Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia

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Cyclic nucleotide phosphodiesterase (PDE) isoforms can influence disease pathogenesis and be novel therapeutic targets. Because lower cAMP levels may contribute to the decreased apoptosis that occurs in chronic lymphocytic leukemia (CLL), we assessed the expression levels of PDE isoforms in peripheral blood mononuclear cells (PBMC) of healthy adults and patients with CLL. We found a unique PDE mRNA signature in CLL: higher levels than in normal PBMC of PDE7B (increased ≈23-fold) and lower levels of PDE3B, 4D, 5A, and 9A mRNA (each decreased ≈30-fold). Increased PDE7B mRNA in CLL correlates with a 10-fold-higher expression of PDE7B protein and results in an increased contribution of PDE7 to total PDE activity. Consistent with the higher level of PDE7B expression, inhibitors of PDE7 (BRL-50481, IR-202) and a dual PDE4/PDE7 inhibitor (IR-284) selectively increase apoptosis in CLL cells compared with normal PBMC or B cells. Apoptosis of CLL cells promoted by inhibitors of PDE7 and PDE4/7 is attenuated by PKA inhibition, occurs via a mitochondrial-dependent process, and is associated with increased cAMP accumulation and down-regulation of the antiapoptotic protein survivin and of PDE7B. The increase in PDE7B expression and PDE7 inhibitor-promoted apoptosis implicates PDE7B as a drug target in CLL. Our findings identify a unique PDE signature in CLL and illustrate the utility of broad analyses of PDE isoform expression in human disease.

apoptosis | B cell | cAMP | survivin

Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia, is characterized by accumulation of $CD5^+$, $CD19^+$, and $CD23^+$ B cells (1). Therapeutic approaches aim to induce apoptosis of these malignant B cells. Because the second messenger cAMP can promote apoptosis of neoplastic lymphocytes by activating protein kinase A (PKA), pharmacological agents that increase cAMP levels have the potential to treat CLL (2–4). The concentration of cAMP and activity of PKA are lower in lymphocytes of CLL patients compared with those of normal subjects, suggesting a disease-related defect in this pathway (5, 6).

The cellular level of cAMP is governed by its formation by adenylyl cyclases and hydrolysis by cyclic nucleotide phosphodiesterases (PDEs). Eleven families of PDEs, comprising multiple isoforms and splice variants, hydrolyze cAMP and cGMP and have unique regulatory characteristics, cellular distribution, subcellular localization, and sensitivities to inhibitors (7, 8). The nonselective PDE inhibitor theophylline increases cAMP, induces apoptosis, and down-regulates expression of the antiapoptotic protein Bcl-2 in CLL cells (9, 10). Administration of theophylline increases the response rate and progression-free survival of CLL patients treated with drugs such as chlorambucil (11, 12), but theophylline has a narrow therapeutic index. Identification of PDE isoforms selectively or highly expressed in CLL cells could provide a basis for the use of isoform-selective PDE inhibitors to promote apoptosis of these leukemic cells.

CLL cells express numerous PDEs, including PDE4 isoforms, whose hydrolytic activity is cAMP-selective (3, 4, 13–15). PDE4-specific inhibitors promote apoptosis of CLL cells and augment killing by glucocorticoids (3). Previous studies have not comprehensively assessed PDE isoform expression in CLL. By performing such an assessment, we find that CLL cells have an expression profile of PDE isoforms that distinguishes them from normal lymphocytes. Our data reveal that CLL cells have increased expression of PDE7B, that this isoform is an important regulator of cAMP-PDE activity in these cells, and that both inhibitors of PDE7 and a dual PDE4/7 inhibitor kill CLL cells at concentrations that have little toxicity on normal B cells, thus highlighting PDE7B as a potential therapeutic target in this disease.

Results

Expression Pattern of PDE Isoforms in Peripheral Blood Mononuclear Cells (PBMC) from CLL Patients. We examined the mRNA expression of PDE isoforms by quantitative PCR (QPCR) after validating the use of two sets of primers specific for 18 PDE isoforms using human reference RNA. We found that QPCR is highly reproducible for investigating PDE isoform expression, provided that the efficiency of the primers and the product sequence are verified, the primers detect all variants of each PDE isoform, and a suitable housekeeping gene is used for normalization. Use of the appropriate primers revealed that PBMC from normal subjects and CLL patients express mRNA for 15 PDE isoforms: PDE1B, 1C, 2A, 3A, 3B, 4A, 4B, 4C, 4D, 5A, 7A, 7B, 8A, 8B, and 9A; we did not detect PDE1A, PDE10A, or PDE11A although our primers detected these isoforms in human reference RNA.

Using QPCR we evaluated PDE expression in PBMC from 7 CLL patients and 7 healthy subjects [supporting information (SI) Fig. S1]. Samples were compared by using the relative cycle threshold (C_t) method; lower C_t values reflect greater mRNA expression. As noted previously (4), we found that PDE4B mRNA is the highest expressed in PBMC from healthy adults and CLL patients. Compared with normal PBMC, CLL cells had higher expression levels of the mRNA encoding PDE7B and lower expression of mRNA encoding PDE3B, PDE4D, PDE5A,

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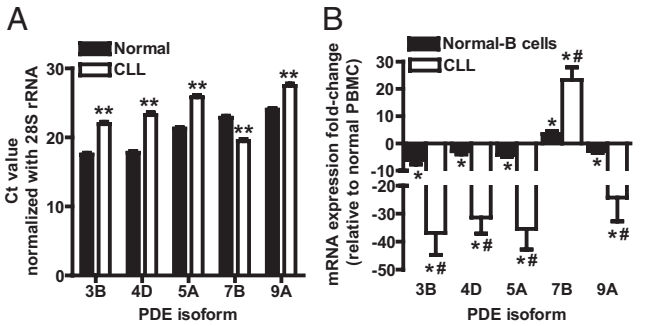

Fig. 1. mRNA expression of multiple PDE isoforms is altered in CLL. (*A*) QPCR analysis of the expression of PDE isoforms that show differences with CLL from 16 normal subjects (normal) and 25 CLL patients (CLL). Data are expressed as C_t normalized to 28S rRNA (mean ± SEM). **, $P < 0.01$. (*B*) QPCR showing the mRNA expression of PDE3B, 4D, 5A, 7B, and 9A from normal B cells [obtained by CD19-positive selection ($n = 10$) or negative isolation ($n = 3$)] and CLL cells ($n = 25$–60) compared with normal PBMC. *, $P < 0.01$. Data are expressed as fold change of each PDE isoform relative to the average expression in normal PBMC. In addition, CLL cells have significantly different expression of each of the PDE isoforms compared with normal B cells. #, $P < 0.05$.

and PDE9A without consistent differences in any of the other PDE isoforms (Fig. 1). The mRNA encoding PDE7B is one of the most abundant PDE transcripts expressed in CLL cells (Fig. S1). Studies of additional subjects revealed that CLL cells have ≈20-fold-higher expression of PDE7B mRNA and lower expression of PDE3B, 4D, 5A, and 9A (37-, 31-, 35-, and 24-fold, respectively) than do normal PBMC (Fig. 1).

Altered PDE mRNA Expression Results from Malignant B Cells in CLL. Because 90% of the CLL cells are B cells, but normal PBMC are composed mostly of T cells, we tested whether the CLL PDE profile might result from the increased number of B cells by assessing B cells isolated from normal PBMC for the expression of PDEs altered in CLL. Compared with normal PBMC, CLL cells and purified B cells have ≈23-fold and ≈3-fold respective increases in the expression of PDE7B mRNA and isolated B cells have lower expression of PDE3B, PDE4D, PDE5A, and PDE9A (≈6-, ≈3-, ≈4-, and ≈2-fold, respectively), albeit to levels not as low as those observed in CLL (Fig. 1*B*). Thus, the increased PDE7B expression and decreased expression of the other PDEs in CLL relate to a minor extent to the B cell enrichment in CLL but much more prominently to the malignancy of the B cells. We focused further analysis on PDE7B because its expression was enhanced in CLL and thus we reasoned that it might be a novel target in this disease.

PDE7B Expression and PDE7 Enzymatic Activity Are Increased in CLL Cells. We assessed the expression of PDE7B mRNA in CLL cells from 60 CLL patients (Fig. 1*B*) and found that 95% of patients had 2-fold or greater expression of PDE7B mRNA in their CLL cells than did PBMC of healthy adults. Moreover, 37% of the patients had >18-fold higher PDE7B mRNA than did normal PBMC. Immunoblot analyses revealed that CLL cells have 10 ± 2-fold more PDE7B protein than do normal PBMC (Fig. 2*A*); 94% of the patients had cells with 3-fold or more PDE7B protein expression with 37% having at least 11-fold greater expression. Increased PDE7B protein expression correlated with the increase in PDE7B mRNA ($r = 0.414$, $P < 0.05$). The PDE7B protein localizes to the membrane and insoluble fractions of CLL cells, unlike PDE4B, which is predominantly cytosolic and primarily represents PDE4B2 (78 kDa) and PDE4B3 (100 kDa) (Fig. 2*B* and Fig. S2). Consistent with the RNA and protein expression data, studies with the PDE7 inhibitor BRL-50481

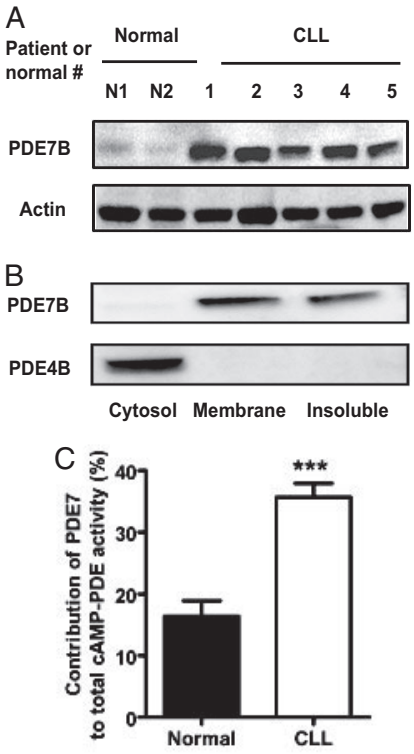

Fig. 2. PDE7B protein expression and the contribution of PDE7 to total cAMP-PDE activity are increased in CLL cells. (*A*) Shown is a representative immunoblot of PDE7B (52 kDa) and β-actin (43 kDa) protein expression in 2 normal subjects (N1 and N2) and 5 CLL patients. Quantification of PDE7B protein expression normalized to β-actin showed a 10.8 ± 1.5-fold increase in CLL cells ($n = 19$) vs. normal PBMC ($n = 5$) ($P < 0.01$; samples from 2 other normal subjects had PDE7B protein levels that were below the level of detection). (*B*) Representative immunoblot of PDE7B (52 kDa) and PDE4B (100 kDa, PDE4B2) expression in cytosol, membrane, and insoluble fractions of CLL cells (data representative of $n = 5$; Fig. S2 shows all PDE4B isoforms detected). (*C*) The contribution of PDE7 to total cAMP-PDE activity was determined by using the PDE7 inhibitor BRL-50481 (30 μM, $n = 9$). Data are mean ± SEM. ***, $P < 0.001$ compared with normal.

revealed that PDE7 contributes more to cAMP-PDE activity in CLL cells than in normal PBMC (Fig. 2*C*).

CLL Cells Are More Sensitive to the Cytotoxic Effects of PDE4 and PDE7 Inhibitors than Are Normal PBMC. Because cAMP levels can influence survival of leukemia cells (2, 14) and PDE7B selectively hydrolyzes cAMP, we examined whether PDE7 inhibitors induce apoptosis of CLL cells. We found that CLL cells are more sensitive than PBMC of healthy donors to proapoptotic effects of PDE7 inhibitors [BRL-50481, IC_{50} 200 nM; and IR-202, IC_{50} 85 nM, for inhibition of cAMP hydrolysis (16, 17)] but were not killed by inhibitors of PDE3 (milrinone) or PDE5 (T-0165) (Fig. 3*A*). Both PDE7 inhibitors, used at concentrations below the IC_{50} for inhibition of other PDEs, selectively induced apoptosis of CLL cells (Fig. 3*A*) (16–18).

PDE7B is an abundantly expressed PDE in CLL cells, but PDE4B is the highest expressed PDE isoform (Fig. S1). Consistent with these data, and confirming previous work, we found that inhibitors of PDE4 (either rolipram or RO20-1724) induce apoptosis in CLL cells (3, 19). We hypothesized that combined inhibition of PDE7 and PDE4 would increase killing of CLL cells. In support of this hypothesis, we found that the selective PDE7 inhibitor BRL-50481 (30 μM), which itself kills CLL cells, enhanced apoptosis induced by the PDE4 inhibitor rolipram (3 μM) (Fig. 3*B*). Moreover, BRL-50481 enhanced

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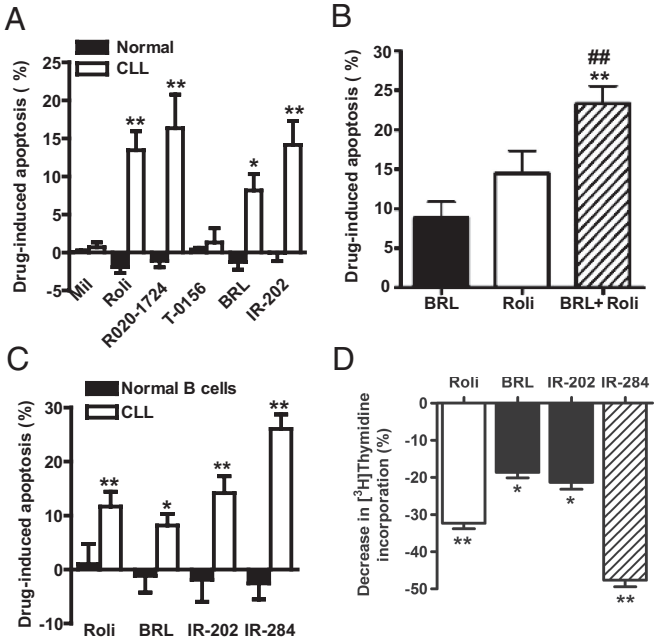

Fig. 3. PDE7 inhibition induces apoptosis in CLL cells but not normal PBMC. (*A*) Apoptosis was assessed in PBMC from normal subjects and CLL patients by staining with annexin V and analysis by flow cytometry after 48 h of incubation with vehicle, 10 μM milrinone (Mil), rolipram (Roli), R020-1724, T-0156, or IR-202 or 30 μM BRL-50481 (BRL). Data are expressed as drug-induced apoptosis (%) for each PDE inhibitor. Each data point represents mean ± SEM of 3–8 normal subjects and 3–10 B-CLL patients. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle. (*B*) Apoptosis of CLL cells induced by 30 μM BRL-54081, 3 μM Roli, or their combination for 48 h ($n = 7$). Data (mean ± SEM) are expressed as drug-induced apoptosis (%). **, $P < 0.01$ for combination of BRL-50481 and Roli vs. BRL-50481 alone; ##, $P < 0.01$ for combination of BRL-50481 and Roli vs. Roli alone. (*C*) Apoptosis in normal B cells (labeled using a PE-conjugated anti-CD19 antibody) and CLL cells after 48 h of incubation with vehicle, 10 μM rolipram (Roli), 30 μM BRL-50481 (BRL), 10 μM IR-202, or 100 nM IR-284. Data (mean ± SEM) are expressed as drug-induced apoptosis (%) ($n = 8$–10). *, $P < 0.05$; **, $P < 0.01$ compared with vehicle. (*D*) Proliferation of EHEB cells incubated for 48 h with vehicle, 10 μM rolipram (Roli), 30 μM BRL-50481 (BRL), 10 μM IR-202, or 100 nM IR-284. Data (mean ± SEM) are expressed as drug-induced decrease in proliferation (%) ($n = 4$). *, $P < 0.05$; **, $P < 0.01$ compared with vehicle.

rolipram-induced apoptosis of freshly isolated CLL cells, which have lower basal apoptosis (Fig. S3): BRL-50481 shifted the concentration–response curve for rolipram-induced apoptosis to the left (i.e., enhancing the apoptotic effect of low-dose rolipram) and increased the maximal response. These data provide further evidence for the potential of PDE7B as a drug target in CLL that can enhance the proapoptotic effect of low-dose PDE4 inhibitors. In accordance with these findings, we found that a dual PDE4/7 inhibitor, IR-284 (patent WO 02/085906, IC_{50} 2.3 nM for PDE4-mediated and 23 nM for PDE7-mediated inhibition of cAMP hydrolysis), induces apoptosis of CLL cells: concentrations from 10 nM to 10 μM selectively kill CLL cells compared with normal PBMC (Fig. S3*B*).

PDE7 and Dual PDE4/PDE7 Inhibition Induce Significantly Less Apoptosis in Normal B Cells than Does Inhibition of PDE4. We tested killing of normal B cells by various PDE inhibitors and found that inhibition of PDE4, PDE7, or dual inhibition of PDE4/7 does not significantly kill these cells, in contrast to the proapoptotic effect observed in CLL cells (Fig. 3*C*). Normal B cells from 3 of 8 subjects showed apoptosis in response to rolipram whereas only one was sensitive to apoptosis in response to BRL-50481, IR-202, or IR-248. PDE7 and dual PDE4/7 inhibitors are thus more selective in killing of CLL than normal B cells, consistent with the higher PDE7B expression in CLL cells.

PDE4, PDE7, and Dual PDE4/PDE7 Inhibitors Decrease the Proliferation of EHEB Cells. Rolipram (10 μM), BRL-50481 (30 μM), IR-202 (10 μM), and IR-284 (100 nM) each inhibited the proliferation of EHEB, a CLL cell line (20), decreasing proliferation 32 ± 1%, 19 ± 1%, 21 ± 2%, and 48 ± 2%, respectively (Fig. 3*D*). IR-284 was more efficacious, perhaps because it inhibits both PDE4 and PDE7. EHEB cells are easy to maintain in culture and thus may be useful for identifying other PDE inhibitors with potential therapeutic utility in CLL.

PDE7 Inhibitor-Induced Apoptosis of CLL Cells Occurs via a cAMP/PKA-, Mitochondrial-Dependent Pathway. Inhibition of PDE4 (10 μM rolipram) and PDE7 (30 μM BRL-50481, 10 μM IR-202) and dual inhibition of PDE4 and PDE7 (100 nM IR-284) increased basal and forskolin-stimulated cAMP accumulation in CLL cells, consistent with the idea that the proapoptotic effect of PDE inhibition occurs, at least in part, via a cAMP-dependent mechanism (Fig. 4*A*). PKA has been shown to be important for cAMP-dependent apoptosis of malignant lymphoid cells (2, 10, 21). Two PKA-selective inhibitors [PKI (14–22, 5 μM) and Rp-cAMP (Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium, 100 μM)] attenuated IR202-induced (54 ± 9% and 75 ± 8%, respectively) and IR-284-induced (24 ± 6% and 28 ± 11%, respectively) apoptosis (Fig. 4*B*). Thus, a cAMP/PKA-dependent pathway likely mediates the killing of CLL cells promoted by PDE7 and PDE4/7 inhibitors.

Mitochondrial depolarization and the release of cytochrome *c* are key events in mitochondrial-dependent apoptosis (22). Rolipram, BRL-50481, IR-202, and IR-284 induced mitochondrial depolarization (25 ± 4%, 13 ± 4%, 17 ± 6%, and 39 ± 5% depolarization, respectively, vs. vehicle) and released cytochrome *c* from mitochondria into the cytosol (Fig. 4*C*), implying that PDE inhibitor-induced apoptosis of CLL cells occurs via a mitochondrial-dependent mechanism.

The proapoptotic and antiapoptotic proteins BIM and survivin, respectively, contribute to cAMP-induced apoptosis in T cell-derived lymphoma (2, 23). We analyzed the impact of PDE inhibitors on their expression and that of PDE7A and PDE7B because inhibition of PDE in CLL cells can secondarily increase PDE protein expression (13, 15). Incubation of CLL cells with IR-284 for 24 h decreased PDE7B protein expression (70 ± 14% decrease vs. vehicle; Fig. 4*D*) without significantly altering expression of PDE7A. Although PDE4, PDE7, or PDE4/7 inhibition did not enhance BIM expression (data not shown), survivin expression decreased (45 ± 10% vs. vehicle) after PDE4/7 inhibition, thus highlighting this antiapoptotic protein as a target for cAMP-promoted apoptosis in CLL.

Discussion

To our knowledge, these results provide the first comprehensive analysis of PDE isoforms in relation to human cancer; previous studies have examined expression of certain PDE isoforms in malignant cells (4, 13, 14, 24–27). We find that PBMC and normal B cells express PDE7B and that CLL cells selectively overexpress this isoform, highlighting PDE7B as a potential therapeutic target in CLL. Expression of other PDE isoforms (PDE3B, 4D, 5A, and 9A) is decreased in CLL cells. Because PBMC are enriched in T cells, whereas CLL cells are predominately B cells, differences in PDE isoform expression may result from differences in expression of the cell populations. However, we find large differences in expression of the PDE isoforms in CLL vs. normal B cells, implying that the PDE expression pattern in CLL cells is characteristic of the malignancy, perhaps as a result of clonal expansion of a subset of B cells. More generally,

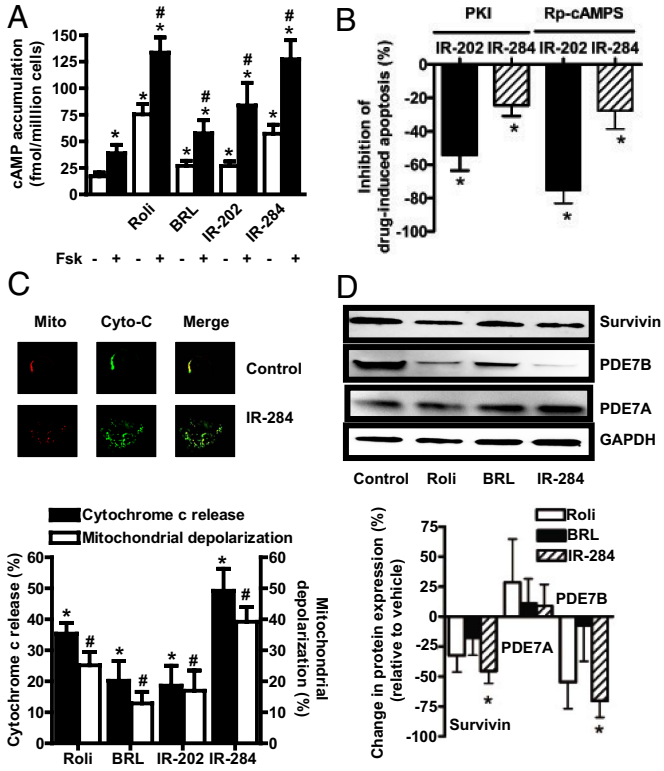

Fig. 4. Apoptosis of CLL cells in response to PDE7 inhibition occurs via a cAMP-, mitochondrial-dependent mechanism. (*A*) cAMP accumulation in CLL cells with or without 10 μM forskolin (Fsk) with or without 10 μM rolipram (Roli), 30 μM BRL-54081 (BRL), 10 μM IR-202, or 100 nM IR-284 for 10 min (n = 8–16), Data are expressed as mean ± SEM cAMP accumulation (femtomoles per million cells). *, $P < 0.05$ compared with basal; #, $P < 0.05$ compared with forskolin alone. (*B*) Apoptosis of CLL cells induced by 10 μM IR-202 and 100 nM IR-284 in the presence of PKA inhibitors (5 μM PKI, 100 μM Rp-cAMPS) or vehicle for 48 h (n = 6–9); data are expressed as mean ± SEM inhibition of drug-induced apoptosis (%). *, $P < 0.05$ compared to vehicle. (*C Upper*) Representative image of mitochondria (Mito, red), cytochrome *c* (Cyto-c, green), and their colocalization (Merge, yellow) before (control) and after 24 h of treatment of CLL cells with 100 nM IR-284. (*C Lower*) Data from multiple experiments showing PDE inhibitor-induced [10 μM rolipram (Roli), 30 μM BRL-54081 (BRL), 10 μM IR-202, or 100 nM IR-284] cytochrome *c* release (percentage of cells showing release, filled bars) and mitochondrial membrane depolarization of CLL cells (percentage of cells showing depolarization by JC-1 assay, open bars). Data are mean ± SEM (n = 5–8). * and #, $P < 0.05$ compared with vehicle. (*D Upper*) Representative immunoblot of survivin (16.5 kDa), PDE7A (50 kDa), PDE7B (52 kDa), and GAPDH (37 kDa) in CLL cells treated with vehicle (control), 10 μM rolipram (Roli), 30 μM BRL-54081 (BRL), or 100 nM IR-284. (*D Lower*) Quantification of immunoblots from CLL cells expressed as percentage change in protein expression (mean ± SEM, n = 5; *, $P < 0.05$ compared with vehicle).

the findings indicate that profiling PDE expression, which we have also used to identify novel increases in PDE1A and PDE1C with pulmonary hypertension (28), may prove useful for understanding pathophysiology and identifying therapeutic targets.

PDE7B, the isoform increased in CLL, together with PDE7A comprise the PDE7 family. After discovery of the PDE7 isoforms and their tissue expression (29–32), human B cells were noted to have PDE7 activity and high expression of PDE7 mRNA (33). PDE7A and 7B are high-affinity cAMP-specific PDEs (K_m for cAMP is <0.2 μM) (7, 29–32). PDE7A is abundant in brain, proinflammatory, and immune cells and is involved in T cell activation and proliferation; by contrast, PDE7B is highly expressed in brain, liver, and skeletal muscle and, based on its up-regulation by dopamine, is thought to contribute to dopaminergic signaling (18, 29, 31, 32, 34–37). PDE7A, but not PDE7B, is a dual inhibitor of cAMP signaling, increasing hydrolysis of cAMP and directly inhibiting PKA activity via a PKA pseudosubstrate site (38). The functional consequence of this site and relationship to transcriptional activation of PDE7B by cAMP/PKA via activation of the cAMP-response element binding (CREB) protein are unclear (31, 39). Investigation of the regulation of PDE7B in B cells should help define its function in these cells, mechanisms involved in its cellular expression, and its role in CLL.

The increase in PDE7B expression in CLL may be therapeutically relevant: We find that two different PDE7 inhibitors promote apoptosis of CLL cells and decrease proliferation of EHEB, a CLL cell line. Because PDE7 inhibitors are nonselective we attempted to use siRNA to inhibit PDE7B as a means to determine its contribution to effects observed with such inhibitors; however, because of poor transfection efficiency and high levels of basal apoptosis of CLL cells, these experiments were not successful (data not shown). PDE7A and PDE7B are 70% identical and thus the design of isoform-specific inhibitors is a challenge, although their differences may be sufficient for identification of inhibitors with specificity for PDE7B (7, 31, 32).

PDE4B, the main cAMP-PDE in PBMC and CLL cells, is the target of the PDE4-selective inhibitor rolipram (3, 4, 19). PDE7 inhibition enhances the proapoptotic effects of rolipram, which led us to synthesize and test IR-284, a PDE4/7 dual inhibitor. PDE4 inhibitors have dose-limiting cardiovascular, gastrointestinal, and central nervous system side effects; thus, combining a low dose of a PDE4 inhibitor with a PDE7 inhibitor or a dual PDE4/7 inhibitor may reduce side effects, increase efficacy for CLL cell killing, and perhaps be useful for the treatment of CLL (35, 40) by providing increased specificity but decreased toxicity of normal B or T cells (Fig. 3*C*) (16, 35). PDE7 and PDE4/7 inhibitors increase killing of CLL cells by dexamethasone (data not shown), suggesting that the PDE inhibitors may be a useful addition to current treatment regimes.

Activation of the cAMP/PKA-dependent pathway contributes to the proapoptotic effects of PDE7 and PDE4/7 inhibitors (Fig. 4). Such results and previous data imply that in CLL cells the activity of PDEs is important in governing cellular cAMP levels (4, 19). PDE7 and PDE4/7 inhibitors show greater killing relative to their ability to raise cAMP than does rolipram, suggesting that cellular compartmentation of cAMP and of PDE isoforms contributes to apoptotic response, perhaps facilitating interaction with signaling pathways (7, 8, 41). Unlike PDE4 inhibition or forskolin, PDE7 inhibition activates PKA but not Epac (exchange protein directly activated by cAMP) (15, 21), a downstream effector of cAMP. Because activation of Epac, but not PKA, is antiapoptotic in CLL cells (21), PDE7 inhibitors would be predicted to be more effective than PDE4 inhibitors in enhancing cell killing. Moreover, PKA inhibition does not completely block the proapoptotic effects of IR-202 or IR-284 [akin to results obtained with theophylline- and rolipram-induced apoptosis (10, 21)], providing evidence that PDE inhibitors may act by non-PKA mechanisms to kill CLL cells.

PDE4 inhibition and, based on data shown here, PDE7 inhibition promote apoptosis of CLL cells by the intrinsic cell death pathway, releasing cytochrome *c* from mitochondria and cleaving caspases 3 and 9 (19). Consistent with evidence that the antiapoptotic protein survivin contributes to cAMP/PKA-dependent apoptosis of murine lymphoma cells (23), IR-284 decreases expression of survivin (Fig. 4*D*), implicating survivin as a target for cAMP-promoted apoptosis, an idea that contrasts with other reports regarding survivin expression in CLL (42, 43).

We were surprised to find that PDE4/7 inhibition decreases PDE7B expression (Fig. 4*D*) because previous data show that cAMP increases the expression of other PDEs in CLL cells (13,

15), perhaps via a compensatory feedback loop (7, 8). However, recent studies have shown that cAMP, via the induction of ICER, can decrease PDE3A and PDE4A10 in cardiac myocytes (44, 45). The decrease in PDE7B expression that we observe with increasing cAMP may be due to a similar transcriptional mechanism or to the increase in cell killing, i.e., a depletion of the CLL cells that express a high level of this PDE isoform.

In summary, the current data indicate that CLL cells have a unique overexpression of PDE7B and that inhibition of PDE7 promotes apoptosis in CLL, a disease associated with defective apoptosis. Selective inhibition of PDE7 or dual PDE4/7 inhibition may provide a novel therapeutic approach for the treatment of CLL by enhancing killing and increasing specificity for CLL cells. More generally, the current data indicate that comprehensive analysis of PDEs in disease settings can reveal patterns of isoform expression that have the potential to serve as diagnostic markers and/or therapeutic targets.

Materials and Methods

Unless stated, all chemicals and cell culture reagents were purchased from Sigma–Aldrich and Gibco BRL, respectively. The PDE7 inhibitor BRL-50481 [3-(*N*,*N*-dimethylsulfonamido)-4-methyl-nitrobenzene] was from TOCRIS. IR-202 [(*Z*)-methyl 4-(5-(3-hydroxycyclohexylimino)-4-methyl-4, 5-dihydro-1, 3,4-thiadiazol-2-yl) phenylcarbamate] and IR-284 [(4-(3-chloro-4-methoxyphenyl)-2-(1-morpholine-4-carbonyl) piperadin-4-yl)-4a, 5,8,8a-tetrahydrophthalazin-1(2*H*)-one (patent WO 02/085906)] were prepared by the University of California at San Diego Medicinal Chemistry Core as described (17). The PKA inhibitor PKI [myristylated PKI (14–22) amide] was from Biomol.

PBMC Isolation. Blood was collected from healthy donors (normal PBMC) and CLL patients (CLL cells) after informed consent, in agreement with institutional guidelines. All use of patient data and samples followed or exceeded the guidelines of the Health Insurance Portability and Accountability Act (46). CLL diagnosis was made by blood cell morphology and immunophenotyping. The patients' median age was 61 yr (range, 44–75 yr), with a median WBC of 142×10^6 cells per milliliter (range, 15–460 $\times$ 10^6 cells per milliliter). PBMC were isolated by density-gradient centrifugation using Ficoll-Paque (Amersham Biosciences), washed, suspended in FCS containing 10% DMSO, then stored in liquid N_2 for subsequent use.

Isolation of B Cells. B cells were isolated from normal PBMC by using either Dynabeads CD19 pan B or a Dynal B cell negative isolation kit (Invitrogen), which resulted in >90% $CD19^+$ cells, as assessed by flow cytometry.

EHEB Cell Culture. EHEB cells (from the German Collection of Microorganisms and Cell Cultures) were grown in 90% RPMI medium 1640 plus 10% FBS and maintained at 0.1–0.5 $\times$ 10^6 cells per milliliter at 5% CO_2 at 37 °C.

Real-Time RT-PCR (QPCR). Total RNA was isolated from PBMC by use of the Versagene RNA Cell Kit (Gentra). SuperScript III First Strand Synthesis System (Invitrogen) was used to synthesize cDNA. Real-time PCR (MJ Research Opticon 2 using Eurogentec QPCR Mastermix Plus SYBR Green Kit) was performed by using 8 ng of RNA per reaction and 100 nM sense/antisense primers. Two primer sets were designed for each PDE isoform, one that we previously published (28) and the other available upon request. All primers were validated by using human reference RNA (Stratagene). Thermal cycling conditions were as follows: incubation 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C. Samples were compared by using the relative cycle threshold (C_t) method normalizing to 28S rRNA.

Western Blot Analysis. Western blotting was performed on 5–10 $\times$ 10^6 cells per sample. Lysates were prepared as previously described (2), separated by gel electrophoresis, immunoblotted by using the NuPage gel system (Invitrogen), and visualized by horseradish peroxidase and enhanced chemiluminescence (Amersham). Antibodies used were directed against PDE7A (FabGennix), PDE7B (FabGennix and Orbigen), PDE4B (FabGennix), survivin (Novus Biologicals), BIM (BD Biosciences), β-actin (Santa Cruz Biotechnology), and GAPDH (Abcam).

Subcellular Fractionation. CLL cells (3×10^7) were separated into cytosolic, membrane, and insoluble fractions. Cells were washed with ice-cold PBS, pelleted, sonicated in low-salt buffer (25 mM Hepes/2 mM EDTA/1 mM DTT, pH 7.4), and centrifuged at 100,000 $\times$ *g* for 20 min, and the supernatant was stored as the cytosolic fraction. The pellet was sonicated in low-salt buffer plus 1% Triton X-100, incubated at 4 °C for 20 min, and centrifuged at 100,000 $\times$ *g* for 20 min. The pellet was used as the insoluble fraction, and the supernatant was used as the membrane, detergent-soluble fraction. Western blotting was performed as above.

PDE Activity Assay. PDE activity was determined by using 1 μM cAMP as substrate via a two-step radioassay method adapted from a published procedure (47). Substrate and protein were incubated over a period that PDE activity was linear, after which samples were boiled. To identify the contribution of PDE7 to total PDE activity, the assay was performed in the presence of the PDE7 inhibitor BRL-50481 (30 μM).

Apoptosis Assay. A total of 1×10^6 cells per milliliter were cultured in 90% RPMI medium 1640 plus 10% FBS at 37 °C in 5% CO_2 and treated with the rolipram, R020-1724, BRL-50481, IR-202, IR-284, milrinone, T-0156, or vehicle for 48 h. To determine the role of PKA PKI, Rp-cAMP or vehicle was added 1 h before the inhibitors then again 24 h after. Apoptosis was assessed by flow cytometry as the percentage of cells stained with annexin V and propidium iodide and analyzed by using CELLQuest software (Becton Dickinson Immunocytometry Systems) (2). B cells were labeled by using a PE-conjugated anti-CD19 antibody (Invitrogen), and apoptosis was measured on the positively stained cells.

Proliferation Assay. [^{3}H]Thymidine incorporation was used to assess DNA synthesis of EHEB cells. Cells (0.5×10^6 per milliliter) were incubated for 24 h with rolipram, BRL-50481, IR-202, IR-284, or vehicle. One microcurie (1 Ci = 37 GBq) of [^{3}H]thymidine per milliliter was added for a further 24 h, and cells were washed with cold PBS and 7.5% TCA and then dissolved in 0.5 M NaOH before liquid scintillation counting.

cAMP Assay. cAMP accumulation was measured as described (28). CLL cells (3×10^6 cells per well) were stimulated with forskolin (10 μM) or vehicle for 10 min in the absence and presence of PDE inhibitors (added 20 min before the addition of forskolin). Reactions were terminated by aspiration of the medium and addition of 60 μL of cold 7.5% (vol/vol) TCA. cAMP content in TCA extracts was determined by RIA and normalized to cell number.

Mitochondrial Membrane Potential. Mitochondrial membrane potential was assessed by using the MitoProbe JC-1 assay kit (Molecular Probes). Cells were treated with PDE4, PDE7, PDE4/7 inhibitors, and 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (a positive control) or vehicle for 24 h, then incubated with 2 μM JC-1 for 30 min. Cells resuspended in PBS were analyzed on a flow cytometer at 488-nm excitation using appropriate emission filters.

Immunohistochemistry. Immunohistochemistry was performed as described (23). In brief, CLL cells were treated with PDE inhibitors for 24 h, incubated with 50 nM Mitotracker Red CMXRos (Molecular Probes) for 30 min, seeded on polyD-lysine-coated coverslips, fixed, permeabilized with 0.1% Triton X-100, incubated for 2 h with an anticytochrome *c* antibody (1:100; Santa Cruz Biotechnology), followed with an FITC-conjugated secondary antibody (1:250) and DAPI (1:5,000), then mounted. Images were captured with a DeltaVision deconvolution microscope system (Applied Precision), deconvolved, and analyzed by using SOFTWORX software (Applied Precision). Cytochrome *c* release from mitochondria into the cytosol was visualized by immunofluorescence and from the loss of colocalization. Cells in 3 separate fields of view were counted, and results are expressed as the percentage of cells showing cytochrome *c* release.

Statistical Analyses. All determinations were performed in duplicate or triplicate; each experiment was repeated at least 3 times. Values are expressed as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni's post hoc test. Comparison between two groups was based on an unpaired Student *t* test or paired *t* test for comparing combined treatments. A value of $P < 0.05$ was considered statistically significant.

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