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Inhibition of calmodulin-dependent phosphodiesterase induces apoptosis in human leukemic cells

(cAMP/leukemia/antisense/cell death)

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ABSTRACT **Cytosolic extracts from a human lymphoblastoid B-cell line, RPMI-8392, established from a patient with acute lymphocytic leukemia, contain two major forms of cyclic nucleotide phosphodiesterase (PDE): Ca^{2+}-calmodulin dependent PDE (PDE1) and cAMP-specific PDE (PDE4). In contrast, normal quiescent human peripheral blood lymphocytes (HPBL) are devoid of PDE1 activity [Epstein, P. M., Moraski, S., Jr., and Hachisu, R. (1987) *Biochem. J.* 243, 533–539]. Using reverse transcription–polymerase chain reaction (RT-PCR), we show that the mRNA encoding the 63-kDa form of PDE1 (PDE1B1) is expressed in RPMI-8392 cells, but not in normal, resting HPBL. This mRNA is, however, induced in HPBL following mitogenic stimulation by phytohemagglutinin (PHA). Also using RT-PCR, the full open reading frame for human PDE1B1 cDNA was cloned from RPMI-8392 cells and it encodes a protein of 536 amino acids with 96% identity to bovine, rat, and mouse species. RT-PCR also identifies the presence of PDE1B1 in other human lymphoblastoid and leukemic cell lines of B- (RPMI-1788, Daudi) and T- (MOLT-4, NA, Jurkat) cell origin. Inhibition of PDE1 or PDE4 activity by selective inhibitors induced RPMI-8392 cells, as well as the other cell lines, to undergo apoptosis. Culture of RPMI-8392 cells with an 18-bp phosphorothioate antisense oligodeoxynucleotide, targeted against the translation initiation region of the RPMI-8392 mRNA, led to a specific reduction in the amount of PDE1B1 mRNA after 1 day, and its disappearance after 2 days, and induced apoptosis in these cells in a sequence specific manner. This suggests that PDEs, particularly PDE1B1, because its expression is selective, may be useful targets for inducing the death of leukemic cells.**

In lymphocytes, cAMP has negative effects on proliferation and cell function (1). Early studies by Tomkins and colleagues (2, 3), using an S49 mouse lymphoma cell line, had shown that cAMP induces these cells to undergo reversible G_1 arrest, followed by cytolysis. Mutants resistant to cAMP-induced death were deficient in cAMP-dependent protein kinase, indicating that this enzyme functions in cAMP-induced cytolysis (2, 3). More recent studies have shown that the death induced by cAMP is apoptotic cell death, and it occurs in normal, as well as transformed, lymphoid cells (4–7).

Selective elevations of cAMP levels in transformed lymphocytes could provide a means to selectively induce apoptosis in these cells. One means of elevating cAMP levels in cells is through the inhibition of cyclic nucleotide phosphodiesterase (PDE) activity. PDE is an isozymic family composed of many different types, grouped into seven broad gene families: Ca^{2+}-calmodulin (CaM)-dependent (PDE1), cGMP-stimulated (PDE2), cGMP-inhibited (PDE3), cAMP-specific (PDE4), cGMP-specific (PDE5), photoreceptor (PDE6), and higher affinity cAMP-specific (PDE7) PDEs (8). Early studies from our laboratory showed that PDE activity is greatly increased in actively growing and transformed lymphocytes (9), that PDE activity is induced in human peripheral blood lymphocytes (HPBL) following mitogenic stimulation (9, 10), and that PDE inhibitors profoundly inhibit mitogenic stimulation of HPBL (10, 11). Initial characterizations of PDE in HPBL suggested it was comprised mainly of PDE4 activity (reviewed in ref. 11), and recent cloning analysis shows expression of PDE4 mRNA in HPBL (12). More recent biochemical analysis of PDE in purified human T lymphocytes using ion exchange HPLC separation (13, 14), and in HPBL by sensitivity to selective PDE inhibitors (15), gives evidence for the presence in these cells of PDE3, as well as PDE4, and inhibitors of PDE3 and PDE4 act synergistically to inhibit proliferation in both human (14) and rat (16) T lymphocytes. The presence of PDE7 in cultured human T lymphocytes was also recently shown by HPLC separation and cloning techniques (17, 18). We documented the presence of PDE1 activity in a human B-lymphoblastoid cell line isolated from a patient with acute lymphocytic leukemia, and showed that PDE1 activity is absent in normal, resting HPBL (19). Others using bovine PBL confirmed an absence of PDE1 activity in resting PBL and showed its appearance in these cells following mitogenic stimulation (20). Characterization with monoclonal antibodies suggested that the induced PDE1 activity in bovine PBL belongs to the PDE1B, 63-kDa Ca^{2+}-CaM-dependent PDE gene family (20).

Recently, the cDNA for PDE1B1 has been cloned from bovine (21), rat (22), and mouse (23) brain cDNA libraries. The expression of the mRNA for PDE1B1 in different tissues as assessed by Northern blot analysis showed it to be restricted largely to brain, where it is enriched in the striatum (21–23). Its expression in the T-lymphoma cell line, S49, was also shown (22). In brain, PDE1B1 mRNA is expressed as a single species of ≈3–4 kb (21–23), whereas in mouse S49 cells three transcripts are seen at 4.4, 7, and 12 kb (22).

In this paper, we report the sequence of the full open reading frame (ORF) of the human form of PDE1B1, obtained by reverse transcription–polymerase chain reaction (RT-PCR) from a human lymphoblastoid cell line, demonstrate the expression of the mRNA for PDE1B1 in several lymphoblastoid and leukemic cell lines, as well as in mitogen-stimulated HPBL, and show that inhibition of PDE1B1 induces apoptosis of these cells.

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Abbreviations: PDE, cyclic nucleotide phosphodiesterase; PBL, peripheral blood lymphocytes; HPBL, human PBLs; ORF, open reading frame; RT-PCR, reverse transcription–polymerase chain reaction; AS ODN, antisense oligodeoxynucleotide; NS ODN, nonsense oligodeoxynucleotide; PHA, phytohemagglutinin; CaM, calmodulin.

The sequence reported in this paper has been deposited in the GenBank data base (accession no. U56976).

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MATERIALS AND METHODS

Growth and Maintenance of Cells. The B-cell lines RPMI-8392, RPMI-1788, and Daudi and the T-cell lines NA and JB, were established from patients and provided by other investigators. The T-cell lines Jurkat and MOLT 4 were obtained from the American Type Culture Collection. HPBL were isolated from 60 ml of freshly drawn blood from normal donors by defibrination with glass beads and separation of the lymphocytes by Ficoll/Hypaque density gradient centrifugation, as described (10). When stimulated by mitogen, HPBL were suspended in RPMI 1640 medium supplemented with 10% autologous serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and stimulated with 10 μg/ml PHA-P (Sigma) for 3 days.

Production and Sequence of Human 63-kDa CaM-PDE cDNA. The cDNA for the human 63-kDa CaM-PDE was produced and amplified from RPMI-8392 cells by RT-PCR. Total RNA (5 μg) isolated from RPMI-8392 cells was reverse transcribed with random primers and the DNA amplified by PCR with reagents and protocol from GIBCO/BRL using forward and reverse primers specific for different regions of PDE1B1 as indicated in the legend to Fig. 2, containing *Not*I restriction sites and 10 random bp on their 5′ ends, for subsequent subcloning. Amplification was for 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min. The PCR products were separated on 1.5% agarose gels, purified, and sequenced directly and then subcloned into p-Bluescript and sequenced again. Sequencing was performed as described (24) using the same primers as for PCR, as well as other oligodeoxynucleotide primers derived from the sequences obtained, and T3 and T7 primers for sequencing of the subcloned inserts.

Detection of Apoptosis by Analysis of DNA Fragmentation. Cells (10^6) were collected by centrifugation at 670 × *g* for 5 min. The cell pellets were rinsed once with 0.2 M dibasic sodium phosphate and lysed in 300 μl of lysis buffer [50 mM Tris·HCl, pH 7.5/10 mM EDTA/1% (vol/vol) Triton X-100] for 20 min on ice. Proteinase K was added to 0.5 mg/ml and DNase-free RNase was added to 50 μg/ml and the extract incubated at 37°C for 1 hr. DNA was then extracted in phenol/chloroform (1:1) and chloroform (1:1) and precipitated in 2.5 vol of ethanol. The extracted DNA was electrophoresed on 2% agarose gels, the gels stained with 0.5 μg/ml ethidium bromide, and photographed under UV light.

RESULTS

Expression of PDE1B1 in Mitogen-Activated and Cultured Lymphoblastoid Cells. Previous studies showed increased PDE activity in growing, cultured lymphoblastoid and leukemic cells, relative to normal, resting HPBL (9). Long-term induction of PDE activity was shown to occur in HPBL following mitogenic stimulation (9, 10), but the specific PDE isozyme(s) induced in HPBL were not fully characterized. CaM-dependent PDE activity (PDE1) was shown to be present in a human B lymphoblastoid cell line, RPMI-8392, isolated from a patient with acute lymphocytic leukemia, but absent from normal, resting HPBL (19). The PDE1 gene family is comprised of at least three different genes, some producing alternate spliced forms as well (8, 25, 26). We asked: (*i*) is PDE1 activity induced in HPBL following mitogen stimulation and (*ii*) if so, what type is it, and how does it relate to the PDE1 found in RPMI-8392 cells? Using quantitative RT-PCR, we examined resting and mitogen-stimulated HPBL and RPMI-8392 cells for the presence or absence of mRNA for the 63-kDa form of PDE1 (PDE1B1), using degenerate primers specific for this form of PDE. As shown in Fig. 1, HPBL stimulated by the mitogen, PHA, for 3 days, show a cDNA fragment of the predicted size of ≈333 bp, whereas unstimulated HPBL do not. Primers specific for human β-actin, included as a control in the same reactions, show similar amounts of β-actin present in both resting and PHA-stimulated HPBL (Fig. 1), indicating that the absence of a fragment corresponding to PDE1B1 in resting HPBL is not due to nonspecific breakdown of the mRNA, and that equal amounts of template mRNA were added to each of the RT-PCR reactions. mRNA isolated from RPMI-8392 cells and similarly subjected to RT-PCR also produced the 333-bp DNA fragment expected for PDE1B1 (not shown). Sequence analysis of the 333-bp fragments from PHA-treated HPBL and RPMI-8392 cells showed the two to be 100% identical to one another and to share 95% nt identity to bovine PDE1B1 (complete sequence is shown in Fig. 2). Hence, the cDNA fragments produced by RT-PCR clearly belong to the PDE1B1 gene family.

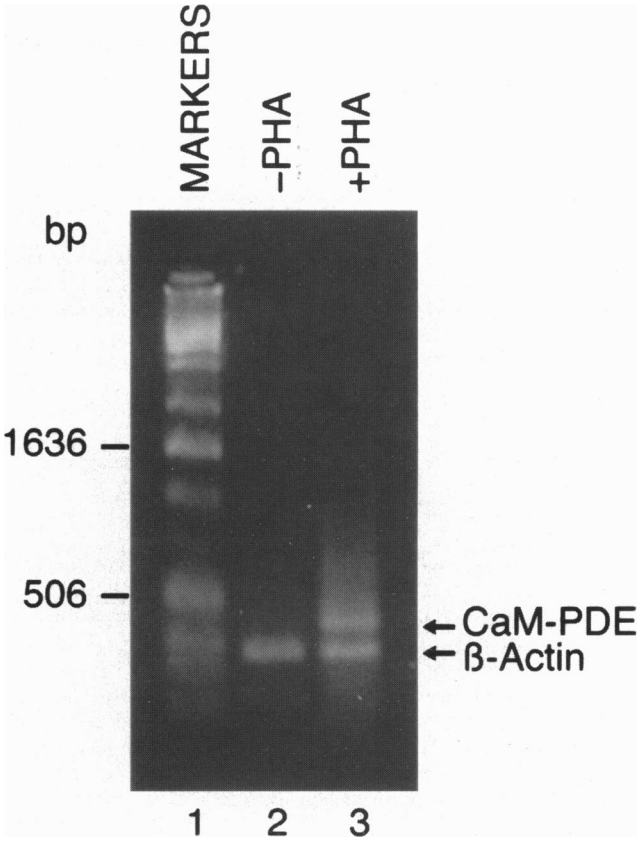

FIG. 1. RT-PCR of the expression of PDE1B1 and β-actin in HPBL. HPBL were isolated from a normal human donor and placed in culture with (+PHA) or without (−PHA) PHA for 3 days, as described. Quantitative RT-PCR was performed with 2 μg of HPBL RNA using reagents and protocol from GIBCO/BRL. Degenerate 23-bp primers were synthesized based on reported sequences for PDE1B1 (21–23). The sequence of the forward primer (Primer 1) corresponds to nt −34 to −12 and the sequence of the reverse primer (Primer 2) is complementary to nt 277–299 of the PDE1B1 cDNA, as presented in Fig. 2. PCR amplification was carried out for five cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, after which forward and reverse primers specific for a 250-bp fragment of β-actin, kindly supplied by K.-C. Cheng (University of Connecticut Health Center), were added to the reactions, and PCR amplification continued for another 20 cycles, followed by a final extension at 72°C for 10 min. Amplified DNA was resolved on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and visualized and photographed under UV light. Samples applied to the agarose gel were as follows: lane 1, 1-kb DNA ladder size markers (GIBCO/BRL); lane 2, unstimulated HPBL (−PHA) RT-PCR product; lane 3, PHA-stimulated HPBL (+PHA) RT-PCR product.

Cloning of the Full cDNA ORF for Human PDE1B1. Since the full sequence for the human form of PDE1B1 has not been reported, to obtain this sequence, we cloned the cDNA for PDE1B1 from RPMI-8392 cells by RT-PCR. cDNAs corre-

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-34                                                                                                    gctrgtccmygccagccgcagaccgtggctgaagc

 1    ATG GAG CTG TCC CCC CGC AGT CCT CCG GAG ATG CTG GAG GAG TCG GAT TGC CCG TCA CCC CTG GAG CTG AAG TCA GCC CCC AGC AAG AAG
 1    Met Glu Leu Ser Pro Arg Ser Pro Pro Glu Met Leu Glu Glu Ser Asp Cys Pro Ser Pro Leu Glu Leu Lys Ser Ala Pro Ser Lys Lys

91    ATG TGG ATT AAG CTT CGG TCT CTG CTG CGC TAC ATG GTG AAG CAG TTG GAG AAT GGG GAG ATA AAC ATT GAG GAG CTG AAG AAA AAT CTG
31    Met Trp Ile Lys Leu Arg Ser Leu Leu Arg Tyr Met Val Lys Gln Leu Glu Asn Gly Glu Ile Asn Ile Glu Glu Leu Lys Lys Asn Leu

181   GAG TAC ACA GCT TCT CTG CTG GAA GCC GTC TAC ATA GAT GAG ACA CGG CAA ATC TTG GAC ACG GAG GAC GAG CTG CAG GAG CTG CGG TCA
61    Glu Tyr Thr Ala Ser Leu Leu Glu Ala Val Tyr Ile Asp Glu Thr Arg Gln Ile Leu Asp Thr Glu Asp Glu Leu Gln Glu Leu Arg Ser

271   GAT GCC GTG CCT TCG GAG GTG CGG GAC TGG CTG GCC TCC ACC TTC ACC CAG CAG GCC CGG GCC AAA GGC CGC CGA GCA GAG GAG AAG CCC
91    Asp Ala Val Pro Ser Glu Val Arg Asp Trp Leu Ala Ser Thr Phe Thr Gln Gln Ala Arg Ala Lys Gly Arg Arg Ala Glu Glu Lys Pro

361   AAG TTC CGA AGC ATT GTG CAC GCT GTG CAG GCT GGG ATC TTC GTG GAA CGG ATG TTC CGG AGA ACA TAC ACC TCT GTG GGC CCC ACT TAC
121   Lys Phe Arg Ser Ile Val His Ala Val Gln Ala Gly Ile Phe Val Glu Arg Met Phe Arg Arg Thr Tyr Thr Ser Val Gly Pro Thr Tyr

451   TCT ACT GCG GTT CTC AAC TGT CTC AAG AAC CTG GAT CTC TGG TGC TTT GAT GTC TTT TCC TTG AAC CAG GCA GCA GAT GAC CAT GCC CTG
151   Ser Thr Ala Val Leu Asn Cys Leu Lys Asn Leu Asp Leu Trp Cys Phe Asp Val Phe Ser Leu Asn Gln Ala Ala Asp Asp His Ala Leu

541   AGG ACC ATT GTT TTT GAG TTG CTG ACT CGG CAT AAC CTC ATC AGC CGC TTC AAG ATT CCC ACT GTG TTT TTG ATG AGT TTC CTG GAT GCC
181   Arg Thr Ile Val Phe Glu Leu Leu Thr Arg His Asn Leu Ile Ser Arg Phe Lys Ile Pro Thr Val Phe Leu Met Ser Phe Leu Asp Ala

631   TTG GAG ACA GGC TAT GGG AAG TAC AAG AAT CCT TAC CAC AAC CAG ATC CAC GCA GCC GAT GTT ACC CAG ACA GTC CAT TGC TTC TTG CTC
211   Leu Glu Thr Gly Tyr Gly Lys Tyr Lys Asn Pro Tyr His Asn Gln Ile His Ala Ala Asp Val Thr Gln Thr Val His Cys Phe Leu Leu

721   CGC ACA GGG ATG GTG CAC TGC CTG TCG GAG ATT GAG CTC CTG GCC ATC ATC TTT GCT GCA GCT ATC CAT GAT TAT GAG CAC ACG GGC ACT
241   Arg Thr Gly Met Val His Cys Leu Ser Glu Ile Glu Leu Leu Ala Ile Ile Phe Ala Ala Ala Ile His Asp Tyr Glu His Thr Gly Thr

811   ACC AAC AGC TTC CAC ATC CAG ACC AAG TCA GAA TGT GCC ATC GTG TAC AAT GAT CGT TCA GTG CTG GAG AAT CAC CAC ATC AGC TCT GTT
271   Thr Asn Ser Phe His Ile Gln Thr Lys Ser Glu Cys Ala Ile Val Tyr Asn Asp Arg Ser Val Leu Glu Asn His His Ile Ser Ser Val

901   TTC CGA TTG ATG CAG GAT GAT GAG ATG AAC ATT TTC ATC AAC CTC ACC AAG GAT GAG TTT GTA GAA CTC CGA GCC CTG GTC ATT GAG ATG
301   Phe Arg Leu Met Gln Asp Asp Glu Met Asn Ile Phe Ile Asn Leu Thr Lys Asp Glu Phe Val Glu Leu Arg Ala Leu Val Ile Glu Met

991   GTG TTG GCC ACA GAC ATG TCC TGC CAT TTC CAG CAA GTG AAG ACC ATG AAG ACA GCC TTG CAA CAG CTG GAG AGG ATT GAC AAG CCC AAG
331   Val Leu Ala Thr Asp Met Ser Cys His Phe Gln Gln Val Lys Thr Met Lys Thr Ala Leu Gln Gln Leu Glu Arg Ile Asp Lys Pro Lys

1081  GCC CTG TCT CTA CTG CTC CAT GCT GCT GAC ATC AGC CAC CCA ACC AAG CAG TGG TTG GTC CAC AGC CGT TGG ACC AAG GCC CTC ATG GAG
361   Ala Leu Ser Leu Leu Leu His Ala Ala Asp Ile Ser His Pro Thr Lys Gln Trp Leu Val His Ser Arg Trp Thr Lys Ala Leu Met Glu

1171  GAA TTC TTC CGT CAG GGT GAC AAG GAG GCA GAG TTG GGC CTG CCC TTT TCT CCA CTC TGT GAC CGC ACT TCC ACT CTA GTG GCA CAG TCT
391   Glu Phe Phe Arg Gln Gly Asp Lys Glu Ala Glu Leu Gly Leu Pro Phe Ser Pro Leu Cys Asp Arg Thr Ser Thr Leu Val Ala Gln Ser

1261  CAG ATA GGG TTC ATC GAC TTC ATT GTG GAG CCC ACA TTC TCT GTG CTG ACT GAC GTG GCA GAG AAG AGT GTT CAG CCC CTG GCG GAT GAG
421   Gln Ile Gly Phe Ile Asp Phe Ile Val Glu Pro Thr Phe Ser Val Leu Thr Asp Val Ala Glu Lys Ser Val Gln Pro Leu Ala Asp Glu

1351  GAC TCC AAG TCT AAA AAC CAG CCC AGC TTT CAG TGG CGC CAG CCC TCT CTG GAT GTG GAA GTG GGA GAC CCC AAC CCT GAT GTG GTC AGC
451   Asp Ser Lys Ser Lys Asn Gln Pro Ser Phe Gln Trp Arg Gln Pro Ser Leu Asp Val Glu Val Gly Asp Pro Asn Pro Asp Val Val Ser

1441  TTT CGT TCC ACC TGG GTC AAG CGC ATT CAG GAG AAC AAG CAG AAA TGG AAG GAA CGG GCA GCA AGT GGC ATC ACC AAC CAG ATG TCC ATT
481   Phe Arg Ser Thr Trp Val Lys Arg Ile Gln Glu Asn Lys Gln Lys Trp Lys Glu Arg Ala Ala Ser Gly Ile Thr Asn Gln Met Ser Ile

1531  GAC GAG CTG TCC CCC TGT GAA GAA GAG GCC CCC CCA TCC CCT GCC GAA GAT GAA CAC AAC CAG AAT GGG AAT CTG GAT TAG ccctggggctg
511   Asp Glu Leu Ser Pro Cys Glu Glu Glu Ala Pro Pro Ser Pro Ala Glu Asp Glu His Asn Gln Asn Gly Asn Leu Asp  *

1623  gcccaggtcttcattgagtccaaagtgtttgatgtcatcagcaccatccatcaggactggctcccccatctgctccaagggagcgtggtcgtggaagaaacaacccacctgaaggccaa
1742  atgccagagatttggggttggggaaagggccccctccccacctgacacccactggggtgcactttaatgttccggcagcaagactggggaacttcaggctcccagtggtcactgtgccca
1871  tccctcagcctctggattctcttcatggccaggtggctgccagggagcggggagcttcctggaggcttcccagggccttggggaaggggtcagagatgccagccccctgggacctccccc
1980  atccttttgcctccaagtttctaagcaatacattttgggggttcccctcagccccccaccccagatcttagctggcaggtctgggtgccccttttcctccccctgggaaggggctggaata
2099  ggatagaaagctgggggttttcagagccctatgtgtggggaggggagtggattccttcagggcatggtacctttctaggatctgggaatggggtggagaggacatcctcttcaccccag
2218  aattgcgggaattc
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FIG. 2. Nucleotide and deduced amino acid sequences of the ORF of the human RPMI-8392 cell PDE1B1. Overlapping RT-PCR products were made from RPMI-8392 cell RNA and sequenced as described. Nucleotide and amino acid numbering are indicated at the left, with positions in the 5′-untranslated region indicated as negative numbers. The 5′-untranslated sequence is presented in lowercase letters, and the ORF sequence in uppercase letters. Primer sequences were based on reported sequences for PDE1B1 from other species (21–23). The forward primers were Primer 3, nt −23–3 and Primer 5, nt 729–752. The reverse primers were Primer 4, complementary to nt 810–834 and Primer 6, complementary to nt 1587–1611. PCR of a human temporal cortex cDNA library (Stratagene) was accomplished with a primer from nt 1509–1532 (Primer 7) and the T7 primer. Primers 2–7 are underlined and Primer 1 is overlined. The TAG termination codon is marked by an asterisk. The 3′-untranslated sequence is given up to the *Eco*RI cloning site in the vector. The nucleotide degeneracy is as follows: R = A or G, M = A or C, Y = C or T.

sponding to regions spanning from nt −23 to 834 and from nt 729 to 1611 of PDE1B1 were generated and sequenced, and together they encompass the full ORF of the human form of PDE1B1. Attempts to do RT-PCR using degenerate primers corresponding to regions of the 3′-untranslated sequence did not produce a product and, thus, a degenerate primer corresponding to the last 25 bp of the 3′ end of the ORF (Fig. 2) was used instead. The sequence of this 3′ end of the ORF and the 3′-untranslated sequence were obtained by PCR of a human brain cDNA library. As shown in Fig. 2, PDE1B1 in RPMI-8392 cells has an ORF of 1611 bp, encoding a predicted protein of 536 aa. The predicted protein shares 96% amino acid identity with PDE1B1 from bovine, rat, and mouse, indicating a very high degree of sequence homology for this protein across species.

Induction of Apoptosis in RPMI-8392 Cells by Pharmacological Inhibitors of PDE1 and PDE4. Analysis of cytosolic extracts of RPMI-8392 cells by DEAE anion exchange chromatography yielded two peaks of activity with properties representative of PDE1 and PDE4 (19). The effect of pharmacological inhibition of each of these PDE activities on induction of apoptosis in RPMI-8392 cells was tested, using the appearance of oligonucleosome length fragments of endonuclease-digested DNA as a measure of apoptosis. As shown in Fig. 3, vinpocetine, a selective inhibitor of PDE1 (27), induced apoptosis in these cells at concentrations ≥30 μM; rolipram and RO 20–1724, selective inhibitors of PDE4 (27), each induced apoptosis at concentrations ≥10 μM. The effects of these inhibitors on PDE activity was examined in whole cell homogenates of RPMI-8392 cells. As seen in Fig. 4, these inhibitors significantly inhibited PDE activity in RPMI cells at the concentrations at which they produced apoptosis. PDE inhibition appeared to plateau at ≈60% with PDE1 and ≈40% with PDE4 inhibitors; however, when added together, the effects of these inhibitors were not completely additive, suggesting that an additional form(s) of PDE may account for some of the activity in these cells. Treatment of other cell lines, which by RT-PCR were shown to express PDE1B1 mRNA were as follows: RPMI-1788, Daudi, MOLT-4, NA, and Jurkat, as well as a human T-lymphoma cell line, JB, with 100 μM of vinpocetine-induced apoptosis in every one of these cell types (data not shown).

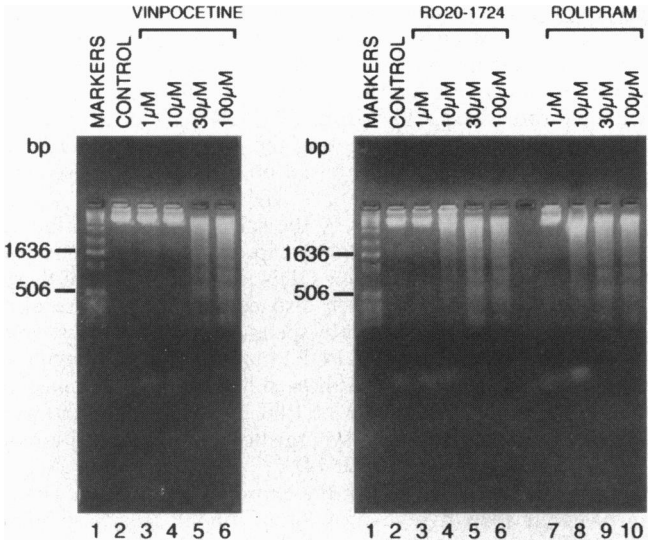

FIG. 3. Induction of apoptosis in RPMI-8392 cells by PDE inhibitors. DNA from RPMI-8392 cells was isolated and analyzed for fragmentation on 2% agarose gels as described, after culture for 24 hr without (Control) or with different concentrations of the PDE inhibitors vinpocetine (lanes 3–6, *Left*), RO 20–1724 (lanes 3–6, *Right*), and rolipram (lanes 7–10, *Right*), as indicated. Markers (lane 1 in both gels) are 1-kb DNA ladder size markers from GIBCO/BRL.

Induction of Apoptosis in RPMI-8392 Cells by Antisense to PDE1B1. Experiments were conducted to determine if inhibition of the expression of the gene for PDE1B1 could induce apoptosis. Based on the nucleotide sequence obtained for PDE1B1 from RPMI-8392 cells (Fig. 2), an 18-bp phosphorothioate antisense oligodeoxynucleotide (AS ODN) was synthesized, starting from 6 bp to the 5′ end of the translation initiation codon and extending over the first four codons of the ORF. As a control, a nonsense oligodeoxynucleotide (NS ODN) containing the same base composition, but in a random, scrambled order, was also synthesized. These synthetic phosphorothioate ODNs were added to RPMI-8392 cells in concentrations from 0.3–30 μM, and the cells were examined for apoptosis. As shown in Fig. 5, 48 hr treatment with 30 μM of AS ODN clearly induced apoptosis in these cells, whereas 30 μM of NS ODN did not. When cells were examined for apoptosis at 1, 2, and 3 days after addition of AS and NS ODNs, it was found that after 3 days, AS ODN induced apoptosis at both 10 μM and 30 μM, whereas in all cases, NS ODN had no effect (Fig. 5*B*).

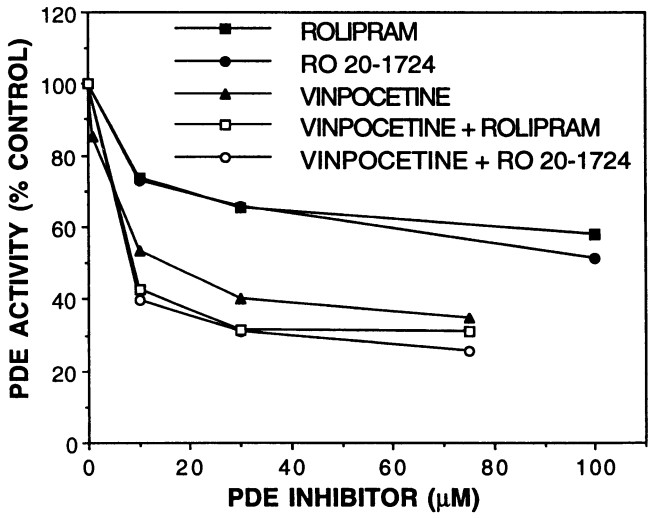

FIG. 4. Inhibition of PDE activity in RPMI-8392 cells by PDE inhibitors. PDE activity in whole cell homogenates of RPMI-8392 cells was tested for sensitivity to inhibition by vinpocetine, RO 20–1724, and rolipram, as indicated. Cells were grown to a density of about 10^6/ml, collected by centrifugation (1200 × *g*, 10 min), resuspended in 1 ml of homogenization buffer containing 40 mM Tris·HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 35 μg/ml phenylmethylsulfonyl fluoride, 15 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin, and 1 μg/ml aprotinin, homogenized with 20 strokes of a Dounce glass-on-glass homogenizer and assayed for PDE activity, using 1 μM cAMP as substrate, as described (19). Data points represent the means of 2–10 separate determinations. When vinpocetine was added, PDE activity was assayed in the presence of 0.2 mM Ca^{2+} and 15 nM CaM. Control activity was 1.66 ± 0.14 pmol/min/10^6 cells (mean ± SEM; n = 8) in the absence and 4.80 ± 0.42 pmol/min/10^6 cells (mean ± SEM; n = 10) in the presence of Ca^{2+} and CaM.

Effect of Antisense on Expression of PDE1B1 mRNA and Enzymatic Activity. Using quantitative RT-PCR, the effect of AS ODN and NS ODN on the level of mRNA for PDE1B1 in RPMI-8392 cells was examined. As seen in Fig. 6, the level of mRNA for PDE1B1 is diminished, relative to control, 1 day after treating cells with 10 μM and 30 μM of AS ODN, and absent after 2 days. The levels of β-actin mRNA, measured as a control, were unaltered by AS ODN. NS ODN had no effect

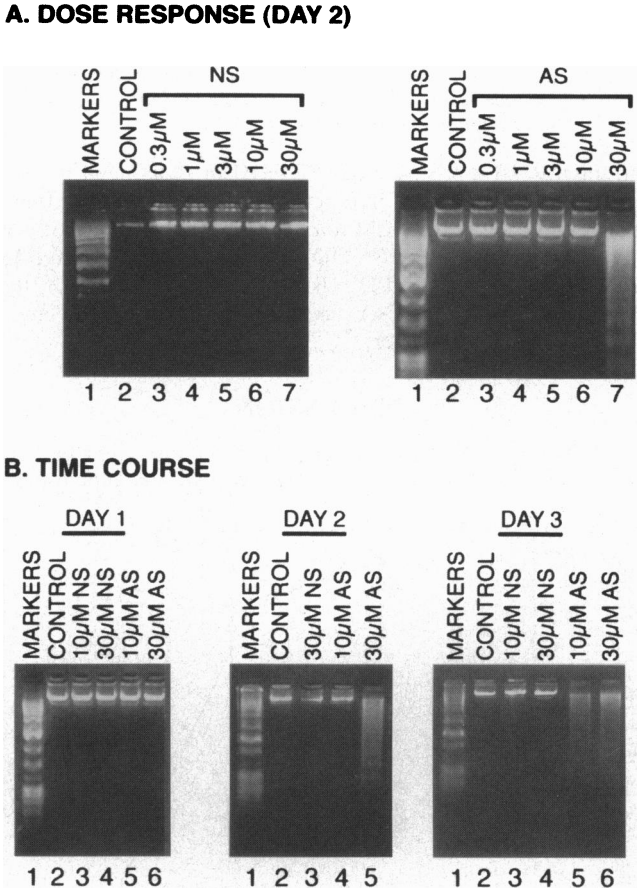

FIG. 5. Induction of apoptosis in RPMI-8392 cells by antisense to PDE1B1. DNA isolated from RPMI-8392 cells was analyzed for fragmentation on 2% agarose gels after the cells were cultured with different concentrations of phosphorothioate AS ODN (AS) or phosphorothioate NS ODN (NS) for 2 days (*A*) or 1, 2, and 3 days (*B*), as indicated. Cell culture was done at a concentration of about 10^6/ml in 1 ml volumes in 24-well plates, in RPMI 1640 growth medium, except that the fetal calf serum was heat inactivated at 65°C for 1 hr to help minimize nuclease activity. The sequence of the 18-bp AS ODN used was 5′-GGACAGCTCCATGCTCAG-3′, and the sequence of the 18-bp NS ODN used was 5′-TACGTGAGGCACCTACGC-3′. Controls (lanes 2 in all gels) represent no additions of ODN to the cells. Markers (lanes 1 in all gels) are *Hae*III digests of φX174 DNA from GIBCO/BRL.

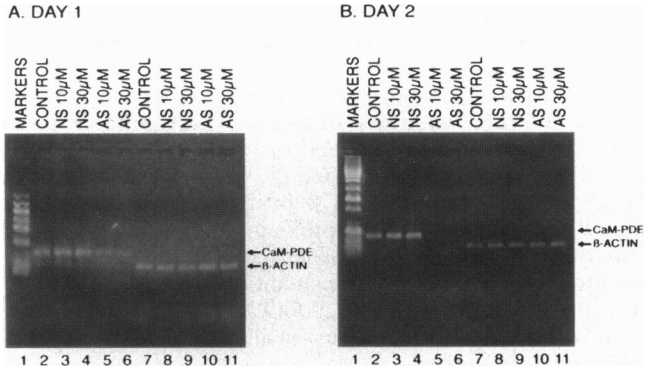

FIG. 6. Effect of antisense to PDE1B1 on expression of β-actin and PDE1B1 mRNAs in RPMI-8392 cells. Quantitative RT-PCR was used to determine the relative amounts of β-actin and PDE1B1 mRNAs present in RPMI-8392 cells after 1 and 2 days in culture with 10 μM and 30 μM of AS ODN (AS) and NS ODN (NS), as indicated. Total RNA (2 μg) isolated from RPMI-8392 cells was reversed transcribed and amplified by PCR as described in the legend to Fig. 1, except that the RT reactions for amplification of PDE1B1 and β-actin were done in separate tubes. PCR amplification was carried out for 19 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The AS and NS ODNs used are those given in the legend to Fig. 5. Positions for PCR products PDE1B1 (CaM-PDE), run in lanes 2–6, and β-actin, run in lanes 7–11, are indicated. Control (lanes 2 and 7 in both gels) represents no additions of ODN. Markers (lane 1 in both gels) are 1-kb DNA ladder size markers from GIBCO/BRL.

on the mRNA levels of either PDE1B1 or β-actin. As seen in Fig. 7, CaM-stimulated PDE activity was undetectable after 3 days in culture with 10 μM and 30 μM of AS ODN, but was relatively unchanged by NS ODN. These results show that AS ODN targeted against PDE1B1 leads to a reduction in the expression of the mRNA and protein for PDE1B1 in a sequence specific manner.

DISCUSSION

In this study, we show that several types of lymphoblastoid and leukemic cells of T- and B-cell origin express the gene for the

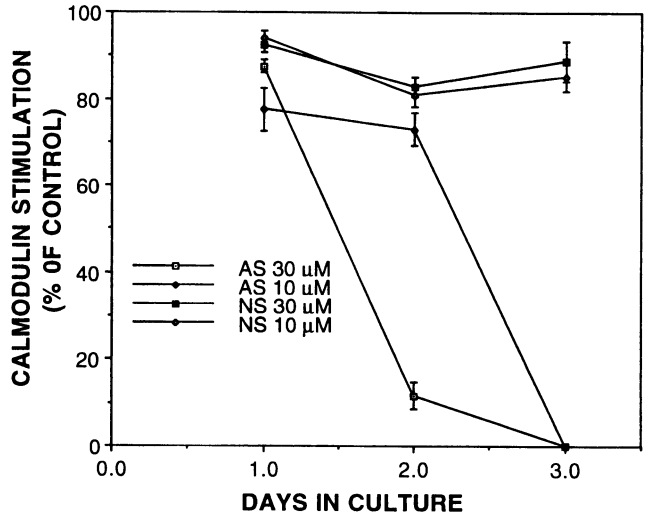

FIG. 7. Effect of antisense to PDE1B1 on the expression of PDE1B1 activity. RPMI-8392 cells were grown and treated with AS and NS ODNs as described in the legend to Fig. 5. Cells were then collected at daily intervals and assayed for PDE activity as described in the legend to Fig. 4. Results represent the mean ± the range of duplicate determinations. Control activities (pmol/min/10^6 cells) were 1.17, 1.34, and 1.50 in the absence, and 3.83, 4.34, and 3.79 in the presence of Ca^{2+} and CaM at days 1, 2, and 3, respectively.

63-kDa CaM-dependent PDE (PDE1B1). Isolated HPBL do not express this gene, but are induced to do so following mitogenic stimulation. An induction of PDE1 has also been observed in Chinese hamster ovary cells, following treatment with phorbol ester (28). Using RT-PCR we cloned and sequenced the cDNA representing the full ORF for PDE1B1 from RPMI-8392 cells, and based on this sequence, we synthesized AS ODN.

Blockage of PDE activity by the selective PDE1 inhibitor, vinpocetine, or blockage of the expression of the gene for PDE1B1 by treatment with AS ODN led to apoptosis of these cells. RPMI-8392 cells, which also contain PDE4, are also induced to undergo apoptosis by specific PDE4 inhibitors. This suggests that both PDE4 and PDE1 may both serve to regulate a common pool of cAMP in these cells. A similar conclusion was reached for the actions of PDE3 and PDE4 in human lymphocytes, based on their synergistic effects on inhibition of lymphocyte proliferation (13, 14).

Use of AS ODNs to block the expression of specific genes involved in growth regulation of lymphoid cells is gaining widespread interest as a result of the potential for such an approach to provide a novel therapeutic strategy for treatment of leukemias (29). Inhibition of proliferation and/or induction of apoptosis in leukemic cells has recently been demonstrated by using AS ODNs targeted to *bcl-2* (30), p53 (31), *bcr-abl* (32), c-*myb* (33), c-*myc* (34), c-*kit* (35), c-*fes* (36), *raf-1* (37), and IL-10 (38). Anti-c-*myb* (39) and *bcl-2* (40) AS ODNs were also shown to increase the survival time of severe combined immunodeficient mice transplanted with human leukemic cells. With the exception of anti-*bcr-abl* AS ODN, which can be targeted to a leukemia-specific sequence in chronic myelogenous leukemia cells resulting from a chromosomal translocation (32), all other AS ODNs used in this manner have the capability to block normal cellular functions, since the targeted genes are also expressed in normal cells as well.

The approach of using anti-PDE AS ODN to induce apoptosis of leukemic cells, as demonstrated in this study, has the potential to be selective for leukemic cells. Although PDE4 could be used as a therapeutic target, as demonstrated by the ability of rolipram and RO 20–1724 to induce apoptosis of RPMI-8392 cells, PDE4 is clearly present in normal, resting HPBL (10–12) and has a widespread distribution in tissues throughout the human body (41). Similarly, although PDE7 has recently been shown to be expressed in cultured human T cells (17, 18), analysis of tissue distribution of PDE7 show it to be fairly widespread and to predominate in skeletal muscle (42). We have concentrated instead, therefore, on PDE1B1 as a target, because, as shown in this study, the mRNA for PDE1B1 is selectively expressed in leukemic and actively growing lymphocytes, and not in resting HPBL. Moreover, the expression of PDE1B1 in tissues other than activated or transformed lymphocytes is largely restricted to areas of the brain (21–23). Because phosphorothioate AS ODNs distribute very poorly into the brain (43), brain function should be little affected by therapeutic AS ODNs targeted to leukemic forms of PDE1B1. A few other tissues express small amounts of PDE1B, either as alternate spliced forms (kidney papilla) (21) or multiple transcripts (testes, thymus) (23) of the mRNA. Hence, very selective AS ODNs, targeted specifically to PDE1B1 in leukemic cells could be produced, especially if polymorphism or variants of PDE1B could be shown in these cells.

In conclusion, these studies show that sequence-specific disruption of the gene for PDE1B1 by AS ODN induces human leukemic cells to die, therefore providing potential promise and a basis for development of a novel therapeutic strategy for the treatment of leukemia.

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