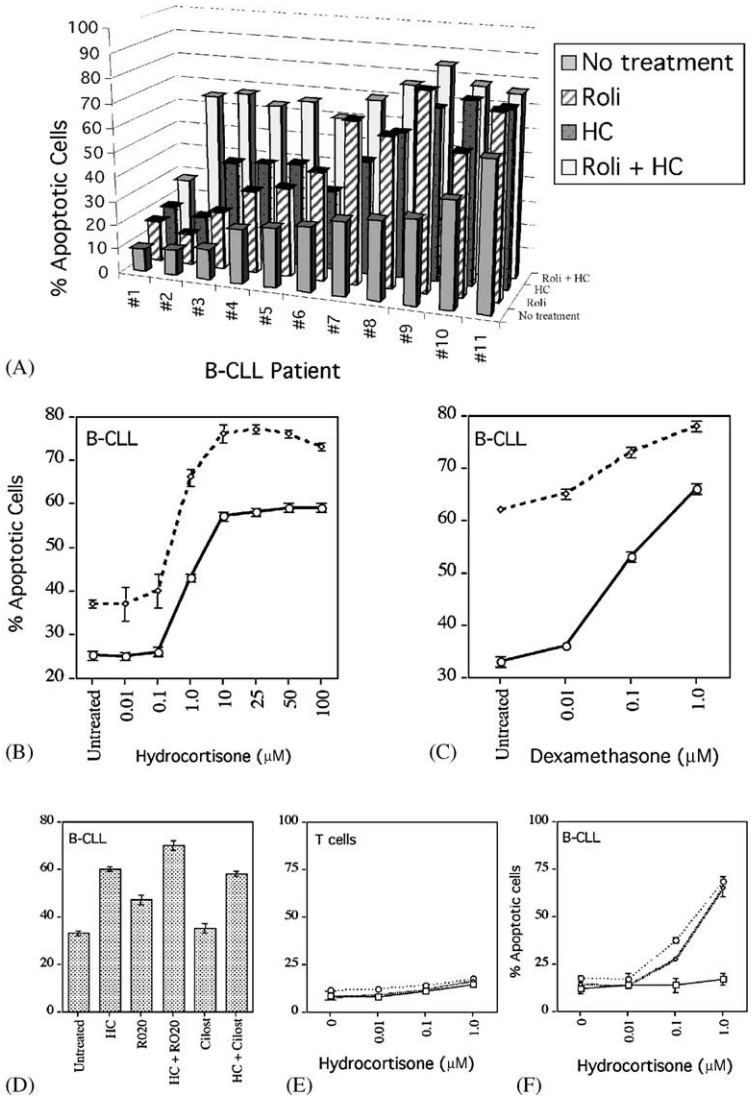

Fig. 1. Effects of the cyclic nucleotide phosphodiesterase inhibitors rolipram, RO20-1724 and cilostamide on hydrocortisone and dexamethasone-induced apoptosis in B-CLL and normal T cells. (A) Leukemic cells from 11 B-CLL patients were assessed for apoptosis by Hoechst 3342 analysis after 48 h culture in media alone (no treatment), 10 μM rolipram (Roli), 1 μM hydrocortisone (HC) or a combination of the two drugs (Roli + HC). The clinical characteristics of the B-CLL patients are summarized in Table 1. (B and C) Leukemic cells from patients with B-CLL were cultured in media alone or with a dose titration of hydrocortisone (B, solid line) or dexamethasone (C, solid line), followed by assessment for apoptosis by Hoechst 33342 FACS analysis at 48 h. Using the same experimental conditions, the effect of addition of 10 μM rolipram (thick dashed line) to the glucocorticoids was also assessed. (D) The apoptotic effects of the PDE4 inhibitor RO20-1724 (RO20) (10 μM) or the PDE3 inhibitor cilostamide (Cilost) (10 μM) were assessed on B-CLL cells when combined with 1 μM hydrocortisone. (E) Magnetic bead purified peripheral blood T cells were cultured in media alone or with a dose titration of hydrocortisone (solid line), followed by assessment for apoptosis. Using the same experimental conditions, the effect of addition of 10 μM rolipram (thick dashed line) or 10 μM rolipram and 40 μM forskolin (dotted line) was also assessed. (F) B-CLL cells were analyzed as for (E). The S.E.M. of triplicate samples is shown; where not visible, the S.E.M. was less than 1.0%.

doses of glucocorticoids were utilized, rolipram was added to leukemic cell samples treated with hydrocortisone concentrations as high as 100 μM. In five patients so analyzed, addition of 10 μM rolipram augmented the induction of apoptosis by 40 ± 18% relative to the increment in apoptosis induced with 100 μM hydrocortisone alone (Fig. 1B). Parallel experiments utilizing $DiOC_6(3)$ to examine mitochondrial depolarization in B-CLL cells treated with glucocorticoids and rolipram confirmed the results obtained above using the Hoechst 33342 apoptosis assay (data not shown).

To generalize these results, we next examined the effects of combined therapy with other drugs within these two classes of compounds. When rolipram was added to the glucocorticoid dexamethasone, the combined treatment once again augmented B-CLL apoptosis beyond the levels observed with either agent alone (Fig. 1C). A structurally distinct PDE4 inhibitor, RO20-1724 (10 μM), also augmented hydrocortisone-induced apoptosis in leukemic cells from the five B-CLL patients tested (Fig. 1D). In contrast, despite prior documented expression of PDE3 in B-CLL cells, inhibition of this PDE with the selective

Table 1
Analysis of hydrocortisone and rolipram-induced apoptosis in leukemic cells from 11 B-CLL patients

Patient number	Stage	Prior therapy	WBC	No Rx	Roli	HC	R/HC	Add.
1	1	No Rx	13	10	17	19	27	26
2	3	C, F, R, Cy, St	295	11	13	16	65	18
3	4	CHOP, F	87	13	24	41	67	52
4	3	F	228	23	34	42	63	53
5	3	C, F, R, St	66	25	37	43	66	55
6	1	No Rx	30	27	45	33	60	51
7	1	No Rx	15	31	67	47	69	83
8	2	No Rx	51	33	62	60	76	89
9	4	No Rx	147	35	81	71	85	100
10	2	No Rx	57	44	58	75	78	89
11	3	C, St	12	61	75	73	76	88

The Rai stage and prior treatment are shown. Rai stage 1 corresponds to lymphocytosis and lymphadenopathy, Rai stage 2 to lymphocytosis and splenomegaly or hepatomegaly, Rai stage 3 to lymphocytosis and anemia, and Rai stage 4 to lymphocytosis and thrombocytopenia [54]. Prior chemotherapy for these patients included chlorambucil (C), fludarabine (F), rituxan (R), cyclophosphamide (Cy), prednisone (St) and combination chemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP). Apoptosis was assessed by Hoechst 3342 analysis after 48 h culture of one million leukemic cells in media alone (no Rx), 10 μM rolipram (R), 1 μM hydrocortisone (HC), or both drugs (R/HC). The predicted percentage of apoptotic cells calculated by adding the (R-basal) + (HC-basal) + basal apoptosis values is shown (Add.) for comparison.

inhibitor cilostamide (10 μM), neither induced apoptosis by itself, above that of control, nor augmented hydrocortisone-induced apoptosis in leukemic cells from the same five B-CLL patients (Fig. 1D) [34].

If intermittent therapy of B-CLL patients with regimens that include PDE4 inhibitors and glucocorticoids is to be of clinical benefit, it would be preferable that such therapy does not induce apoptosis in normal T cells, as drug regimens that induce T cell apoptosis are associated with an increased risk of opportunistic infections. As previously published, treatment with 10 μM rolipram failed to induce apoptosis in peripheral blood T cells (Fig. 1E) [27]. Further, in contrast to B-CLL cells, rolipram treatment also failed to augment the apoptotic effect of 1 μM hydrocortisone on T cells (Fig. 1E). In prior studies of B-CLL, we found that PDE4 inhibitors induce apoptosis and activate PKA and the Rap1 GDP exchange factor EPAC in the absence of additional adenylyl cyclase stimulation [27–29]. Forskolin, a direct activator of adenylyl cyclase, when added at 40 μM, augmented rolipram-induced apoptosis in B-CLL to a minor degree, but had little or no further effect on the combination of rolipram and hydrocortisone in B-CLL cells or peripheral blood T cells (Fig. 1E and F).

3.2. PDE4 inhibitors augment glucocorticoid-mediated GRE transactivation in B-CLL

The experiments described above demonstrating that PDE4 inhibitors can synergize with glucocorticoids in inducing apoptosis are consistent with recent studies reporting that PDE4 inhibitors can increase GR-mediated signaling [36]. To test such a hypothesis, we examined the effects of rolipram on hydrocortisone-induced transactivation of GRE luciferase constructs transiently transfected into B-CLL cells. In five patients tested, the addition of 10 μM rolipram significantly augmented hydrocortisone-induced transactivation by an average of 33% (Fig. 2A; $p < 0.02$). Although this increase in transactivation is clearly modest, the transfection efficiency of the B-CLL cells was low (10–20% viable transfected cells) which would be expected to limit the augmentation observed. As deletion of the GR has been demonstrated to inhibit cAMP-induced apoptosis in CCRF-CEM cells, we also examined the effect of rolipram itself on GRE transactivation [8]. Although we consistently observed minor augmentation (10%) of GRE transactivation by rolipram (7/8 experiments), the augmentation was not statistically significant ($p < 0.23$). Thus, these data do not support the hypothesis that PDE4 inhibitors induce apoptosis in vitro in B-CLL cells by augmenting basal GRE transactivation through the GR.

3.3. Inhibition of PKA blocks both glucocorticoid-mediated apoptosis and GRE transactivation in B-CLL

Given the evidence that PDE4 inhibitors, previously shown to induce PKA-mediated signaling in B-CLL cells, can synergize with glucocorticoids to induce apoptosis in B-CLL, we asked whether conversely PKA was required for glucocorticoid-mediated apoptosis. Our prior studies of PKA inhibitors had demonstrated that treatment of B-CLL cells with Rp-8Br-cAMPS (1 mM), an enantiomeric cAMP-binding site competitive antagonist, blocked rolipram-induced CREB phosphorylation and significantly reduced both basal and rolipram-induced B-CLL apoptosis [29]. Remarkably, we found that co-treatment of B-CLL cells with 1 mM Rp-8Br-cAMPS inhibited hydrocortisone-induced apoptosis by 86 ± 14% in the six B-CLL patients tested (Fig. 2C). Treatment with 1 mM Rp-8Br-cAMPS also reduced hydrocortisone-induced transactivation of GRE luciferase constructs by 83% in eight B-CLL samples tested (Fig. 2A; $p < 0.02$). PKA-mediated signal transduction can also be disrupted by Ht31 peptide, a 23-residue peptide derived from an AKAP that binds with 4.0 nM dissociation constant to PKA RIIα subunits [37,38]. Preincubation of the B-CLL cells with 10 μM St-Ht31 also significantly reduced hydrocortisone-induced transactivation by an average of 33% in three patients tested ($p < 0.006$). These studies suggest that PKA activity is required for both glucocorticoid-mediated GRE transactivation and B-CLL apoptosis.

3.4. Adenylyl cyclase activation but not PDE4 inhibition augments glucocorticoid-induced apoptosis and GRE activation in CCRF-CEM cells

As the CCRF-CEM cell line has been utilized for seminal studies on glucocorticoid and cAMP-mediated

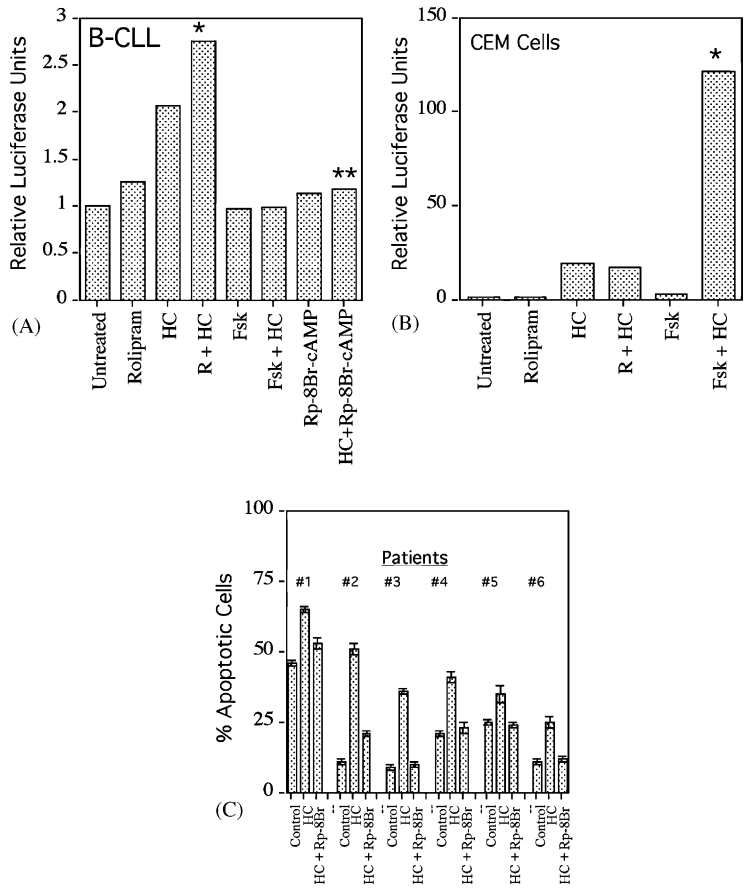

Fig. 2. Analysis of the effects of rolipram, forskolin, hydrocortisone and the PKA antagonist Rp-8Br-cAMPS on transactivation of GRE elements and induction of apoptosis in B-CLL and CCRF-CEM cells. (A and B) B-CLL cells (A) or CCRF-CEM cells (B) were transiently transfected with a luciferase construct in which expression is regulated by GRE elements, followed by culture in 10 μM rolipram (R), 40 μM forskolin (Fsk), 1 μM hydrocortisone (HC), 1 mM Rp-8Br-cAMPS (Rp-8Br) or combinations of these agents. Twelve hours after addition of the drugs, the relative luminescence of the samples was determined. The data shown are the mean of eight experiments for B-CLL and two experiments for CCRF-CEM cells. The single asterisk denote a significant difference by paired Student's *t*-test between HC alone and either R/HC (A) or F/HC (B) ($p < 0.02$). The double asterisk denotes a significant difference between Rp-8Br-cAMPS/HC and HC alone ($p < 0.02$). (C) B-CLL cells from six patients were cultured for 48 h in either media alone (control), 1 μM hydrocortisone (HC), or 1 μM hydrocortisone in combination with 1 mM Rp-8Br-cAMPS (HC + Rp-8Br). Cells were assessed for apoptosis by Hoechst 33342 flow cytometry. Data represent the mean ± S.E.M. of triplicate samples.

apoptosis, we next sought to determine whether this cell line resembled B-CLL cells with respect to the ability of PDE4 inhibitors to augment glucocorticoid-mediated apoptosis and GRE transactivation [8,11]. As prior work on PDE activity and the effect of PDE4 inhibitors in CCRF-CEM cells have utilized CEM clones of varying glucocorticoid sensitivity, we isolated both dexamethasone-sensitive and dexamethasone-resistant subclones for our studies (Fig. 3A) [11,39]. By MTS assay, we found that, unlike B-CLL cells, 10 μM rolipram alone had no discernable effect on the viability of either dexamethasone-resistant or sensitive CCRF-CEM cells (Fig. 3B). Forskolin, either as a single agent or combined with rolipram, also had no effect on cell viability. In contrast, forskolin (10 μM), but not rolipram (10 μM), dramatically enhanced the glucocorticoid sensitivity of dexamethasone-sensitive CCRF-CEM cells, an observation that stands in striking contrast to the results previously obtained in B-CLL cells (Fig. 3B). Forskolin also induced glucocorticoid sensitivity in the glucocorticoid-resistant CCRF-CEM cell clone. When the CCRF-CEM cells were analyzed by the Hoechst 33342 apoptosis assay, comparable results were obtained (data not shown). 1,9-Dideoxyforskolin, a forskolin analog that does not stimulate adenylyl cyclase, failed to augment glucocorticoid sensitivity in CCRF-CEM cells, suggesting that forskolin augments glucocorticoid-mediated CCRF-CEM apoptosis by its activity on adenylyl cyclase (Fig. 3B).

To further support the hypothesis that it is cAMP signaling and not a non-cAMP-mediated effect of forskolin that accounts for forskolin's ability to enhance glucocorticoid-mediated apoptosis in CCRF-CEM cells, we also examined the effects of combined treatment with hydrocortisone and the cAMP analog dibutyryl cAMP (dbcAMP). Apoptosis following treatment with a range of doses of dbcAMP as a single agent was similar in dexamethasone-sensitive and resistant CCRF-CEM cells, suggesting that whatever mechanism induced glucocorticoid resistance in CCRF-CEM cells did not lead to simultaneous resistance to cAMP analogs (Fig. 3C). Addition of 1 μM hydrocortisone to the same dbcAMP dose response

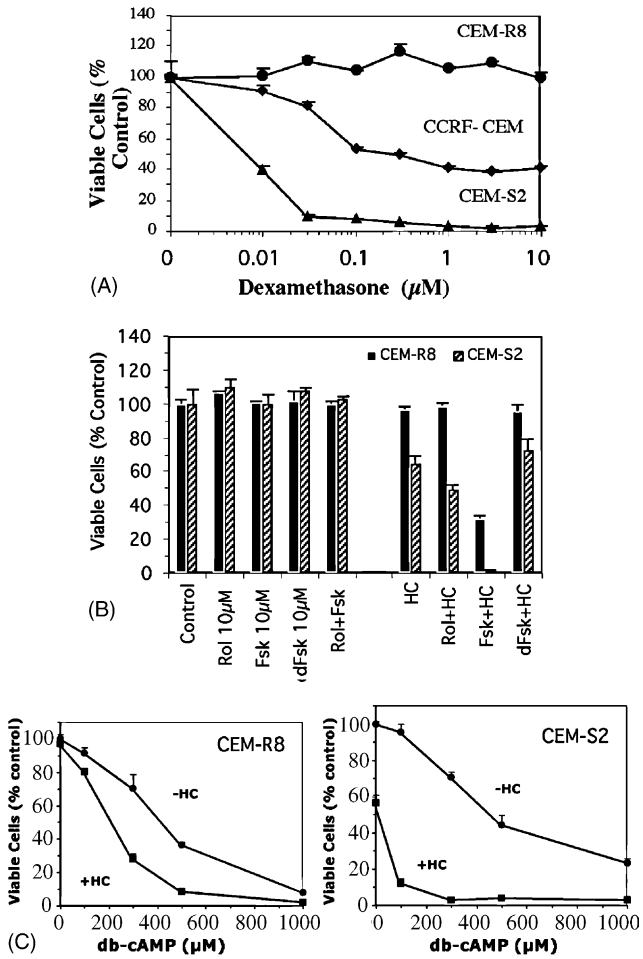

Fig. 3. The diterpene adenylyl cyclase stimulant forskolin synergizes with glucocorticoids in inducing apoptosis in dexamethasone-sensitive and resistant CCRF-CEM cells. (A) The polyclonal parental cell line (CCRF-CEM), the single cell-derived glucocorticoid-resistant subclone (CEM-R8) and the single cell-derived glucocorticoid-sensitive subclone (CEM-S2) were cultured for 72 h in the presence of vehicle (ETOH) or dexamethasone at the concentrations indicated and cell viability was assessed by the MTS assay as described in Section 2. (B) The glucocorticoid-resistant subclone (CEM-R8) and the glucocorticoid-sensitive subclone (CEM-S2) were cultured for 72 h in the presence of hydrocortisone (HC) (10 μM HC for the CEM-R8 cells and 1 μM HC for the CEM-S2 cells), 10 μM forskolin (Fsk), 10 μM rolipram (Rol), or combinations of these agents as indicated, and cell viability was assessed by the MTS assay as described in Section 2. (C) The glucocorticoid-resistant subclone (CEM-R8) and the glucocorticoid-sensitive subclone (CEM-S2) were cultured for 72 h in the presence of varying amounts of dibutyryl cAMP (db-cAMP) in the absence (−HC) or presence (+HC) of 1 μM hydrocortisone as indicated, and cell viability was assessed by the MTS assay as described in Section 2. The data shown were normalized to the vehicle control. Results represent the mean ± S.D. of triplicate determinations. Similar results were obtained in a total of three experiments performed.

assay resulted in marked synergy in the apoptotic effects of these two compounds for both dexamethasone-sensitive and resistant CCRF-CEM cells. These data support the hypothesis that cAMP-mediated signal transduction augments glucocorticoid-mediated apoptosis in CCRF-CEM cells regardless of the initial sensitivity of the leukemic clone to glucocorticoids.

Given the above marked discrepancy in the type of cyclic nucleotide-associated stimuli that synergize with glucocorticoids in inducing apoptosis in B-CLL and CCRF-CEM cells, we examined the effects of rolipram and forskolin on glucocorticoid-induced GRE transactivation in CCRF-CEM cells. In concurrence with the results of the apoptosis studies, we found that forskolin, but not rolipram, markedly augmented GRE transactivation in CCRF-CEM cells (Fig. 2B). Forskolin as a single agent had modest effects in this assay, while rolipram as a single agent had none. The level of transactivation observed in CCRF-CEM cells was markedly higher than that observed in the B-CLL studies, most likely as a result of a higher transfection efficiency in this cell line relative to the primary leukemic cells. These studies suggest that while GR-mediated signaling is augmented by PDE4 inhibition but not adenylyl cyclase stimulation in B-CLL cells, the converse is true in the T-ALL cell line CCRF-CEM.

3.5. cAMP levels and PDE4 isoforms are regulated by rolipram and forskolin differently in B-CLL and CCRF-CEM cells

As the studies above demonstrate that B-CLL and CCRF-CEM cells differ in their response to PDE4 inhibitors and adenylyl cyclase activation, we measured cAMP levels in these two types of cells following treatment with rolipram or forskolin. As prior studies have demonstrated that glucocorticoids raise levels of cAMP in lymphocytes and potentiate the cAMP response to agents that activate adenylyl cyclase, we also examined whether glucocorticoid treatment altered the cAMP response to rolipram or forskolin [40–42]. In leukemic cells from six B-CLL patients examined, treatment with 10 μM rolipram for 30 min augmented cAMP levels 2.5 ± 0.7-fold above that observed in untreated cells (Fig. 4A) (single factor ANOVA, $p < 0.04$). In contrast, in three experiments performed, treatment of CCRF-CEM cells with rolipram had no effect while treatment with forskolin caused a marked increase in cAMP levels at the 30 min time point that was not observed in B-CLL cells (Fig. 4B). Glucocorticoid treatment had no significant effect on cAMP levels in either cell type.

Given that B-CLL cells, but not CCRF-CEM cells, respond to PDE4 inhibitors with increased intracellular cAMP levels, glucocorticoid receptor-mediated GRE activation and glucocorticoid-mediated apoptosis, we next examined the expression of PDE4 isoforms in these two cell types. Our prior studies had demonstrated that inhibition of PDE4 with rolipram resulted in marked up-regulation of PDE4B2 levels in B-CLL cells as judged by RT-PCR and Western analysis [34]. Such an observation was in keeping with studies demonstrating that pharmacologic agents result in cAMP-induced increases in levels of PDE4B and PDE4D short forms through cAMP-activated intronic enhancers [43]. B-CLL cells were incubated with

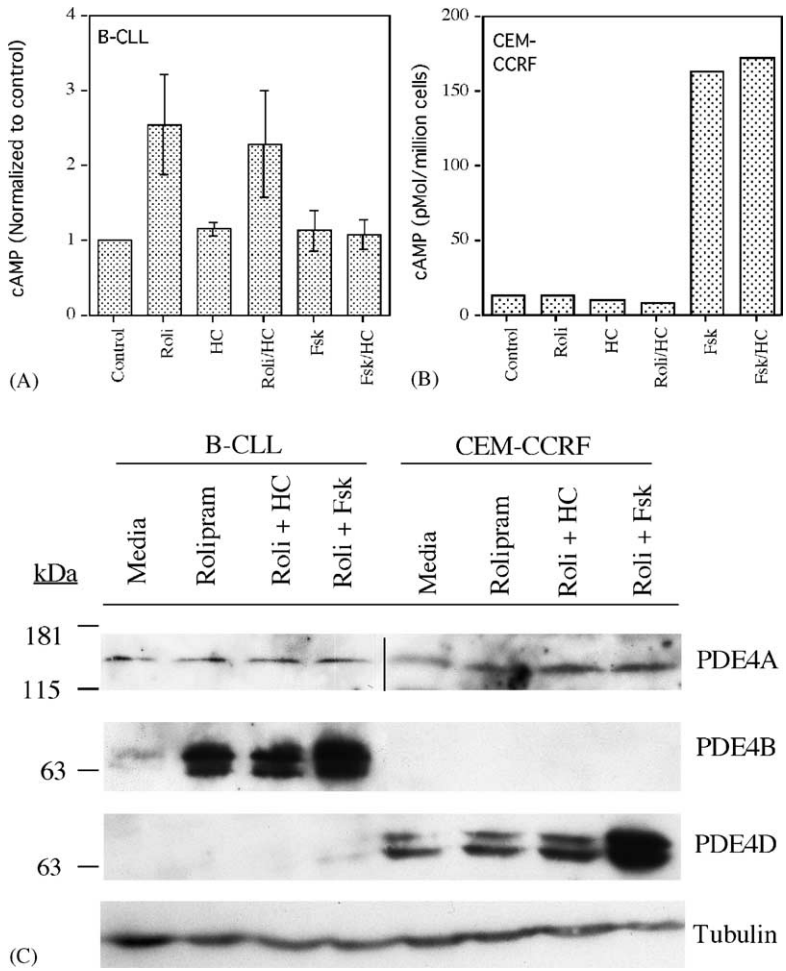

Fig. 4. Effect of PDE4 inhibitors or forskolin on cAMP levels and PDE4 isoform expression in B-CLL and CCRF-CEM cells. (A and B) Leukemic cells from six B-CLL patients or CCRF-CEM cells were incubated for 30 min with media alone (CT), 10 μM rolipram (Roli), 10 μM forskolin (Fsk), 1 μM hydrocortisone (HC), or the same concentration of hydrocortisone combined with rolipram (Roli/HC) or forskolin (Fsk/HC). Lysates of the cells were then assayed for cAMP using a RIA. The cAMP level obtained from each treated B-CLL sample was normalized to that observed in the untreated leukemic cells. The mean cAMP level for the B-CLL samples incubated in media alone (CT) was 3.0 pmol/million cells. The CCRF-CEM data shown are representative of three experiments performed. (C) B-CLL and CCRF-CEM cells were incubated for 18 h with media alone, 10 μM rolipram, 10 μM forskolin (Fsk), 1 μM hydrocortisone (HC), or the same concentration of rolipram combined with 1 μM hydrocortisone (Roli + HC) or 10 μM forskolin (Roli + Fsk). Lysates of the cells were then assessed for expression of PDE4A, PDE4B or PDE4D by Western analysis. Equal loading of samples was verified by immunoblotting for tubulin.

media alone, rolipram (10 μM) or rolipram combined with either hydrocortisone (1 μM) or forskolin (10 μM), followed by Western analysis for expression of PDE4A, PDE4B and PDE4D. In B-CLL cells, there was constitutive expression of a 130 kDa long form of PDE4A, rolipram-inducible expression of 63 and 68 kDa forms of PDE4B (both consistent with PDE4B2) and either no or very low level rolipram-induced expression of 67 and 72 kDa forms of PDE4D (consistent with PDE4D1/D2) (Fig. 4C). In contrast, in CCRF-CEM cells, we detected the same constitutive 130 kDa form of PDE4A, no PDE4B and constitutive expression of PDE4D1/D2 that was markedly increased by forskolin treatment (Fig. 4C). Thus, for at least these two cell types, rolipram-mediated inhibition of PDE4B but not PDE4D correlates with augmentation of intracellular cAMP levels, glucocorticoid receptor-mediated GRE activation and apoptosis.

4. Discussion

Although synergistic effects of cAMP signaling and glucocorticoid signaling have been studied for years in lymphoid cells, a critical missing element has been a therapeutically plausible means by which to synchronously activate these two pathways in targeted cells [8,44]. The major finding of this study is that treatment of B-CLL cells with PDE4 inhibitors, in the absence of exogenous stimulation of adenylyl cyclase, augments killing of these primary leukemic cells beyond that observed by adding the apoptotic effects observed with each drug class alone. Importantly, the same combined treatment does not induce apoptosis in primary human T cells. As at least two PDE4 inhibitors, roflumilast (Daxas, Altana Pharma) and cilomilast (Ariflo; GlaxoSmithKline) are in late stages of clinical development in Europe and the US, respectively,

it should become practical to test the efficacy of combined PDE4 inhibitor/glucocorticoid therapy in treatment-resistant lymphoid malignancies in the near future.

What factors determine whether a normal or malignant lymphoid cell will undergo apoptosis following treatment with either PDE4 inhibitors alone or in combination with glucocorticoids? In the current study, we examine two quite different examples of lymphoid cells in which activation of cAMP-mediated signaling in combination with glucocorticoid treatment induces a synergistic apoptotic effect. In B-CLL cells, the cell lineage sensitive to combined PDE4 inhibitor/glucocorticoid-induced apoptosis, treatment with rolipram alone augments cAMP levels and induces PDE4B2. In CCRF-CEM cells, a T-ALL-derived cell line that was insensitive to combined PDE4 inhibitor/glucocorticoid-induced apoptosis, treatment with rolipram neither augmented cAMP nor induced any PDE4 isoform. Notably, CCRF-CEM cells were extremely sensitive to forskolin/glucocorticoid or dbcAMP/glucocorticoid-induced apoptosis. From these results, we conclude that at least two requirements for PDE4 inhibitor-mediated lymphoid apoptosis are sufficient basal adenylyl cyclase activity to drive cAMP accumulation in a subcellular compartment (so-called ''flux-mediated sensitivity'') and control of that subcellular cAMP pool by a PDE4 enzyme. Given that CCRF-CEM cells express constitutive PDE4A2, it is clearly not sufficient for a lymphoid cell to express a PDE4 isoform for it to respond to PDE4 inhibition with elevation of cAMP, compensatory up-regulation of PDE4 enzymes or apoptosis. Furthermore, given that both CCRF-CEM and B-CLL cells express PDE4A2, it seems likely that the PDE4 isoform regulating the critical subcellular pro-apoptotic cAMP compartment in B-CLL cells is PDE4B2.

However, it is also clear that adequate adenylyl cyclase activity and the presence of PDE4B2 in a lymphoid cell is not sufficient for augmentation of glucocorticoid-mediated apoptosis following treatment with PDE4 inhibitors. We have previously found that while rolipram alone does not augment cAMP levels in human whole mononuclear cells, a population consisting predominantly of T cells, combined treatment with rolipram and forskolin results in markedly higher cAMP levels than those observed with forskolin alone [27]. Despite this, in the current studies we find that the same combined treatment of purified human T cells with rolipram and forskolin does not have any significant effect on glucocorticoid-mediated apoptosis. Furthermore, multiple studies in T cells have demonstrated that they contain PDE4B enzyme and that activation or inhibition of PDE4 induces important functional changes in this cell lineage [45,46]. Thus, in addition to adenylyl cyclase activity and a regulating PDE4 isoform, lymphoid cells must require specific ''downstream'' signaling targets in order for PDE4 inhibitors to activate an apoptotic cascade. While our prior work has implicated PP2A activation and BAD dephosphorylation as potentially relevant rolipram-induced events in B-CLL, a clear picture of the molecular targets that differ between these leukemic cells and peripheral T cells remain to be established [28]. Given that PDE4 inhibitors increase T cell cAMP levels but not glucocorticoid-mediated apoptosis, it will be of interest to determine whether, as with CCRF-CEM cells, PDE4 inhibitors fail to augment T cell GRE transactivation.

Why do cAMP-mediated and glucocorticoid-mediated signaling synergize in killing susceptible lymphoid populations? In one model, cAMP and glucocorticoids could activate non-interdependent signaling pathways that positively interact as a result of distinct signaling outcomes that collaborate to induce apoptosis. In another model, one signaling pathway could increase either the magnitude (quantity) or the character (quality) of signaling by the other pathway. Most, but not all, studies of the interactions between these two pathways in lymphoid cells favor the hypothesis that PKA signaling positively regulates glucocorticoid-mediated apoptotic signaling. Kiefer et al. demonstrated that a GR-deficient CCRF-CEM subclone was resistant to apoptosis induced by cAMP analogs [8]. GR signaling may be not only necessary but also possibly sufficient for cAMP-mediated apoptosis in lymphoid cells, as inhibition of CRE-mediated transcriptional activation by transfection with CRE ''decoy'' oligonucleotides failed to protect T cell hybridomas from cAMP potentiation of GC-mediated apoptosis [47].

Unfortunately, we have been unable to test the role of the GR in PDE4 inhibitor-mediated apoptosis as we find that the best characterized GR antagonist, mefipristone (RU486), while not apoptotic in B-CLL cells when used alone, behaves as a GR agonist in the setting of co-treatment with PDE4 inhibitors or other drugs that activate PKA signaling in B-CLL cells (data not shown). Gruol et al. have previously reported similar findings in immature murine T cells, where RU486 synergized with cAMP to induce apoptosis but had no activity when used alone [48]. While we cannot therefore yet confirm that glucocorticoid-mediated signaling is required for PKA-mediated apoptosis in B-CLL, using the enantiomeric cAMP antagonist Rp-8Br-cAMPS, we do find evidence suggestive that PKA-mediated signaling is required for glucocorticoid-mediated B-CLL apoptosis. Consistent with this observation, the modest PDE4 inhibitor-induced augmentation in glucocorticoid-induced GRE transactivation we observed in transfected B-CLL cells was also inhibited by Rp-8Br-cAMPS treatment. In the more efficiently transfected CCRF-CEM cells, the augmentation in GRE transactivation following co-treatment with forskolin and glucocorticoids was far more pronounced. These data support the hypothesis that the synergistic apoptosis we observe in these two models following treatment with rolipram or forskolin combined with glucocorticoids are the result of augmented signaling through the glucocorticoid receptor.

If the glucocorticoid receptor is the relevant target of PKA in combined PDE4 inhibitor/glucocorticoid therapy,

what does PKA phosphorylate and how does the resulting transcriptional complex trigger apoptosis? Although PKA has been reported to be associated with the GR and to modulate GR-mediated transcriptional activity, consistent evidence of functionally relevant phosphorylation of the GR itself by PKA is lacking [13,49]. Alternatively, PKA may phosphorylate other transcription factors or co-activators or co-repressors with which the GR interacts [13,50]. Studies of glucocorticoid-induced lymphoid apoptosis have come to differing conclusions with regard to the role of GR-mediated transactivation through GREs or transrepression of NFκB or AP1 [6]. A murine ''knock-in'' for the A458T dimerization-defective GR that cannot activate GRE-containing promoters demonstrated that glucocorticoids no longer induce apoptosis in the thymocytes of such mice, while glucocorticoid-mediated AP-1 and NFκB transrepression remains intact [51]. In contrast, glucocorticoid-mediated killing of Jurkat cells has been argued to be independent of GR-mediated transcriptional activation [52]. A readily transfectable cell line in which PDE4 inhibition induces apoptosis will be required in order to examine more critically whether PDE4 inhibitor-associated synergy with GR signaling proceeds through a transactivation or transrepression pathway.

Recent studies by other groups have supported the concept that PDE4 may prove to be an important therapeutic target in resistant human lymphoid malignancies. A chip analysis by Shipp et al. demonstrated that mRNA expression of one PDE4 gene family member, PDE4B, correlates with resistance of diffuse large B cell lymphomas (DLBCL) to standard CHOP chemotherapy [53]. Given our finding that inhibition of PKA in B-CLL cells with Rp-Br-cAMPS leads to striking resistance to hydrocortisone-induced apoptosis, we hypothesize that high level expression of PDE4B reduces PKA activation in DLBCL cells, thereby rendering them less sensitive to the apoptotic effects of prednisone, a component of the CHOP regimen. Regardless of the mechanism by which PDE4 inhibitor/glucocorticoid synergy occurs, the coming availability of PDE4 inhibitors in the clinic should eventually allow us to test the concept that this class of drugs may improve clinical responses to glucocorticoid-containing chemotherapy regimens.

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References

[1] McConkey DJ, Orrenius S, Jondal M. Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. J Immunol 1990;145:1227–30.

[2] Kizaki H, Suzuki K, Tadakuma T, Ishimura Y. Adenosine receptor-mediated accumulation of cAMP-induced T-lymphocyte death through internucleosomal DNA cleavage. J Biol Chem 1990;265:5280–4.

[3] Lomo J, Blomhoff HK, Beiske K, Stokke T, Smeland EB. TGF-B1 and cyclic AMP promote apoptosis in resting human B lymphocytes. J Immunol 1995;154:1634–43.

[4] Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251–306.

[5] Cohen JJ, Duke RC. Glucocorticoid activation of a calcium dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol 1984;132:38–42.

[6] Greenstein S, Ghias K, Krett NL, Rosen ST. Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies. Clin Cancer Res 2002;8(6):1681–94.

[7] Harrigan MT, Baugman G, Campbell NF, Bourgeois S. Isolation and characterization of glucocorticoid and cyclic-AMP-induced genes in T lymphocytes. Mol Cell Biol 1989;9:3438–46.

[8] Kiefer J, Okret S, Jondal M, McConkey DJ. Functional glucocorticoid receptor expression is required for cAMP-mediated apoptosis in a human leukemic T cell line. J Immunol 1995;155:4525–8.

[9] Dowd DR, Miesfield RL. Evidence that glucocorticoid and cyclic AMP-induced apoptotic pathways in lymphocytes share distal events. Mol Cell Biol 1992;12(8):3600–8.

[10] Medh RD, Saeed MF, Johnson BH, Thompson EB. Resistance of human leukemic CEM-C1 cells is overcome by synergism between glucocorticoid and protein kinase A pathways: correlation with myc suppression. Cancer Res 1998;58:3684–93.

[11] Ogawa R, Streiff MB, Bugayenko A, Kato GJ. Inhibition of PDE4 phosphodiesterase activity induces growth suppression, apoptosis, glucocorticoid sensitivity, p53, and p21 WAF1/CIP1 proteins in human acute lymphoblastic leukemia cells. Blood 2002;99:3390–7.

[12] Refojo D, Liberman AC, Giacomini D, Carbia Nagashima A, Graciarena M, Echenique C, et al. Integrating systemic information at the molecular level: cross-talk between steroid receptors and cytokine signaling on different target cells. Ann N Y Acad Sci 2003;992:196–204.

[13] Doucas V, Shi Y, Miyamoto S, West A, Verma I, Evans RM. Cytoplasmic catalytic subunit of protein kinase A mediates cross-repression by NF-kB and the glucocorticoid receptor. Proc Natl Acad Sci USA 2000;97:11893–8.

[14] Heck S, Bender K, Kullmann M, Gottlicher M, Herrlich P, Cato AC. I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. EMBO J 1997;16(15):4698–707.

[15] Nissen RM, Yamamoto KR. The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev 2000;14(18):2314–29.

[16] De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr Rev 2003;24(4):488–522.

[17] Reichardt HM, Tuckermann JP, Gottlicher M, Vujic M, Weih F, Angel P, et al. Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. EMBO J 2001;20(24):7168–73.

[18] Epstein PM, Hachisu R. Cyclic nucleotide phosphodiesterase in normal and leukemic human lymphocytes and lymphoblasts. Adv Cyclic Nucleotide Protein Phosphorylation Res 1984;16:303–24.

[19] Lerner A, Kim B, Lee R. The cAMP signaling pathway as a therapeutic target in lymphoid malignancies. Leuk Lymphoma 2000;37(1–2):39–51.

[20] Jiang X, Paskind M, Weltzien R, Epstein PM. Expression and regulation of mRNA for distinct isoforms of cAMP-specific phosphodiesterase (PDE4) in mitogen-stimulated and leukemic human lymphocytes. Cell Biochem Biophys 1998;28:135–60.

[21] Francis SH, Turko IV, Corbin JD. Cyclic nucleotide phosphodiesterases: relating structure and function. Prog Nucleic Acid Res Mol Biol 2001;65:1–52.
[22] Beavo JA, Brunton LL. Cyclic nucleotide research—still expanding after half a century. Nat Rev Mol Cell Biol 2002;3(9):710–8.
[23] Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C. Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. J Biol Chem 2003;278(8):5493–6.
[24] Houslay MD, Adams DR. PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. Biochem J 2003;370(Part 1):1–18.
[25] Michel JJ, Scott JD. AKAP mediated signal transduction. Annu Rev Pharmacol Toxicol 2002;42:235–57.
[26] Burnouf C, Pruniaux MP. Recent advances in PDE4 inhibitors as immunoregulators and anti-inflammatory drugs. Curr Pharm Des 2002;8(14):1255–96.
[27] Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood 1998;92(7):2484–94.
[28] Moon E-Y, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood 2003;101:4122–30.
[29] Tiwari S, Felekkis K, Moon E, Flies A, Sherr D, Lerner A. Among circulating hematopoietic cells. B-CLL uniquely expresses functional EPAC1 but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood 2004;103: 2661–7.
[31] Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 1965;18:522–9.
[32] Tiwari S, Felekkis K, Moon E, Flies A, Sherr D, Lerner A. Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1 but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood 2004;103:2661–7.
[33] Umesono K, Evans RM. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 1989;57(7):1139–46.
[34] Moon E, Lee R, Near R, Weintraub L, Wolda S, Lerner A. Inhibition of PDE3B augments PDE4-inhibitor-induced apoptosis in leukemic cells from a subset of patients with chronic lymphocytic leukemia. Clin Cancer Res 2002;8:589–95.
[35] Bolger GB, Erdogan S, Jones RE, Loughney K, Scotland G, Hoffmann R, et al. Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. Biochem J 1997;328:539–48.
[36] Miller A, Vogt G, Pearce B. The phosphodiesterase type 4 inhibitor, rolipram, enhances glucocorticoid receptor function. Neuropsychopharmacology 2002;27:939–48.
[37] Carr DW, Hausken ZE, Fraser ID, Stofko-Hahn RE, Scott JD. Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein. Cloning and characterization of the RII-binding domain. J Biol Chem 1992;267(19):13376–82.
[38] Klussmann E, Maric K, Wiesner B, Beyermann M, Rosenthal W. Protein kinase A anchoring proteins are required for vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells. J Biol Chem 1999;274(8):4934–8.
[39] Epstein PM, Mills JS, Ross CP, Strada SJ, Hersh EM, Thompson WJ. Increased cyclic nucleotide phosphodiesterase activity associated with proliferation and cancer in human and murine lymphoid cells. Cancer Res 1977;37:4016–23.
[40] Marone G, Lichtenstein LM, Plaut M. Hydrocortisone and human lymphocytes: increases in cyclic adenosine 3′,5′-monophosphate and potentiation of adenylate cyclase-activating agents. J Pharmacol Exp Ther 1980;215(2):469–78.
[41] Michel MC, Knapp J, Ratjen H. Sensitization by dexamethasone of lymphocyte cyclic AMP formation: evidence for increased function of the adenyl cyclase catalyst. Br J Pharmacol 1994;113(1):240–6.
[42] Baus E, Van Laethem F, Andris F, Rolin S, Urbain J, Leo O. Dexamethasone increases intracellular cyclic AMP concentration in murine T lymphocyte cell lines. Steroids 2001;66(1):39–47.
[43] Vicini E, Conti M. Characterization of an intronic promoter of a cyclic adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase gene that confers hormone and cAMP inducibility. Mol Endocrinol 1997;11(7):839–50.
[44] McConkey DJ, Orrenius S, Okret S, Jondal M. Cyclic AMP potentiates glucocorticoid-induced endogenous endonuclease activation in thymocytes. FASEB J 1993;7(6):580–5.
[45] Baroja ML, Cieslinski LB, Torphy TJ, Wange RL, Madrenas J. Specific CD3 epsilon association of a phosphodiesterase 4B isoform determines its selective tyrosine phosphorylation after CD3 ligation. J Immunol 1999;162(4):2016–23.
[46] Arp J, Kirchhof MG, Baroja ML, Nazarian SH, Chau TA, Strathdee CA, et al. Regulation of T-cell activation by phosphodiesterase 4B2 requires its dynamic redistribution during immunological synapse formation. Mol Cell Biol 2003;23(22):8042–57.
[47] Muller Igaz L, Refojo D, Costas MA, Holsboer F, Arzt E. CRE-mediated transcriptional activation is involved in cAMP protection of T-cell receptor-induced apoptosis but not in cAMP potentiation of glucocorticoid-mediated programmed cell death. Biochim Biophys Acta 2002;1542(1–3):139–48.
[48] Groul DJ, Altschmied J. Synergistic induction of apoptosis with glucocorticoids and 3′–5′-cyclic adenosine monophosphate reveals agonist activity by RU486. Mol Endocrinol 1993;7:104–13.
[49] Rangarajan P, Umesono K, Evans R. Modulation of glucocorticoid receptor by protein kinase A. Mol Endocrinol 1992;6:1451–7.
[50] Kurihara I, Shibata H, Suzuki T, Ando T, Kobayashi S, Hayashi M, et al. Expression and regulation of nuclear receptor coactivators in glucocorticoid action. Mol Endocrinol 2002;189:181–9.
[51] Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, et al. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 1998;93:531–41.
[52] Helmburg A, Auphan N, Caelles C, Karin M. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. EMBO J 1995;14:452–60.
[53] Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC, et al. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med 2002;8(1):68–74.
[54] Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood 1975;46(2):219–34.