Inhibition of PDE3B Augments PDE4 Inhibitor-induced Apoptosis in a Subset of Patients with Chronic Lymphocytic Leukemia[1]

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ABSTRACT

Purpose: cAMP phosphodiesterase (PDE) 4 is a family of enzymes the inhibition of which induces chronic lymphocytic leukemia (CLL) apoptosis. However, leukemic cells from a subset of CLL patients are relatively resistant to treatment with the PDE4 inhibitor rolipram, particularly when this drug is used in the absence of an adenylate cyclase stimulus such as forskolin. Elevated cAMP levels induce compensatory up-regulation of several cyclic nucleotide PDE families in other model systems. We here examine the hypothesis that CLL cells that survive treatment with rolipram do so as a result of residual PDE activity that is not inhibited by this drug.

Experimental Design: We examined by Western analysis the effect of rolipram treatment on CLL expression of PDE3B, PDE4A, PDE4B, PDE4D, and PDE7A. We also examined the ability of rolipram (PDE4 inhibitor) or cilostamide (PDE3 inhibitor), alone or together, to induce apoptosis or elevate cyclic AMP in leukemic cells from patients with CLL.

Results: Rolipram increased levels of PDE4B and, to a variable extent, PDE4D. When combined with forskolin, rolipram also increased levels of a second family of PDEs, PDE3B. Addition of the specific PDE3 inhibitor, cilostamide, modestly augmented rolipram-induced apoptosis in five of seven "rolipram-resistant" CLL samples.

Conclusions: Although this work confirms that PDE4 appears to be the most important PDE target for induction of apoptosis in CLL, combination therapy with PDE3 and PDE4 inhibitors or use of dual-selective drugs may be of benefit in a subset of relatively PDE4-inhibitor resistant CLL patients.

INTRODUCTION

Methylxanthines such as theophylline, a drug widely used for treatment of asthma and neonatal apnea, induce apoptosis in CLL[3] cells *in vitro* (1). The sensitivity of CLL cells to other agents that raise cAMP levels such as dibutyryl cAMP or forskolin has suggested that the proapoptotic activity of methylxanthines may arise, at least in part, because of their activity as nonspecific cyclic nucleotide PDE inhibitors (2). A Phase II clinical trial by Binet *et al.* (3) in patients with chlorambucil-resistant CLL, as well as case reports (4), have suggested that adding theophylline to chlorambucil may be of clinical value in this disease. The efficacy of theophylline as a single agent in early stage CLL is currently being examined by Makower *et al.* (4) in a Phase II Eastern Cooperative Oncology Group trial in Rai stage 0 or 1 patients (E-4998 and NCCTG-988151).

Identification of the putative PDE target(s) of methylxanthines may improve the efficacy of PDE inhibitor therapy for CLL. Methylxanthines cannot be used clinically at dosages that potently inhibit PDEs because they are also adenosine receptor antagonists, a property that can induce seizures in patients whose serum theophylline levels rise above a therapeutic window of 10–20 μg/ml. Lymphoid cells have been reported to express a variety of PDEs that can catabolize cAMP and are inhibited by methylxanthines, including PDE3B, PDE4A/B/D, and PDE7A (5–9). Although PDE3 and PDE4 enzymes can be specifically inhibited with cilostamide (IC$_{50}$, 5 nM) and rolipram (IC$_{50}$, 0.1–1 μM), respectively, PDE7-specific inhibitors are still in development (10, 11).

We recently screened CLL cells for PDE isoform expression by RT-PCR and Northern analysis and examined the ability of the corresponding family-specific PDE inhibitors to induce CLL apoptosis *in vitro* (12). PDE4 transcript and enzymatic activity was present in CLL cells, and in 10 of 14 CLL patients, the PDE4 inhibitor rolipram (10 μM) induced apoptosis in 60 $\pm$ 15% of leukemic cells (12). In contrast, interleukin-2 cultured whole mononuclear cells, a population made up predominantly of peripheral T cells, were resistant to rolipram-induced apoptosis. The ability of a given dose of PDE4 inhibitor to induce apoptosis in CLL correlates well with its ability to raise intracellular cAMP levels (r^2 = 0.998; Ref. 13).

In this report, we have begun to examine the mechanism by which cells from a subset of CLL patients survive rolipram treatment. Prolonged exposure of cells to physiological signals that activate adenylate cyclase and raise intracellular cAMP

[3] The abbreviations used are: CLL, chronic lymphocytic leukemia; cAMP, cyclic AMP; PDE, phosphodiesterase; RT-PCR, reverse transcription-PCR; GST, glutathione *S*-transferase.

levels elicit a variety of adaptive responses in cells that subsequently down-regulate cAMP-mediated signal transduction. One mechanism for such "desensitization" is up-regulation of cyclic nucleotide phosphodiesterase levels. In this study, we hypothesized that rolipram-induced up-regulation of cAMP phosphodiesterase families not inhibited by this drug results in catabolism of cAMP and escape from apoptosis. We present data that support the hypothesis that in a subset of CLL patients, combined therapy with PDE3 and PDE4 inhibitors is more effective in inducing apoptosis than PDE4 inhibitors alone.

MATERIALS AND METHODS

Reagents.   Rolipram was from RBI (Natick, MA). Forskolin and IBMX were from Sigma Chemical Co. (St. Louis, MO). Theophylline was obtained as a 3.2-mg/ml solution in 5% dextrose (Baxter Healthcare Corporation, Deerfield, IL).

Cell Purification and Culture.   Primary leukemic cells were isolated from the peripheral blood of patients with CLL after obtaining Institutional Review Board-approved informed consent. The diagnosis of each patient's malignancy was confirmed by characteristic immunophenotype. Primary splenic B cells were isolated from the discarded splenic tissue of a patient who underwent splenectomy for idiopathic thrombocytopenic purpura. Normal or leukemic cells or WSU-CLL cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin (Sigma Chemical Co.), and 10 mM HEPES (pH 7.4).

Antibodies.   Monoclonal antibodies directed against PDE3B, PDE4A, PDE4B, and PDE7A were generated using fusion proteins derived from the GST expression system (Pharmacia). The PDE3B antibody 281K was generated from a GST-fusion protein with cDNA corresponding to amino acids 520–879 of PDE3B (14). The PDE4A antibody 66C12H was generated from a GST fusion protein with cDNA corresponding to amino acids 718–886 of PDE4A3 (15). The PDE7A1-specific antibodies 144N and 144R were generated from a GST fusion protein with cDNA corresponding to amino acids 18–190 of PDE7A1 (16). The GST-fusion constructs described above were transformed into the *Escherichia coli* strain XL-1 Blue (Stratagene), and protein expression was induced with isopropyl-β-D-thiogalactoside as described by the manufacturer (Pharmacia). The expressed fusion proteins were isolated from bacterial inclusion bodies by SDS-PAGE and electroelution. The PDE4B monoclonal antibody 96G7A was generated using full-length PDE4B2 expressed in and purified from *E. coli* (15).[4] A PDE7A pan-reactive antibody (184O) that detects both PDE7A1 and PDE7A2 was generated from gel-purified, full-length PDE7A1 expressed in *E. coli*. Monoclonal antibodies selective for the appropriate PDEs were generated in mice using standard procedures (17). Each antibody was tested by Western analysis for its selective reactivity against the respective full-length recombinant PDE from which it was derived. Each antibody was also tested by Western analysis for its lack of reactivity with other PDE family members. Each of the monoclonal antibodies

described (with the exception of 281K) was purified from mouse ascites by protein A chromatography. The source of the monoclonal antibody 281K was a hybridoma culture supernatant. The antibody against PDE4D (61D10E) has been described previously (18).

Protein Extraction and Western Blot Analysis.   Cells were washed once with ice-cold PBS. After centrifugation at 4000 rpm for 5 min (relative centrifugal force, 1310), the cell pellet was lysed for 20 min in lysis buffer [50 mM Tris (pH 7.4), 1% NP40, 125 mM NaCl, 2 μg/ml aprotinin, leupeptin, and pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF]. The protein supernatant was collected after centrifugation at 14,000 rpm (relative centrifugal force, 16,000) at 4°C for 5 min. Protein concentration was measured with bicinchoninic acid reagents (Pierce, Rockford, IL). The proteins were separated on 7.5 or 10% SDS-PAGE using 10–30 μg of protein/lane and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked in 5% skim milk in PBS/0.05% Tween 20 at room temperature for 1 h. Anti-PDE or anti-tubulin antibodies (final concentration, 1 μg/ml) were added in blocking buffer and incubated at room temperature for 1 h with shaking. The membrane was washed four times each for 5 min with PBS/0.05% Tween 20. Horseradish peroxidase-conjugated antimouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was then added (1:1000 dilution), incubated, and washed in a manner identical to that described for the first antibody. Immunoreactive protein was then detected by the ECL technique according to the vendor's protocol (Pierce). Equal loading was verified by reprobing the blots with anti-tubulin antibodies. Where indicated, the developed films were scanned with a Fluor-S MultiImager (Bio-Rad).

Apoptosis Assay.   Apoptosis in primary CLL cells was assessed using a FACS Hoechst 33342 assay as described previously (12).

cAMP Assay.   One million CLL cells were incubated with medium, cilostamide, rolipram, or a combination of the two drugs for various periods of time. The cells were centrifuged, transferred to 1 ml of PBS, and mixed while vortexing with 1 ml of 80% ethanol. After 5 min on ice, cell debris was removed, and the supernatants were dried with a Speedivac. The samples were then assayed for cAMP by RIA (NEN) using the acetylated protocol.

RESULTS

To determine whether treatment with the PDE4 inhibitor rolipram alters cAMP phosphodiesterase levels in human splenic B cells or CLL cells, we performed immunoblot analysis of such cells after 8 h of incubation with medium alone, with the adenylate cyclase activator forskolin (40 μM), with rolipram (10 μM), or with a combination of the two drugs. A M_r 64,000 form of PDE4B, which comigrated with recombinant PDE4B2 (GenBank L20971), was the most commonly expressed PDE4 species in both B cells and CLL cells (Fig. 1). RT-PCR experiments confirmed the presence of PDE4B2 transcripts in CLL cells (data not shown). Levels of this enzyme rose after rolipram treatment but were not altered by treatment with forskolin alone (Fig. 1). Induction was dose dependent and apparent in cells treated with rolipram concentrations as low as 0.1–0.3 μM (Fig. 2A). PDE4B levels increased as early as 1 h after addition of drug (Fig. 2B).

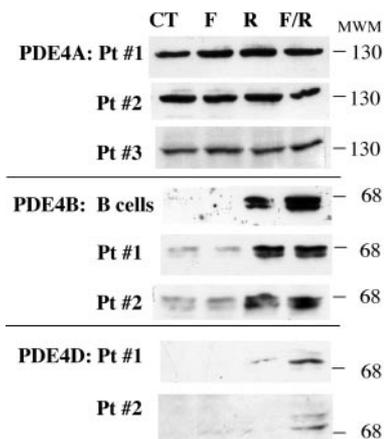[4] L. Uher and V. Florio, unpublished data.

Fig. 1 Effect of rolipram and forskolin on PDE4 family expression in CLL. Twenty to thirty million CLL cells or peripheral blood B cells were incubated in medium (*CT*), 10 μM rolipram (*R*), 40 μM forskolin (*F*), or both drugs (*F/R*) for 8 h. Cells were lysed, protein concentrations were determined, and equal amounts of protein were analyzed for PDE expression by immunoblotting with monoclonal antibodies specific for human PDE4A, 4B, and 4D. These immunoreactive species migrated similarly to their respective recombinant PDEs (data not shown). *Right*, approximate molecular weight. *Pt*, patient.

In a subset of CLL patients, constitutive expression of PDE4A was detectable, predominantly as a M_r 130,000 band that comigrated with PDE4A5 (GenBank L20965; Fig. 1). Treatment with rolipram and/or forskolin did not alter expression of this protein. In contrast, PDE4D was detectable in a subset of patients only after treatment of cells with rolipram or rolipram and forskolin (Fig. 1). The M_r 70,000 immunoreactive PDE4D species migrated slightly faster than recombinant PDE4D1 (GenBank U50157).

Rolipram Treatment Up-Regulates PDE3B in CLL Cells. PDE3B was constitutively expressed as a M_r 130,000 protein at low levels in both normal splenic B cells and in leukemic cells from three CLL patients (Fig. 3) that comigrated with recombinant PDE3B. Although treatment with either an adenylate cyclase activator, forskolin, or a PDE3 inhibitor, cilostamide (10 μM), as single agents did not alter PDE3B levels, combination of these two agents augmented this enzyme in the two patients thus tested (Fig. 3). Similarly, treatment with forskolin and the PDE4 inhibitor rolipram (10 μM) augmented PDE3B levels in B cells and in cells from the three CLL patients tested. Treatment with rolipram alone augmented PDE3B levels in splenic B cells and in 1 CLL patient (patient 2). Thus, despite its ineffectiveness as a PDE3 inhibitor, rolipram augments PDE3B levels, presumably by an indirect mechanism.

A M_r 55,000 protein immunoreactive with anti-PDE7A antisera that comigrates with recombinant PDE7A is constitutively expressed in B cells and primary leukemic CLL cells (Fig. 4). PDE7A transcripts are also present in CLL cells.[5]

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[5] R. Lee, S. Wolda, E. Moon, J. Esselstyn, C. Hertel, and A. Herner. PDE7A is expressed in human B-lymphocytes and is up-regulated by elevation of intracellular cAMP. Cellular Signalling, in press, 2002.
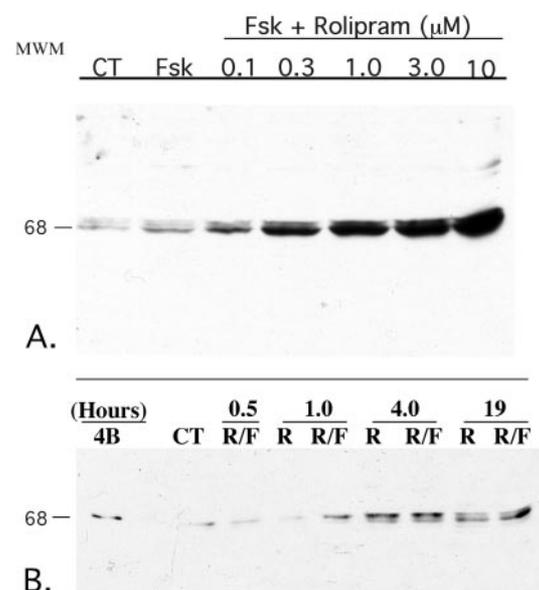

Fig. 2 PDE4B is up-regulated in CLL cells by rolipram/forskolin treatment in a time- and dose-dependent manner. *A*, CLL cells were exposed for 8 h to forskolin (*Fsk*; 40 μM) with or without the indicated concentration of rolipram. Lysates were immunoblotted with a PDE4B-specific monoclonal antibody. *B*, CLL cells from a second patient were exposed to rolipram (*R*; 10 μM) alone or a combination of rolipram and forskolin (*R/F*; 40 μM) for the indicated times, lysed, and immunoblotted for PDE4B. Recombinant PDE4B2 was run as a control.

PDE7A levels were not altered by treatment with rolipram, forskolin, or the drug combination (Fig. 4).

Cilostamide Augments Rolipram-induced Apoptosis in Cells from a Subset of CLL Patients. Because PDE3B is not inhibited by rolipram, one explanation for the relative resistance to rolipram-induced apoptosis of leukemic cells from a subset of CLL patients may be that PDE3 enzymatic activity allows such cells to escape cAMP-induced apoptosis. To test this hypothesis, we incubated leukemic cells obtained from the peripheral blood of 12 patients with CLL for 72 h with rolipram alone, cilostamide alone, or a combination of the two drugs. As expected from our prior experience, both basal apoptotic rates and the response to rolipram were variable (Fig. 5). Using a criterion of ≥50% apoptosis after treatment with 10 μM rolipram to define sensitivity, we identified 5 sensitive patients (mean apoptosis, 70 ± 4) and 7 resistant patients (mean apoptosis, 35 ± 9). Treatment with cilostamide alone had little effect: 2 ± 5% augmentation of apoptosis at 10 μM (range, −6 to 12%) and 1 ± 4% at 1 μM (range, −6 to 8%). Interestingly, however, addition of 10 μM cilostamide augmented apoptosis in 5 of 7 patients in the resistant population ($P < 0.03$). In contrast, addition of cilostamide did not augment apoptosis in any of the five patients in the sensitive group. When analyzed in the entire group of 12 patients, the effect of the addition of cilostamide on rolipram-induced apoptosis was not statistically significant ($P < 0.06$). Augmentation of rolipram-induced apoptosis was also seen in the same five patients when cilostamide was used at 1 μM rather than 10 μM (data not shown).

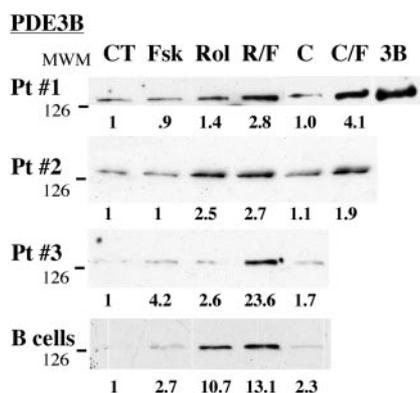Given that resistant patients had high levels of apoptosis

Fig. 3 PDE3B is up-regulated in CLL cells and peripheral blood B cells by rolipram/forskolin treatment. CLL cells or primary B cells were incubated 8 h with 10 μM cilostamide (*C*), 10 μM rolipram (*Rol*), 40 μM forskolin (*Fsk*), or combinations of these drugs (*R/F* and *C/F*) as indicated. Cells were lysed and immunoblotted with a polyclonal anti-PDE3B antibody. Recombinant PDE3B was run as a control (3B). The numbers under the lanes are derived from densitometry and are normalized relative to the control.
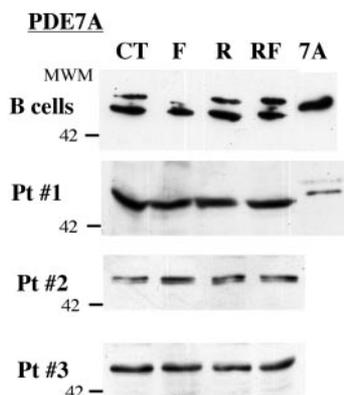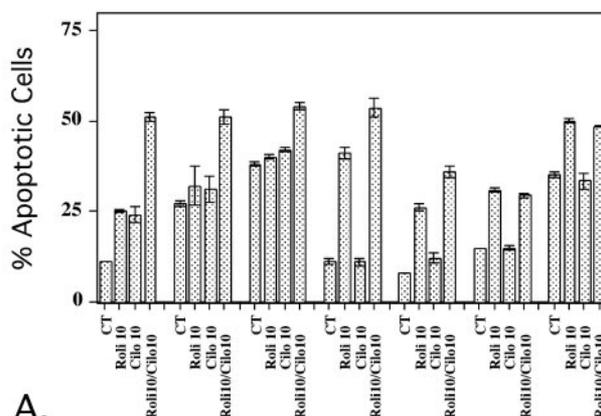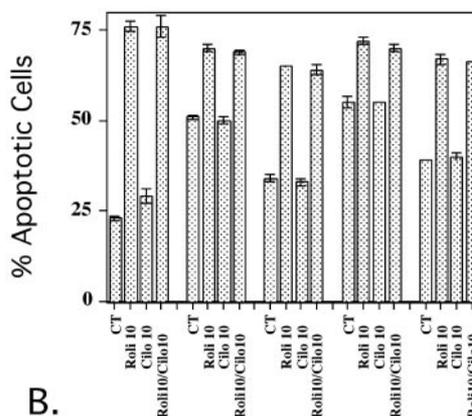

Fig. 4 PDE7A is expressed constitutively in CLL cells. CLL cells or primary B cells were incubated 8 h with 10 μM rolipram (*R*), 40 μM forskolin (*F*), or a combination of these drugs (*RF*) as indicated. Cells were lysed and immunoblotted with a monoclonal anti-PDE7 antibody. Recombinant PDE7A was run as a control (7A).

Fig. 5 Cilostamide augments rolipram-mediated apoptosis in a subset of CLL patients. One million CLL cells were incubated in triplicate cultures for 72 h in medium or the indicated concentrations of rolipram (*Roli*) and/or cilostamide (*Cilo*). Cells were then assayed for apoptosis using Hoechst 33342 dye FACS analysis. The SE (*bars*) of the percentage of apoptotic cells is shown. *A,* induction of apoptosis in 7 resistant patients (<50% apoptosis after treatment with 10 μM rolipram). *B,* induction of apoptosis in 5 sensitive patients.

when treated with 10 μM rolipram alone, it was possible that cilostamide might have a more general ability to augment rolipram-mediated apoptosis when rolipram is used at a suboptimal dosage (1 μM). However, when the same patient samples were treated with a combination of 10 μM cilostamide and 1 μM rolipram, we observed less rather than greater synergy between these two classes of drug. Among the original five patients in whom addition of cilostamide to 10 μM rolipram had increased apoptosis, similar augmentation was detected in only three patients and at a reduced level when rolipram was used at 1 μM (data not shown). One patient not identified previously as sensitive to combined inhibitor treatment now demonstrated augmented apoptosis with the addition of cilostamide.

In prior work, we found that treatment with rolipram as a single agent elevates cAMP levels in CLL cells. In four patients in whom addition of cilostamide augmented (Fig. 6, *A* and *B*) or

failed to augment (Fig. 6, *C* and *D*) rolipram-induced apoptosis, addition of 10 μM cilostamide to 10 μM rolipram did not increase total cAMP levels at 5 min, 30 min, or 12 h. Thus, cilostamide augments PDE4 inhibitor-induced apoptosis by a mechanism that does not alter total cellular cAMP levels.

DISCUSSION

A remarkable feature of cAMP-mediated signal transduction is the variety of PDEs that regulate intracellular concentrations of cAMP, now numbering at least seven distinct gene families (19). Although these enzymes all catalyze the same reaction, they differ in subcellular localization, posttranslational modification by other signaling molecules, and in their ability to regulate specific subsets of cyclic nucleotide-mediated responses. Experiments in which treatment of a homogeneous population of cells with family-specific PDE inhibitors induce different cAMP-mediated responses suggest that cAMP signaling is compartmentalized. This hypothesis has now been put on
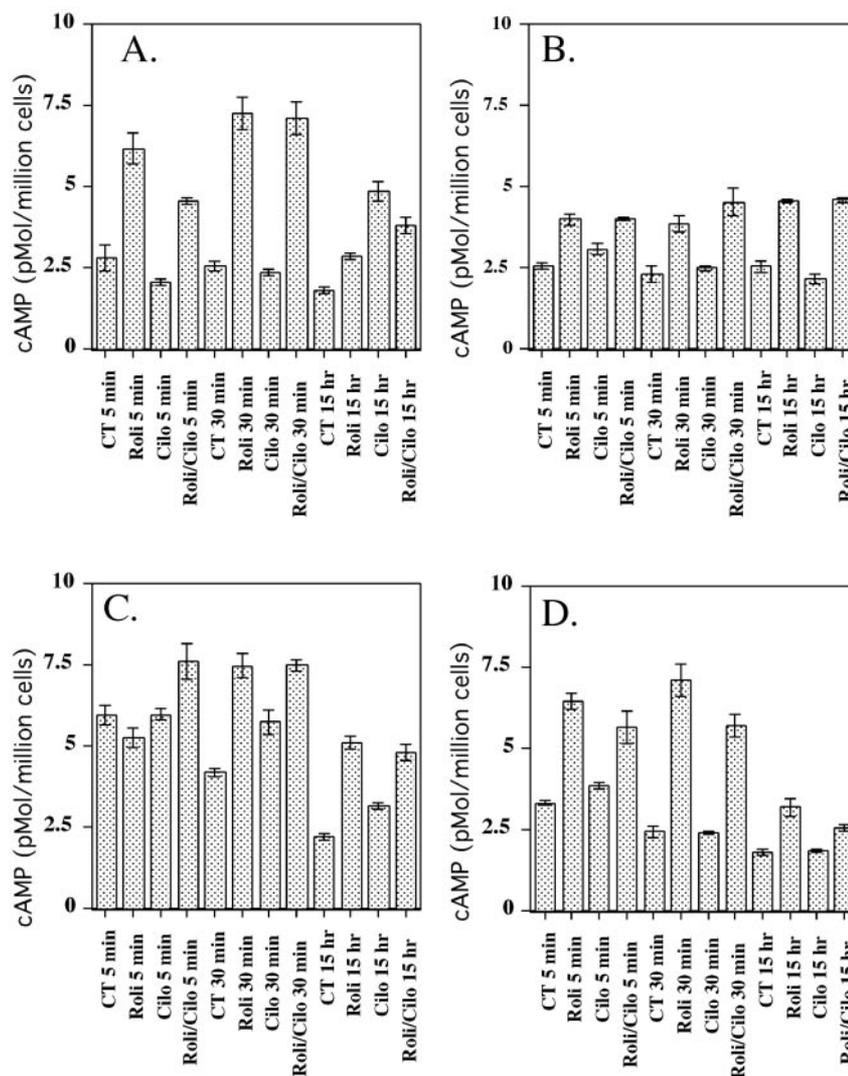
Fig. 6 Cilostamide does not consistently augment cAMP levels in CLL cells, either alone or in combination with rolipram. One million CLL cells were incubated for the indicated periods of time with 10 μM rolipram (*Roli*), 10 μM cilostamide (*Cilo*), or the combination (*Roli/Cilo*). Total cellular cAMP was determined in triplicate by RIA. Cilostamide augmented rolipram-mediated apoptosis in patients *A* and *B,* although it did not in patients *C* and *D*. *Bars,* SE.

a firm molecular basis with the discovery that enzymes influencing cAMP-mediated signal transduction may be physically associated in signaling modules, such as the association of cardiac PDE4D3 with protein kinase A through the adapter molecule mAKAP (20).

Previous reports that have surveyed the expression of cAMP PDEs in lymphoid cells have primarily performed RT-PCR in lymphoid cell lines and in whole mononuclear cells (6, 9). In this study, we have used a series of isoform-specific monoclonal antibodies and antisera to determine PDE expression in CLL cells at the protein level, because variations in translational efficiency or protein turnover may render RT-PCR results misleading. Our study did not examine PDE activity, which can be altered by posttranslational modifications such as phosphorylation by protein kinase A or extracellular signal-regulated kinase 2 (21, 22).

Knockout studies have demonstrated that the physiological function of PDE4 enzymes vary as a function of the gene from which they are derived (*i.e.,* A–D; Refs. 11, 23, 24). In addition,

specific PDE4 "long" and "short" splice isoforms that contain or lack an upstream conserved region denoted UCR1, respectively, differ in their association with the plasma membrane or the cytoskeleton (11, 25). We found constitutive expression of a M_r 135,000 ("long") form of PDE4A and a M_r 65,000 ("short") form of PDE4B in CLL cells, whereas PDE4D was not constitutively expressed. Treatment with the PDE4 inhibitor rolipram consistently augmented expression of the M_r 65,000 form of PDE4B and variably augmented a M_r 70,000 form of PDE4D.

Previous studies have documented that PDE3B is the principal form of PDE3 expressed in human lymphoid cells (7, 9). A study examining the response of the human T-cell line Jurkat to the adenylate cyclase activator forskolin showed a sustained increase in PDE3 activity after 3 h that could be inhibited by cotreatment with actinomycin D (6). Similarly, another group reported that 8-bromo-cAMP augments PDE3B transcript and Org 9935-inhibitable PDE activity (a measure of PDE3 activity) in human T lymphocytes (9). Our work differs from these studies in that we found that

treatment with a PDE4 specific inhibitor and forskolin, but not forskolin alone, can up-regulate immunoreactive PDE3B in primary CLL cells. This result suggests that the intracellular pool of cAMP augmented by PDE4 inhibitors can drive compensatory up-regulation of PDE3B levels.

In a subset of CLL patients, addition of the PDE3 inhibitor cilostamide at 1 or 10 μM modestly augmented rolipram-mediated apoptosis when the PDE4 inhibitor was used at 10 μM but not at 1 μM. Cilostamide as a single agent, in contrast, had no effect. These results confirm that PDE4 is the dominant PDE that regulates pools of cAMP that can drive apoptosis in CLL cells but suggest that residual PDE3 activity in a subset of patients may reduce the ability of PDE4 inhibitor-induced elevation of intracellular cAMP to induce apoptosis. Numerous studies have examined the relative contribution of PDE3 and PDE4 to the regulation of cAMP-mediated processes (8, 26–35). Although distinct roles for these two classes of PDEs have been noted for some cAMP-mediated processes (20, 21), in most cases the combination of PDE3 and four inhibitors have had additive or synergistic effects (22–30). In our study, assays of total intracellular cAMP failed to demonstrate higher levels of cAMP in those CLL patients that were sensitive to the addition of cilostamide to rolipram. Similarly, others have reported that cilostamide can synergize with rolipram in altering cAMP-mediated processes in the absence of a change in total cellular cAMP (36).

Our observations suggest that induction of apoptosis in lymphoid malignancies by monotherapy with PDE4-specific inhibitors may be compromised by PDE activity derived from other PDE families. In particular, if hormonal stimuli normally activate adenylate cyclase *in vivo* but not *in vitro*, PDE4 inhibition may augment levels of PDE3B in CLL patient's leukemic cells. Such a hypothesis could have practical implications for the design of trials to investigate the efficacy of treatment of CLL with PDE inhibitors. Several drugs that inhibit both PDE3 and PDE4 have been identified; such dual selective drugs are of particular interest in the treatment of asthma (37). Zardaverine, a mixed PDE3/4 inhibitor, is a more potent inhibitor of T-cell proliferation than rolipram and improved FEV$_1$ in patients with mild to moderate asthma (38, 39). Similarly, the mixed PDE3/4 inhibitor benafentrine has demonstrated clinical activity in asthmatics (40).

Alternatively, as PDE3 inhibitors have proven to be relatively well-tolerated drugs for other indications such as claudication, combined therapy with specific PDE3 and PDE4 inhibitors may be feasible (41). Although clinical trials examining the use of PDE3 inhibitors and PDE4 inhibitors as monotherapy have not noted myeloid progenitor cell suppression as judged by peripheral blood counts, the effect of dual PDE3/4 inhibition on human marrow function remains unknown because no clinical trials have used therapeutic concentrations of both drug types. Although not yet Food and Drug Administration approved, PDE4 inhibitors are an active area of pharmaceutical research. When rolipram was examined in clinical trials as potential therapy for depression or Parkinson's disease, nausea was noted as a side effect in many patients, despite the relatively low doses used (0.5–1.0 mg p.o. three times/day; Refs. 42–45). Unfortunately, serum levels of rolipram were not measured in these trials. Subsequent studies have suggested that the emetic properties of PDE4 inhibitors can be at least partially dissociated

from their anti-inflammatory activities (46). Establishing the efficacy of PDE inhibition as therapy for CLL will have to await the development of a safe and well-tolerated PDE4 inhibitor.

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