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Cyclic Nucleotide Phosphodiesterases (PDE) 3 and 4 in Normal, Malignant, and HTLV-I Transformed Human Lymphocytes

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ABSTRACT. Intracellular cyclic AMP, determined in part by cyclic nucleotide phosphodiesterases (PDEs), regulates proliferation and immune functions in lymphoid cells. Total PDE, PDE3, and PDE4 activities were measured in phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC-PHA), normal natural killer (NK) cells, Jurkat and Kit225-K6 leukemic T-cells, T-cell lines transformed with human T-lymphotropic virus (HTLV)-I (a retrovirus that causes adult T-cell leukemia/lymphoma) and HTLV-II (a nonpathogenic retrovirus), normal B-cells, and B-cells transformed with Epstein-Barr virus (EBV). All cells exhibited PDE3 and PDE4 activities but in different proportions. In EBV-transformed B cells, PDE4 was much higher than PDE3. HTLV-I$^+$ T-cells differed significantly from other T-lymphocyte-derived cells in also having a higher proportion of PDE4 activities, which apparently were not related to selective induction of any one PDE4 mRNA (judged by reverse transcription–polymerase chain reaction) or expression of the HTLV-I regulatory protein Tax. In MJ cells (an HTLV-I$^+$ T-cell line), Jurkat cells, and PBMC-PHA cells, the tyrosine kinase inhibitor herbimycin A strongly inhibited PDE activity. Growth of MJ cells was inhibited by herbimycin A and a protein kinase C (PKC) inhibitor, and was arrested in G_1 by rolipram, a specific PDE4 inhibitor. Proliferation of several HTLV-I$^+$ T-cell lines, PBMC-PHA, and Jurkat cells was inhibited differentially by forskolin (which activates adenylyl cyclase), the selective PDE inhibitors cilostamide and rolipram, and the nonselective PDE inhibitors pentoxifylline and isobutyl methylxanthine. These results suggest that PDE4 isoforms may be functionally up-regulated in HTLV-I$^+$ T-cells and may contribute to the virus-induced proliferation, and that PDEs could be therapeutic targets in immune/inflammatory and neoplastic diseases. BIOCHEM PHARMACOL **58**;6: 935–950, 1999. © 1999 Elsevier Science Inc.

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In general, cAMP‖ inhibits or attenuates proliferation, differentiation, and functions of immune/inflammatory cells [1]. By catalyzing cAMP and cGMP hydrolysis, PDEs regulate intracellular concentrations and, thus, biological effects of these second messengers. Nine structurally related but independent PDE families (PDE1–9), each consisting of one or more genes, have been identified [2–6]. Although representatives of several PDE families are present in immune/inflammatory cells [7], PDE4 isoenzymes are dominant contributors to cAMP hydrolysis in monocytes and eosinophil, basophil, and neutrophil granulocytes, whereas lymphocytes and macrophages express both PDE3 and PDE4 [7]. Peripheral human CD4$^+$ and CD8$^+$ T-lymphocytes contain PDE3, 4, and 7, which exhibit a high affinity for cAMP [8, 9].

Inhibition of phosphodiesterase activity, resulting in increased cAMP levels, long has been known to inhibit lymphocyte proliferation induced by mitogens such as PHA [10, 11]. We reported recently that in human TCC specifically sensitized to MBP, both rolipram, a PDE4 specific inhibitor, and cilostamide, a specific PDE3 inhibitor, inhibited antigen-induced proliferation, and in some TCC

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‖ *Abbreviations*: ATLL, acute T-cell leukemia/lymphoma; cAMP and cGMP, cyclic AMP and GMP, respectively; c-JNK, c-jun N-terminal kinase; EBV, Epstein-Barr virus; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; HTLV, human T-lymphotropic virus; IBMX, isobutyl methylxanthine; IGF, insulin-like growth factor; IL, interleukin; IP3, inositol-tris-phosphate; IRS, insulin receptor substrate; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MBP, myelin basic protein; NK, natural killer; PBMC, peripheral blood mononuclear cells; PBMC-PHA, PHA-stimulated PBMC; PDE, cyclic nucleotide phosphodiesterase; PHA, phytohemagglutinin; PI3-K, phosphatidyl inositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; RT–PCR, reverse transcription–polymerase chain reaction; STAT, signal transducer and activator of transcription; TCC, T-cell clone(s); TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; TNF, tumor-necrotic factor; and TSP/HAM, tropical spastic paraparesis/HTLV-associated myelopathy.

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the combination of both was more effective than either inhibitor alone [12]. In peripheral human $CD4^+$ and $CD8^+$ T-lymphocytes, rolipram increased cAMP and inhibited PHA- and anti-CD3-induced proliferation and production of IL-2 [8]. Specific PDE3 inhibitors enhanced the effects of PDE4 inhibitors in this [8] and another study [13]. Conversely, mitogenic stimulation of peripheral lymphocytes with PHA [10, 11] or of TCC specific for MBP with antigen [12] increased total PDE activities [10–12] as well as the activities of PDE1 [14] and PDE3 and PDE4 [12] isoforms. These effects of mitogens on PDE activities could reduce intracellular cAMP and mitigate effects of this antiproliferative factor.

The mechanisms by which cAMP inhibits mitogenesis are not understood completely. They could be related to direct interference with mitogenic signaling pathways and/or inhibition of production of IL-2 [8, 15, 16] or other autocrine growth factors. PKA-1 translocates to the T-cell receptor during antigen stimulation [17], and PKA-dependent phosphorylation of signaling proteins could interfere with T-cell activation/proliferation. PKA-dependent phosphorylation of Raf [18, 19] and of c-JNK [20, 21] could inhibit activation of the MAPK cascade [18, 19] and of transcription factors, and phosphorylation of PLC could inhibit production of IP3 [22, 23].

HTLV-I, a retrovirus endemic in some Asian, Caribbean, and African countries, is the cause of a hematopoietic malignancy, ATLL, as well as a progressive myelopathy, TSP/HAM [24]. In HTLV-I asymptomatic carriers, chronic immune stimulation is evidenced by the frequent detection of "spontaneous proliferation" in primary cultures of PMBC [25]. Accordingly, clonal expansion of specific T-cells carrying HTLV-I provirus can occur at any given time in HTLV-I-infected individuals. Such chronic T-cell proliferation may lead to the accumulation of genetic mutations, which may, after a latency of 20–30 years in a small number of infected individuals (1–2%), culminate in ATLL, a rapidly progressive, usually fatal, mature $CD4^+$ T-cell leukemia/lymphoma [24]. The pathogenesis of TSP/HAM is less clear, but autoreactive immunity has been invoked as a potential mechanism [26]. The related HTLV-II retrovirus, originally isolated from patients with hairy cell leukemia, is prevalent among intravenous drug users, endemic in some Amerindian populations, and, in most cases, not pathogenic [24, 27].

The mechanisms by which HTLV-I induces T-cell proliferation are largely unknown [24]. Transformation of T-cells with HTLV-I (HTLV-I^+ cells) *in vitro* has been found to result in constitutive activation of the JAK/STAT pathway [28, 29]. The HTLV-I genome codes for a regulatory protein, Tax, which activates a multitude of host cell genes including cytokine genes and cAMP-responsive genes, but the role of Tax in induction of proliferation is not clear [24]. Infection of B-lymphocytes with EBV results in permanent transformation, which also appears to correlate with constitutive JAK/STAT activation [30].

In this work, we first examined PDE3 and PDE4 in HTLV-I^+ cells and other lymphoid cells, including T-cells transformed with HTLV-II (HTLV-II^+ cells), leukemic T-lymphocytes, normal PBMC-PHA, NK cells, normal B cells, and B cells infected with EBV. The relative proportion of PDE4 was much greater in HTLV-I^+ T-cells and EBV-transformed B-cells than in the other lymphoid cells.

Second, mechanisms for regulation of PDEs in some HTLV-I^+ cells and other cells were studied using a panel of kinase inhibitors, since we recently have found that, in FDCP2 hematopoietic cells, IGF-1 and IL-4 (via JAK kinases) may activate PDE3 via IRS-2, PI3-K, and PKB-dependent pathways [31, *]. In these cells, IL-3 (via JAK and PI3-K) and phorbol esters (via PKC) selectively activate PDE4 by MAPK-dependent signals [31, *]. In adipocytes and pancreatic β-cells, insulin and IGF-1 may also activate PDE3 via PI3-K and PKB-dependent mechanisms [32–35]. In addition, herbimycin A, a src kinase inhibitor, has been reported to down-regulate PDE4 in rat theca cells [36]. We find that, of the kinase inhibitors tested, herbimycin A decreased PDE activity in MJ cells, an HTLV-I^+ cell line, Jurkat leukemic cells, and PBMC-PHA. Not all HTLV-I^+ cells were affected by herbimycin. Herbimycin A and the PKC inhibitor Ro 31–7549 inhibited proliferation of the HTLV-I^+ cells MJ and HUT-102 as well as Jurkat cells and PBMC-PHA.

Third, since agents that increase cAMP, including PDE inhibitors, are known to inhibit lymphocyte proliferation, we investigated the effects of forskolin, an activator of adenylyl cyclase, and of nonspecific and isoenzyme-specific PDE inhibitors on proliferation. Although proliferation of MJ cells was arrested in G_1 by rolipram, proliferation of other HTLV-I^+ cell lines, Jurkat cells, and PBMC was inhibited differentially by these agents. Whether these cellular phenotypic differences reflect differences in patients and whether PDEs might provide useful targets in specific patients with lymphoproliferative diseases is not known at this time.

MATERIALS AND METHODS

Separation and Culture of PBMC

Human PBMC were separated from heparinized buffy coat by Ficoll-Hypaque density-gradient centrifugation. After washing three times with PBS, cells were suspended (10^7 cells/mL) in RPMI 1640 supplemented with 10% FBS (Gibco-BRL), penicillin, streptomycin, and l-glutamine. PHA (Boehringer Mannheim) was added at 4 μg/mL, and cells were cultured for 72 hr. After stimulation, cells (PBMC-PHA) were washed three times in PBS and resuspended in complete medium containing 20 U/mL of IL-2 (Boehringer Mannheim). PBMC-PHA proliferated vigorously for about 2 weeks; all studies in this report were carried out during this period.

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Cell Lines

Eight HTLV-I$^+$ and two HTLV-II$^+$ T-cell lines were studied (see Table 2). The HTLV-I$^+$ T-cell lines included five IL-2-independent HTLV-I$^+$ T-cell lines (C8166–45 [37], MT-2 [38], MJ [39], C91/PL [40], and HUT-102 [41]) and three IL-2-dependent HTLV-I$^+$ T-cell lines (E55/PL, PR-11, and 1186 [38, 40]); of the IL-2-independent cell lines, three (MJ, MT-2, and HUT-102) exhibited constitutive JAK-STAT activation [28, 29]; the others did not [42]. The HTLV-II$^+$ T-cell lines, 96.II and MOT [43], were IL-2 independent and did not exhibit constitutive JAK-STAT activation [42]. We also used two HTLV-I-negative, malignant T-cell lines, Jurkat and Kit225-K6 (from Dr. J. Hakimi, Hoffmann-LaRoche Inc.), and Taxl-1 cells (from Dr. R. Grassman, Institut für Klinische und Molekulare Virologie).

Purification of NK Cells

Purified NK cells were a gift from Dr. Jeffrey Miller of the University of Minnesota. Briefly, NK cells were prepared by Ficoll gradient centrifugation of whole blood followed by enrichment of CD34$^+$ cells by FACS. Then the cells were cultured in RPMI 1640 with 10% heat-inactivated human serum and 1000 U/mL of IL-2.

Generation of MBP-Specific Human TCC

MBP-specific human TCC were generated by the split-well technique, cultured, and stimulated with antigen (MBP) as described previously [12, 44].

Purification of B-Lymphocytes from Human Blood

Human leukocytes were isolated from peripheral blood by leukapheresis (NIH blood bank). B-cells then were purified by incubating cells with magnetic beads coated with monoclonal antibodies against the B-cell marker CD19 [Dynabeads® M-450 Pan-B (CD19) (Dynal A.S.)]. The rosetted CD19$^+$ cells were separated with a magnetic particle concentrator (Dynal) and washed, and the beads detached from B-cells using Detach-a-Bead (Dynal) according to the manufacturer's instructions. The purity of the B-cells was analyzed by FACS.

Transformation of B-Cells with EBV

B-cells, transformed with EBV, were supplied by Dr. Roland Martin, NINDS, NIH. EBV was prepared from EBV-transformed B95–8 cells (5×10^6 cells) cultured in B-cell medium (RPMI 1640 with 5% FBS, 2 μM L-glutamine, 10 mM HEPES, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 50 μg/mL of gentamycin). Cells were pelleted, and the supernatant, which contained EBV, was used to infect peripheral blood lymphocytes (5×10^6 PBL), prepared separately from several healthy donors (NIH blood bank) and resuspended in 4.5 mL of B-cell medium. EBV supernatant (0.5 mL) and 40 μL of anti-CD3 (OKT3) ascites (diluted x10 with PBS) was added. After propagation in B-cell medium, cells (10^5/mL) were seeded into new flasks.

Cell Culture

Cells were maintained and propagated at 37° in 5% CO_2 as suspension cultures in RPMI 1640 (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL), penicillin, streptomycin, L-glutamine, and, for IL-2-dependent cell lines, 20 U/mL of IL-2. After staining with trypan blue (0.1%), cells were counted using a hemocytometer.

For PDE assay, cells ($1–2 \times 10^6$) were harvested by centrifugation (600 *g*, 10 min), suspended in 500 μL of homogenization buffer [100 mM TES, pH 7.4, 5 mM $MgSO_4$, 1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 1 mM benzamidine (Aldrich Chemical Co.), 10 μg/mL each of pepstatin and aprotinin (ICN Biomedical), 10 μg/mL of leupeptin, and 0.5 mM pefabloc (both Boehringer Mannheim)], and sonified on ice (2×20 pulses, output 2, 40% duty cycle) using a Sonifier Cell Disruptor 350 (Branson Sonic Power Co.).

After cells were washed twice in 3 mL of cold PBS and sonified in homogenization buffer as above, protein was assayed using the Bio-Rad Protein assay reagent (Bio-Rad Laboratories) and bovine serum albumin as standard [45].

Assay of PDE3 and PDE4

As previously described [12], samples (75 μL of homogenate) were incubated at 30° for 10 min in a total volume of 300 μL containing 50 mM HEPES, pH 7.4, 0.1 mM EDTA, 8.3 mM $MgCl_2$, and, unless otherwise stated, 0.1 μM [^{3}H]cAMP (New England Nuclear) (20,000–30,000 cpm) as substrate. The product, 5′-AMP, was dephosphorylated to adenosine with *Crotalus atrox* venom (Sigma) (30°, 20 min), separated from substrate using ion exchange chromatography (QAE-Sephadex, Pharmacia), and quantified by scintillation counting. Total activity represents activity in the absence of inhibitors; activities of PDE3 and PDE4 are defined as the difference in activities with/without 1 μM cilostamide (a specific PDE3 inhibitor) and 5 μM rolipram (a specific PDE4 inhibitor) [46], concentrations that give almost complete and specific inhibition of lymphocyte PDE3 and PDE4 [12]. All samples were assayed in duplicate. Variation between duplicates was usually $< 5\%$. PDE activity was linear with respect to time (at least 20 min) and enzyme concentration; hydrolysis of substrate was usually below 20%. Inhibitor vehicle (DMSO, ethanol), included in equal quantities in samples without inhibitor, did not influence PDE activities.

TABLE 1. Primers used for RT-PCR

PDE	Primer	Product length (bp)	Ref.
3A	5′-TCA CCT CTC CAA GGG ACT CCT-3′ 5′-CAG CAT GTA AAA CAT CAG TGG C-3′	709	[47]
3B	5′-AAT TCT TCC AAC CAT CGA CC-3′ 5′-GCT TGT AGC ACA TCT GTG GC-3′	696	[48]
4A	5′-AAC AGC CTG AAC AAC TCT AAC-3′ 5′-CAA TAA AAC CCA CCT GAG ACT-3′	907	[49]
4B	5′-AGC TCA TGA CCC AGA TAA GTG-3′ 5′-ATA ACC ATC TTC CTG AGT GTC-3′	625	[49,50]
4C	5′-TCG ACA ACC AGA GGA CTT AGG-3′ 5′-GGA TAG AAG CCC AGG AGA AAG-3′	289	[51]
4D	5′-CGG AGA TGA CTT GAT TGT GAC-3′ 5′-CGT TCC TGA AAA ATG GTG TGC-3′	641	[49]
7	5′-ATA ATG GAC AAG CCA AGT GT-3′ 5′-TCC AGC TGG CTT TAT TCA GC-3′	936	[52]
1	5′-CAG TCC TCC GGA GAT GCT GGA G-3′ 5′-CCA GTC CCG CAC CTC CGA AGG CAC-3′	264	[53]

Reverse Transcription and Amplification of PDE cDNA by Polymerase Chain Reaction (RT–PCR)

Total RNA was prepared using RNAzol B (Biotec) according to the manufacturer's instructions. Specific oligonucleotide primer sets (PS), designed (Table 1) from PDE1B1, PDE3A–B, PDE4A–D, and PDE7 sequences [47–53], were synthesized using an Oligo 1000 M DNA synthesizer (Beckman). First-strand cDNA was generated from ~2 μg of total RNA using Superscript reverse transcriptase and random hexamers (Gibco-BRL) to prime the reverse transcription. cDNA was amplified directly by PCR following the addition of specific primers and PCR Master, including *Taq* DNA polymerase (Boehringer Mannheim). The PCR reaction included initial denaturation at 94° for 3 min, then denaturation at 94°, 1 min, annealing at 55°, 1 min, and extension at 72°, 1 min, for 30 cycles, and final extension at 72°, 7 min. Products, separated by electrophoresis (1% agarose), were visualized by ethidium bromide staining. No fragments were amplified in the absence of RT or RNA. Amplification of actin PCR product was used to estimate relative amounts of PCR products.

Effects of Kinase Inhibitors on PDE Activity and Cell Proliferation

Cells (~10^6), resuspended in 3 mL of medium, were incubated at 37° in 6-well plates with various inhibitors or vehicle (DMSO) for the indicated times. The DMSO concentration usually did not exceed 0.1%. Cells were harvested for PDE assay as described above, and a portion was stained with trypan blue and counted using a hemocytometer.

The PKC inhibitor Ro 31–7549 was obtained from the Alexis Corp.; the MAPKK-1 inhibitor PD98059, from New England Biolabs; the PI3-K inhibitor wortmannin and the JAK inhibitor AG-490, from Biomol Research Laboratories; and the tyrosine kinase inhibitors herbimycin A, PP1, genistein, tyrphostin A25, and erbstatin analog, all from Calbiochem. All inhibitors were reconstituted in DMSO.

Proliferation Assay Using [^{3}H]Thymidine Uptake

Cells (5000 cells/well) were cultured in 96-well microtiter plates (Falcon) in medium (100 μL) containing the indicated drugs. Sixty-six hours later [^{3}H]thymidine (NEN Dupont) (10 μL, ~1 μCi/well) was added, and cultures were incubated for another 6 hr. Then cells were harvested with a microcell harvester (Skatron), and [^{3}H]thymidine incorporation was determined on a Wallac liquid scintillation counter. All conditions were assayed in triplicate.

Cell Cycle Analysis

Cells were washed in PBS, centrifuged, and 80% ethanol (1 mL) was added to the cell pellet. After 30 min on ice, cells were washed once in PBS, suspended in 0.5 mL PBS, and DNAse-free RNAse (7 μg/mL) (Boehringer Mannheim) was added. The cell suspension was incubated for 25 min at 37°, washed once in PBS, and resuspended in propidium iodide (50 μg/mL) (Sigma). DNA fluorescence was measured with a FACScan Flow Cytometer (Becton Dickinson); cell cycle analysis was performed using Modfit LT 2.0 software.

TABLE 2. Total PDE and PDE3 and PDE4 activities in lymphoid cells

Cells	HTLV	IL-2	JAK-STAT	N	Total PDE	PDE3	PDE4	Ratio 4/3
MJ	I	indep	+	4	275 ± 43.5	7.5 ± 8.0	253 ± 39.8	33.7
MT-2	I	indep	+	6	29.7 ± 16.9	1.1 ± 0.4	26.6 ± 15.2	24.2
HUT-102	I	indep	+	7	86.8 ± 13.1	9.0 ± 1.8	70.8 ± 13.3	7.9
C8166-45	I	indep	−	4	37.1 ± 13.6	5.8 ± 3.9	22.9 ± 6.1	3.9
C91/PL	I	indep	−	4	74.7 ± 56.4	47.8 ± 61	20.0 ± 15.3	0.4
E55/PL	I	dep	−	4	51.8 ± 19.1	4.2 ± 0.5	42.9 ± 15.2	10.2
PR-11	I	dep	−	2	20.3 ± 0.2	2.4 ± 1.4	14.3 ± 0.6	5.6
1186	I	dep	−	4	22.0 ± 2.5	5.7 ± 2.0	16.0 ± 2.0	2.8
MOT	II	indep	−	4	17.6 ± 4.8	4.6 ± 2.7	10.0 ± 1.2	2.2
96.II	II	indep	−	2	25.3 ± 5.7	6.0 ± 3.1	17.0 ± 2.5	2.8
Tax1-1	neg	dep	−	4	57.2 ± 16.3	16.4 ± 2.8	40.1 ± 14.3	2.4
Jurkat	neg	indep	−	4	10.7 ± 3.3	5.9 ± 2.3	2.2 ± 0.5	0.4
Kit225-K6	neg	dep	−	2	31.2 ± 3.0	9.5 ± 4.8	17.6 ± 3.2	1.9
3A6*	neg	dep	−	6	14.1 ± 7.5	6.4 ± 6.5	5.9 ± 3.4	0.9
NK	neg	dep	−	4	8.5 ± 5.4	3.5 ± 2.6	3.1 ± 1.7	0.9
PBMC-PHA	neg	dep	−	4	6.4 ± 4.3	2.8 ± 2.0	3.1 ± 2.6	1.1
Pur BC	neg		−	3	7.0 ± 3.2	2.3 ± 2.0	5.2 ± 3.2	2.3
EBV BC	neg	indep		15	6.4 ± 4.5	0.8 ± 1.2	3.8 ± 3.4	4.8

PDE activities were assayed (with 0.1 μM [^{3}H]-cAMP as substrate) in lysates from the indicated cell lines. N refers to the number of independent experiments. Means ± standard deviation (or ± half range if N = 2) of cAMP PDE activities (pmol/min/10^7 cells) are shown in columns 6–8. In column 9, the ratio between the means is given. The difference in ratios between HTLV-I infected T-cells and other T-cells was significant, as determined with Wilcoxons rank-sum test ($P < 0.025$). Columns 3 and 4 indicate whether cells are IL-2 dependent/independent or exhibit constitutive JAK-STAT activation, respectively.

*Values from Ref. 12.

RESULTS

PDE Activities in Normal PBMC-PHA, NK Cells, Jurkat and Kit225-K6 Leukemic T-Cells, Normal B-Cells, and Virally Transformed T- (HTLV-I and HTLV-II-Infected) and B- (EBV-Transformed) Cells

Total PDE and PDE3 and PDE4 activities were assayed (with 0.1 μM [^{3}H]cAMP as substrate) in lysates from PBMC-PHA (normal PBMC stimulated with PHA and IL-2), NK cells, Jurkat and Kit225-K6 leukemic T-cells, HTLV-I$^+$ T-cells (IL-2-dependent/independent, expressing wild type/constitutively activated JAK-STAT) and HTLV-II$^+$ T-cells, purified B-cells, and EBV-transformed B-cells (Table 2). Previously published results with 3A6 T-cells [12], a TCC specifically sensitized to MBP, are included for comparison.

As seen in Table 2 and graphically presented in Fig. 1, and as has been reported for preparations of peripheral CD4$^+$/CD8$^+$ T-lymphocytes [8, 9] and human TCC sensitized to MBP [12], all T-lymphocyte-derived cells exhibited both PDE3 and PDE4 activities, but in different proportions (PDE4/PDE3 ratios). Whereas there is considerable variation in PDE activities in different preparations of individual cell lines, there is less variation in PDE4/3 ratios (except in cells with ratios > 10, i.e. where PDE4 accounts for at least ~90% of PDE activity and small changes in PDE3 markedly influence the PDE4/3 ratio). In Jurkat cells the relative amount of PDE3 was greater than PDE4, with a PDE4/3 ratio < 1. In PBMC-PHA and most T-lymphocyte-derived cells, including NK cells, 3A6 TCC (sensitized to MBP), Kit225-K6 leukemic T-cells, and HTLV-II$^+$ T-cell lines, PDE4 activity was somewhat greater than PDE3, with a PDE4/3 ratio ~1. We recently reported PDE4/3 ratios ranging from ~0.8 to 3 in individual TCC (sensitized to MBP) [12]. The relative proportion of PDE4 activity was greater in EBV-transformed B cells than in purified human peripheral B-lymphocytes (Fig. 1, Table 2).

As seen in Fig. 1 and Table 2, total PDE activity (based on cell number) was much higher in some HTLV-I$^+$ T-cell lines (especially MJ cells) than in most of the other lymphoid cells. When expressed on the basis of protein content, however, the difference in total PDE activities between HTLV-I$^+$ T-cells and other cells was less (except for MJ cells), and thus, probably primarily due to differences in cell size, since, for example, PBMC-PHA are much smaller than several other HTLV-I$^+$ T-cell lines studied (data not shown). The most striking difference between HTLV-I$^+$ T-cells and other cells of T-cell origin, including HTLV-II$^+$ cells, however, was the relative proportions of PDE4 and PDE3 activities. Except for C91/PL cells, the PDE4/3 ratio was higher in most HTLV-I$^+$ T-cells than in other T-lymphocyte derived cells (Fig. 1, Table 2). Although these results reflect studies with a limited number of cell lines, the PDE4/3 ratio in HTLV-I$^+$ T-cells, as a group, was higher than in the other T-lymphocyte-derived cells (Table 2). In some preparations of HTLV-I$^+$ MJ and MT-2 cells (and EBV-transformed B-cells as well), PDE4 accounted for essentially all cAMP hydrolyzing activity. While these results are consistent with "functional" up-regulation of PDE4 in HTLV-I$^+$ cells, we cannot dismiss the possibility that "down-regulation" of PDE3 also contributes to the elevated PDE4/3 ratio.

Amplification of PDE mRNA by RT–PCR

Since two different genes encode PDE3s (PDE3A–B) and four genes encode PDE4 (PDE4A–D) isoforms [2–4, 54,

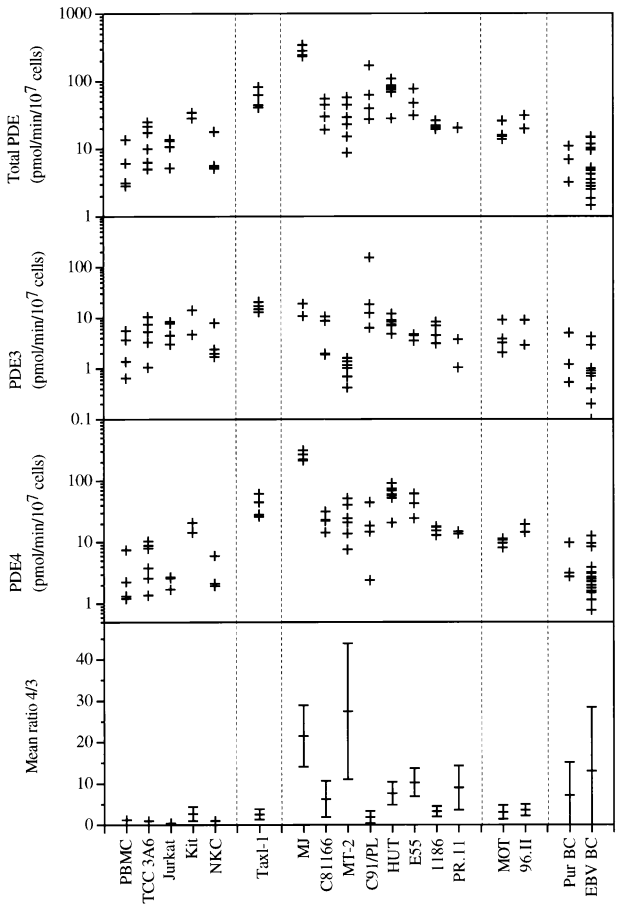

FIG. 1. Activities of total PDE, PDE3, and PDE4 in PBMC-PHA, malignant and HTLV-transformed T-cells, and normal and EBV-transformed B-cells. Cells were cultured, harvested, and counted, and total PDE, PDE3, and PDE4 activities were assayed in duplicate as described in Materials and Methods. PDE activities are presented in logarithmic scale because of the differences in activities between the different cell lines. Data from individual experiments (+) are shown. Numbers of experiments (N) are given in Table 2. In two preparations of MJ cells, PDE3 activity was virtually absent. The bottom panel shows the PDE4/PDE3 ratios as mean ± SD (or ± half range if N = 2) for each cell line.

55], we utilized RT–PCR to identify which mRNAs were expressed in PBMC-PHA, Jurkat cells, and several HTLV-I$^+$ T-cell lines, including HUT-102, MT-2, C91/PL, and MJ cells. Results in Fig. 2 would suggest that the increased proportion of PDE4 in several HTLV-I$^+$ T-cell lines may not be related to differential up-regulation of a specific PDE4 mRNA. With RNA prepared from PBMC-PHA and HTLV-I$^+$ T-cell lines, PCR products of the expected sizes were amplified consistently from PDE1B1, PDE3B, PDE4A, B, and D, and PDE7 mRNAs. PDE4C mRNA was amplified less consistently in different preparations from PBMC-PHA and HTLV-I$^+$ T-cells, especially MJ and C91/PL cells (Fig. 2). We have cloned PDE3B cDNA from a Jurkat cell cDNA library (unpublished) and detected PDE3B (not PDE3A) mRNA in TCC sensitized to MBP [12]. As seen in Fig. 2, PDE3B mRNA was detected in RNA from PBMC-PHA, Jurkat cells, and HTLV-I$^+$ T-cells. PDE3A mRNAs, not detected in preparations from normal TCC sensitive to MBP [12], normal PBMC-PHA and Jurkat cells, were amplified readily in preparations from HTLV-I$^+$ T-cells C91/PL and MJ and amplified weakly in MT-2 preparations. Little or no PDE4B and PDE4D mRNAs were amplified from Jurkat cell RNA (Fig. 2).

Effects of Tax Expression on PDE Activities

The HTLV-I virus encodes the transactivator protein Tax, which transcriptionally regulates the expression of several cellular genes [24, 29], including IL-2 and the α-subunit of its high affinity receptor complex (IL-2Rα) [56]. This may contribute to HTLV-I-stimulated cell proliferation via an autocrine loop. Since mitogenic stimuli can up-regulate PDE activities [11, 12, 14], we investigated whether Tax expression could up-regulate PDE activities in an inducible Tax expression system (Jurkat Jpx9 cells) [57].

In Jpx9 cells (a Jurkat-derived cell line with the *Tax* gene under control of a cadmium-sensitive promoter), basal PDE activities were low and did not change when Tax expression was induced (Fig. 3). Successful induction of Tax was demonstrated by transfection of the same cells with a luciferase reporter gene controlled by a Tax-sensitive promoter (data not shown). In Taxl-1 cells, a T-cell derived cell line that permanently expresses Tax [58], PDE4/PDE3 ratios did not differ significantly from PBMC-PHA (Fig. 1 and Table 2).

Effects of IL-2 Withdrawal on PDE Activities

Considering the importance of cAMP to lymphocyte proliferation [1], and since some HTLV-I$^+$ T-cells were IL-2-dependent (Table 2), we investigated effects of this T-cell growth factor on PDE activities in PBMC-PHA and 1186 cells, an IL-2-dependent HTLV-I$^+$ T-cell line (Table 2).

PBMC were prepared and incubated with PHA for 3 days. Then PHA was removed, and PBMC-PHA were cultured in the presence of IL-2. Removal of IL-2 for 24 or 48 hr resulted in a marked decrease in cell number and PDE activities. Addition of IL-2 for 24 hr increased PDE activities to previous levels (data not shown).

In the presence of IL-2, HTLV-I$^+$ 1186 cells grew vigorously (almost doubling each 24-hr period); in the absence of IL-2, growth was inhibited almost completely (~50% increase in 72 hr). As seen in Fig. 4, PDE activities (both PDE3 and PDE4) decreased markedly as cells were cultured in the absence of IL-2; re-addition of IL-2 after 24- or 48-hr withdrawal of IL-2 resulted in recovery of PDE activities. It is not known if these changes in PDE activities are related to direct effects of IL-2 on PDE gene expression, or on signaling pathways that regulate PDE activities, or are secondary to effects of IL-2 on cell proliferation.

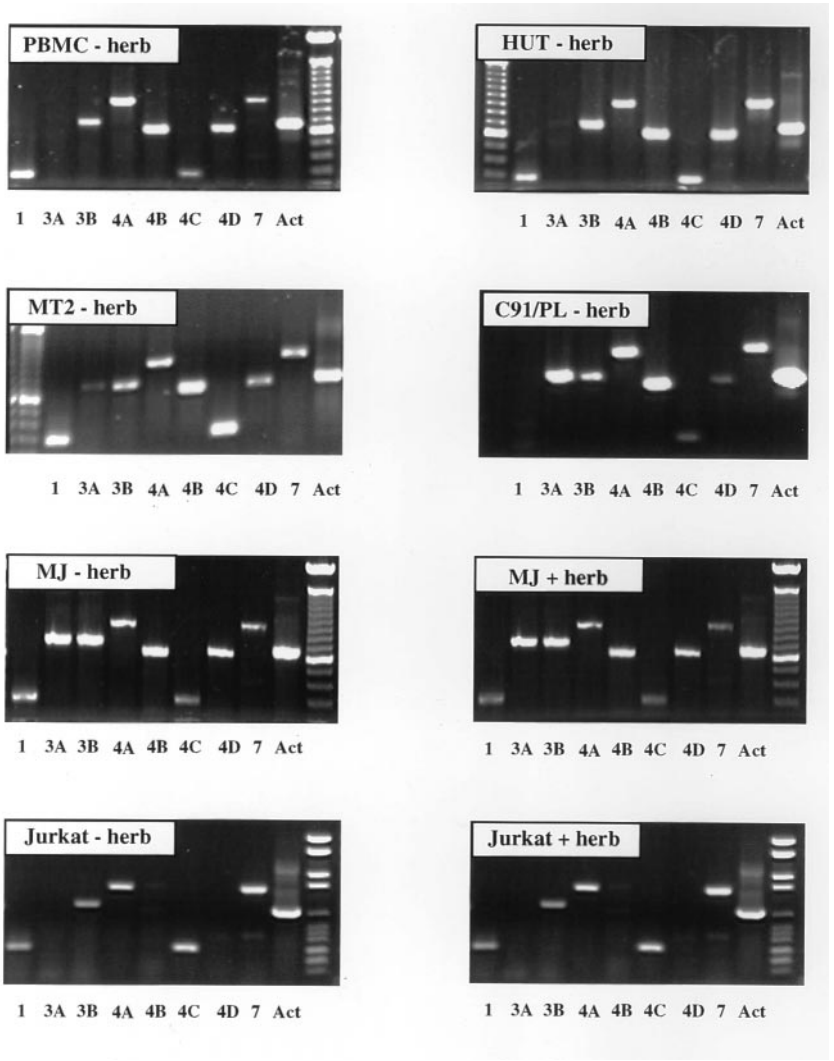

FIG. 2. Amplification of PDE mRNA by RT–PCR in PBMC-PHA, HTLV$^+$ T-cell lines, and Jurkat cells. Total RNA was extracted from the indicated cell lines (5–10 × 10^6 cells) as described in Materials and Methods. MJ cells and Jurkat cells were incubated for 8 hr without and with 1 μM herbimycin A prior to isolation of RNA. cDNA was generated from 1 μg of total RNA, and mRNA was amplified by PCR, using oligonucleotide primer sets (PS) (Table 1) based on sequences from PDEs 1C, 3A, 3B, 4A, 4B, 4C, 4D, 7, and actin. The products were separated on agarose gels and photographed after ethidium bromide staining. The experiments were repeated at least three times, with similar results.

Effects of Herbimycin A, Other Tyrosine Kinase Inhibitors, and Protein Kinase Inhibitors on PDE Activities and Cell Proliferation

Effects of inhibitors of various signaling pathways were also utilized to study potential mechanisms for regulation of PDE3 and PDE4 in some HTLV-I$^+$ cells and other T-lymphocyte derived cells. The tyrosine kinase inhibitor herbimycin has been reported to reduce PDE activity in cultured cells [36]. As shown in Fig. 5A, incubation of MJ cells, Jurkat cells, and PBMC-PHA with herbimycin A (1 μM) for 8 hr decreased PDE activity by 50–85% without any change in cell number. Herbimycin did not alter PDE activities in all HTLV-I$^+$ T-cells; it exerted much smaller effects on PDE activity in HUT-102 cells (Fig. 5A) and variable effects in MT-2 and C91/PL cells (data not shown). Inhibition was reversible; upon removal of herbimycin A from MJ cells, PDE activity returned to control values (Fig. 5B). Inhibition of PDE activities was dependent on the duration of incubation with (Fig. 5C) and the concentration of (Fig. 5D) herbimycin A. Amplification of PDE mRNA from total RNA prepared from MJ and Jurkat cells indicated that herbimycin did not selectively decrease mRNAs for specific PDE3B and PDE4 isoforms (Fig. 2).

Concentrations of herbimycin that inhibited PDE activity in intact MJ cells (≤ 1.0 μM) had little direct effect on PDE activity in cell lysates (Fig. 6A). Higher concentrations of genistein (~100 μM), another tyrosine kinase inhibitor, were reported to inhibit PDE activities in intact cells [59–61]. At these concentrations, however, genistein and the tyrosine kinase inhibitors tyrphostin and erbstatin dramatically inhibited PDE activity in cell lysates (Fig. 6A) and inhibited growth of MJ cells (data not shown). In light of these findings, conclusions as to the role of tyrosine kinases in the effects of genistein on PDE activities in intact cells [59–61] must be reconsidered, since genistein might act by directly inhibiting PDEs as well as tyrosine kinases.

In contrast to herbimycin A (1 μM), which reduced PDE activity in MJ cells by ~60% within 12 hr, other kinase inhibitors, including the PKC inhibitor Ro 31–7549 (10 μM), the MAPKK inhibitor PD98059 (50 μM), the PI3-K

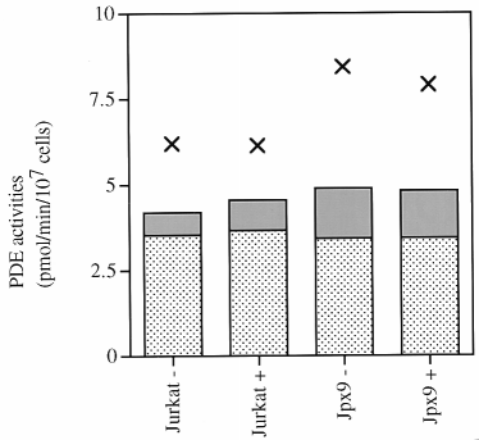

FIG. 3. Effects of Tax induction on PDE activities in Jpx9 cells. Jpx9 cells (a Jurkat-derived cell line with the *Tax* gene under control of a cadmium-sensitive promoter) and, as a control, Jurkat cells, were cultured with (+) or without (-) addition of 20 μM $CdCl_2$ for 16 hr. The cells were harvested and counted, and total PDE (x), PDE3 (dotted bar), and PDE4 (dark bar) activities were assayed in duplicate as described in Materials and Methods. Successful induction of Tax was demonstrated by transfection of the Jpx9 cells with a luciferase reporter gene controlled by the HTLV-I LTR, a Tax-sensitive promoter (data not shown). The experiment was repeated with similar results.

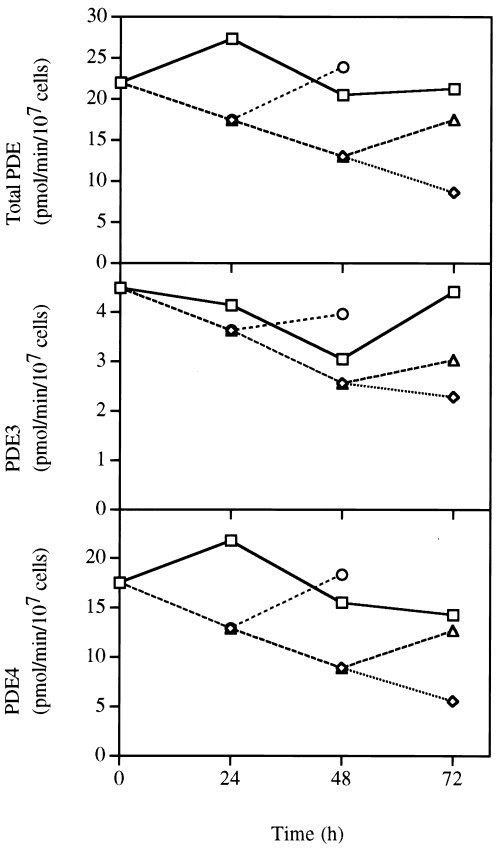

FIG. 4. Effects of IL-2 withdrawal on PDE activities in 1186 cells. 1186 cells (5×10^5/mL) were washed twice and resuspended in RPMI 1640 medium with 10% FBS with (□) or without (◇]) 20 U/mL of IL-2. At 24 hr, the cells that had been cultured without IL-2 were divided into two portions, which were incubated for 24 hr more with (○) or without (◇) IL-2. Then, the latter portion was again divided into two portions, which were incubated for 24 hr more with (△) or without (◇) IL-2. At the indicated times, cells were harvested, stained with trypan blue, and counted. Activities of total PDE, PDE3, and PDE4 were assayed in duplicate; mean values of duplicates (which differed by < 5%) are presented. The experiment was repeated with similar results.

inhibitor wortmannin (100 nM), the JAK inhibitor AG-490 (5 μM), and the tyrosine kinase inhibitor PP1 (0.5 and 5.0 μM), did not reduce PDE activity (Fig. 6B). (These concentrations of Ro 31–7549, PD98059, wortmannin, and AG-490 were found recently to block activation of PDEs by cytokines and phorbol ester in FDCP2 myeloid cells) [31, *]. After exposure to these drugs for 2–3 days, however, herbimycin A and Ro 31–7549 inhibited proliferation of MJ cells; the other kinase inhibitors did not (Fig. 6C). Herbimycin A and Ro 31–7549 also inhibited proliferation of HUT-102 cells, Jurkat cells, and PBMC-PHA. The effect of Ro 31–7549 was greater than that of herbimycin A in HUT-102 and Jurkat cells (Fig. 6, D and E); herbimycin A was less effective in Jurkat and HUT-102 cells than in MJ cells (Fig. 6, C–E).

In three HTLV-I^+ cell lines that exhibited constitutive JAK activation (MT-2 [38], MJ [39], and HUT-102 [41]), and three cell lines that did not (C8166–45 [37], C91/PL [40], and E55/PL [40]), incubation with the JAK inhibitor AG-490 (5 μM) for 30 or 60 min or 16 hr did not inhibit PDE activity or cell proliferation (data not shown).

Effects of Forskolin and Nonspecific and Isoenzyme-Specific PDE Inhibitors on Proliferation

Increased cAMP (achieved by adenylate cyclase stimulation and/or PDE inhibition) is known to inhibit lymphocyte proliferation [1, 54, 55]. Therefore, we examined the proliferative response of some HTLV-I^+ T-cell lines, PBMC-PHA, and Jurkat cells exposed to cAMP-elevating agents, including forskolin (which activates adenylyl cyclase), cilostamide (a specific PDE3 inhibitor [46, 55]), rolipram (a specific PDE4 inhibitor [46, 55]), and IBMX and pentoxifylline (which are nonselective PDE inhibitors). In lysates from MJ cells and HUT-102 cells, the IC_{50} for rolipram inhibition of PDE4 activity (measured in the presence of 1 μM cilostamide) was ~0.03 to 0.1 μM; in Jurkat cells, the IC_{50} for cilostamide inhibition of PDE3 activity (measured in the presence of 5 μM rolipram) was ~0.05 μM (data not shown). Both these results are in agreement with previous results from MBP-sensitive TCC [12].

As shown in Fig. 7, responses to different agents differed in different cell lines. Whereas IBMX alone markedly inhibited thymidine incorporation in MJ cells, 1186 cells, and PBMC-PHA, IBMX was much less effective in Jurkat

* Ahmad F, Cong L-N, Stenson Holst L, Wang L-M, Rahn-Landstrom T, Pierce JH, Quon MJ, Degerman E and Manganiello VC, Manuscript submitted for publication.

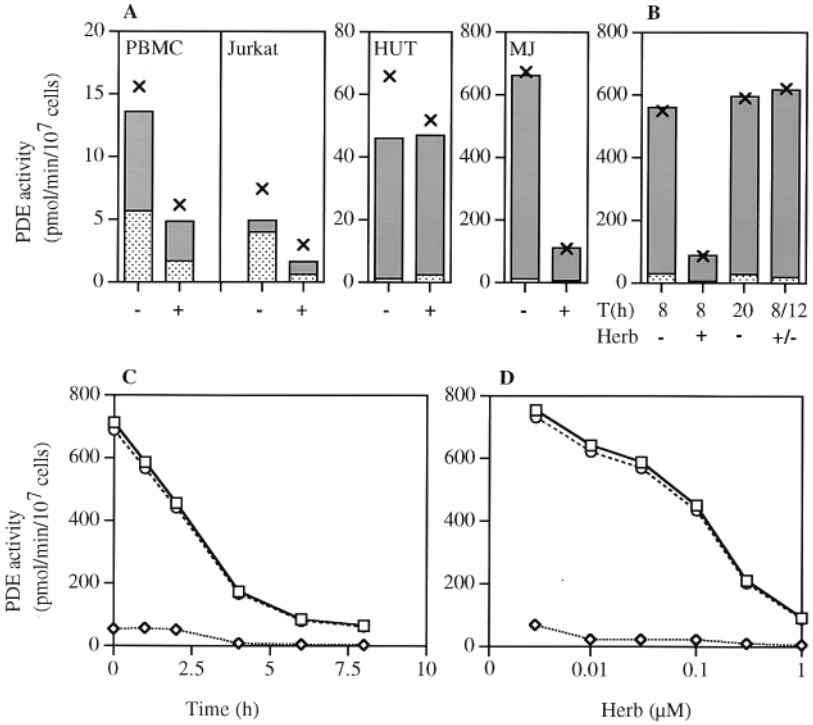

FIG. 5. Effects of herbimycin on PDE activity in different cell lines (A); and on reversibility (B), time course (C), and concentration-dependence (D) of effects in MJ cells. Panel A: The indicated cell lines (10^6 cells) were incubated for 8 hr in 3 mL of RPMI 1640 medium with 10% FBS and 1 μM herbimycin A (+) or vehicle, 0.07% DMSO (−), and harvested, stained with trypan blue, and counted prior to assay of PDE activities. Panel B: MJ cells (10^6 cells) were incubated for 8 hr with 1 μM herbimycin A or for 8 and 20 hr with 0.07% DMSO. A portion (1 mL) of the herbimycin-treated cells was withdrawn, stained with trypan blue, counted, and frozen. The remainder of the herbimycin-treated cells was washed twice in PBS and then resuspended in 2 mL of RPMI 1640 medium with 10% FBS and 0.07% DMSO. After another 12 hr, 1 mL each of the control cells and the treated cells were harvested, stained with trypan blue, and counted. For panels A and B, activities of total PDE (x), PDE3 (dotted bar), and PDE4 (shaded bar) were assayed in duplicate; mean values are presented. Panel C: At t = 0, 2, 4, 6, and 7 hr herbimycin A (final concentration 1 μM) was added; at t = 0 hr 0.07% DMSO was added to control cells. All cells were harvested at t = 8 hr. Panel D: MJ cells were incubated for 8 hr with the indicated concentrations of herbimycin A or 0.07% DMSO. For panels C and D, cells were stained with trypan blue and counted. Activities of total PDE (□), PDE3 (◇), and PDE4 (○) were assayed in duplicate. Experiments in panels A, B, C, and D were repeated with similar results.

cells and HUT-102 cells (Fig. 7A). Only in 1186 cells did forskolin produce a concentration-dependent inhibition of thymidine incorporation. In Jurkat, MJ, HUT-102 cells, and MT-2 cells, forskolin alone had little or no effect except in MT-2 cells at higher concentrations (10 μM). Rolipram had little effect in PBMC-PHA, except at 10 μM forskolin. Effects of forskolin and rolipram were additive in 1186 cells.

Rolipram inhibited proliferation of MJ cells in the absence or presence of forskolin (Fig. 7B). In the cell lines tested, cilostamide was ineffective or much less effective than rolipram. In some cells, i.e. MT-2, 1186, and PBMC-PHA, the effect of PDE inhibitors was obvious only in the presence of 10 μM forskolin. C91/PL cells were relatively insensitive to forskolin, cilostamide, and rolipram; proliferation was inhibited by IBMX. As seen in Fig. 7C, pentoxifylline, at 0.3 and 1.0 mM, inhibited proliferation in three of four cell lines tested. MT2 cells did not respond to pentoxifylline. Jurkat cells were sensitive to the higher concentrations of pentoxifylline, but not forskolin, rolipram, or cilostamide, and were relatively insensitive to IBMX (Fig. 7, A–C).

FACS analysis of MJ cells incubated with 50 μM rolipram for 20 hr showed accumulation of cells in the G_1 phase of the cell cycle (Fig. 8). No evidence of apoptosis was seen. This effect of rolipram most likely is related to inhibition of PDE4, since cAMP has been reported to block proliferation of macrophages in G_1 [62].

DISCUSSION

Since intracellular concentrations of cAMP, which are determined in part by PDEs, regulate proliferation and immune functions in lymphocytes, this work was designed to study the relationship between PDEs and proliferation in lymphocytes transformed with the oncogenic retrovirus HTLV-I and other lymphoid cells. We attempted to address three specific points: (i) whether PDE activities are up-regulated in HTLV-I^+ cells and, if so, which PDE isoenzymes are affected; (ii) potential mechanisms involved in regulation of PDE activities in HTLV-I^+ cells; and (iii) the effects of nonspecific and isoenzyme-specific PDE inhibitors on proliferation of HTLV-I^+ cells.

PDE3 and PDE4 activities were assayed in a number of

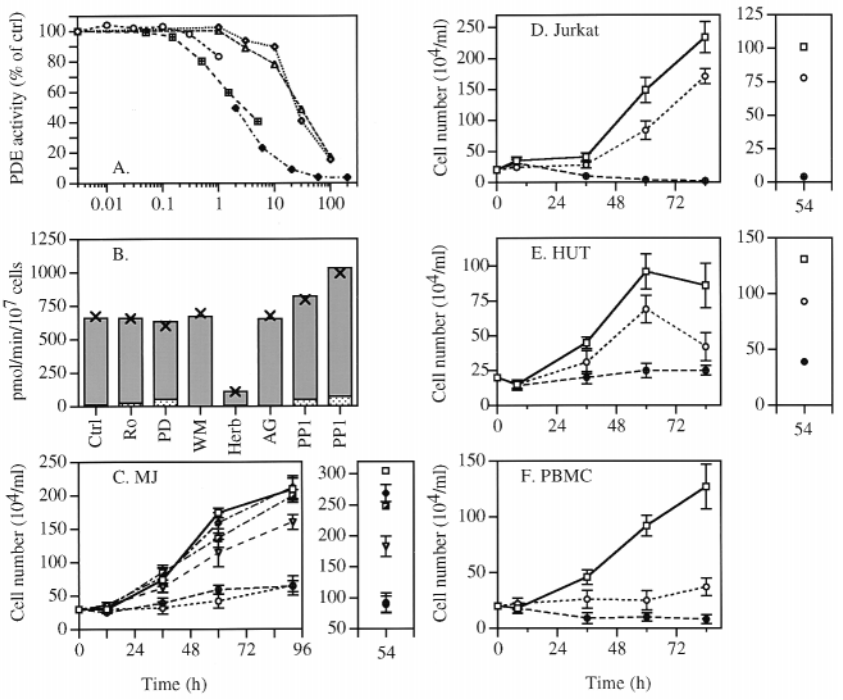

FIG. 6. Effects of tyrosine kinase inhibitors on PDE activities in MJ cell lysates (A) and of different kinase inhibitors on PDE activities in intact MJ cells (B) and on cell proliferation in MJ cells (C), Jurkat cells (D), HUT-102 cells (E), and PBMC (F). Panel A: MJ cells (10^6 cells) were harvested and homogenized as described in Materials and Methods. To portions of the same lysate, tyrosine kinase inhibitors [herbimycin A (○), PP1 (⊞), erbstatin analogue (◇), tyrphostin A25 (△), and genistein (◆)] were added to the indicated final concentrations. The concentration of vehicle (DMSO) did not exceed 0.2%. Total PDE activities were assayed in duplicate as described in Materials and Methods. Mean values are presented as percent activity with vehicle alone; the experiment was repeated with similar results. Panels B and C: MJ cells, 10^6/well, were incubated for 8 hr (panel B) or the indicated times (panel C) in 3 mL of RPMI 1640 medium with 10% FBS and different kinase inhibitors, including 10 μM Ro 31–7549 (a PKC inhibitor, ⊕), 50 μM PD98059 (a MAPKK-inhibitor, ▽), 100 nM wortmannin (a PI3-kinase inhibitor, ◪), 1 μM herbimycin A, (○) and 5 μM AG-490 (a JAK inhibitor, ▲) or 0.1% DMSO (□) alone. In panel B, total PDE (x), PDE3 (dotted bar), and PDE4 (shaded bar) were assayed in duplicate as described in Materials and Methods. Mean values are presented; the experiment was repeated with similar results. Panels D–F: 6×10^5 Jurkat cells (D), HUT-102 cells (E), and PBMC-PHA cells (F) were incubated in 3 mL of RPMI 1640 medium with 10% FBS and 10 μM Ro 31–7549 (●), 1 μM herbimycin A (○) or 0.1% DMSO (□) alone. For panels C–F, cells were harvested at the indicated times, stained with trypan blue, and counted using a hemocytometer. In panels C–E, the experiments were repeated, and cells were counted at t = 54 hr. Results are shown in side boxes. (Control cells were counted also at 30 hr to confirm that cell growth was in log phase; data not shown.) Cell counts (10^4 cells/mL) reflect means ± SD of 6 hemocytometer fields.

HTLV-I^+ cell lines, some IL-2-dependent, some IL-2-independent. Of the IL-2-independent cell lines, three exhibited constitutive JAK/STAT activation; others did not. PDE activities were also assayed in HTLV-II^+ cell lines, in malignant, HTLV-negative cell lines (Jurkat and Kit), in PHA-PBMC (which are mainly T-lymphocytes), in MBP-reactive TCC [12], in purified NK cells, in EBV-transformed B-lymphocytes, and in normal B-lymphocytes. PDE3 and PDE4 activities were detected in all cell lines studied. In Jurkat cells, PDE3 > PDE4; in most other T-lymphocyte-derived cells, i.e. normal (PMBC-PHA, NK cells, MBP-specific TCC), malignant (Kit225-K6), or virus-transformed (HTLV-II infected), PDE4 was ≈ PDE3. In EBV-transformed B-lymphocytes PDE4 was ≫ PDE3. Subfamily isoform expression of PDE3 and PDE4 mRNAs in normal, malignant, and HTLV-I^+ T-cells was analyzed via RT–PCR. In general, all PDE4 isoforms, 4A–D, were detected. However, amplification of PDE4C PCR products was more variable than the other PDE4 isoforms. PDE4A, B, and D (not 4C) and PDE7 mRNAs have been detected in lymphocytes [8] and other immune/inflammatory cells [63], and PDE1B1 mRNAs in human leukemic cells of T- and B-cell origin [63]. However, in antigen-specific Th1 TCC, PDE4C mRNA was detected by RT–PCR but at lower amounts than PDE4A, B, or D mRNAs [64]. Thus, the expression of PDE4C mRNA in lymphocytes is still an open question.

Total PDE activities (per cell) were in most cases higher in HTLV-I^+ than in other lymphoid cells. In one cell line, MJ cells, PDE4 activity was extremely high (~80-fold higher than in normal T-cells). Some of the increase in PDE activities could be ascribed to the increased size of the HTLV-I^+ cells. However, the PDE4/3 ratio, which is independent of cell size, was higher in HTLV-I^+ cells, suggesting that PDE4 is functionally up-regulated in these cells. RT–PCR gave no evidence that this was caused by selective induction of any of the PDE4A–D isoenzymes. Whereas PDE3B, not 3A, mRNA was detected by RT–PCR in MBP-specific TCC, PMBC-PHA, and Jurkat cells, both PDE3A and B mRNAs were detected in several

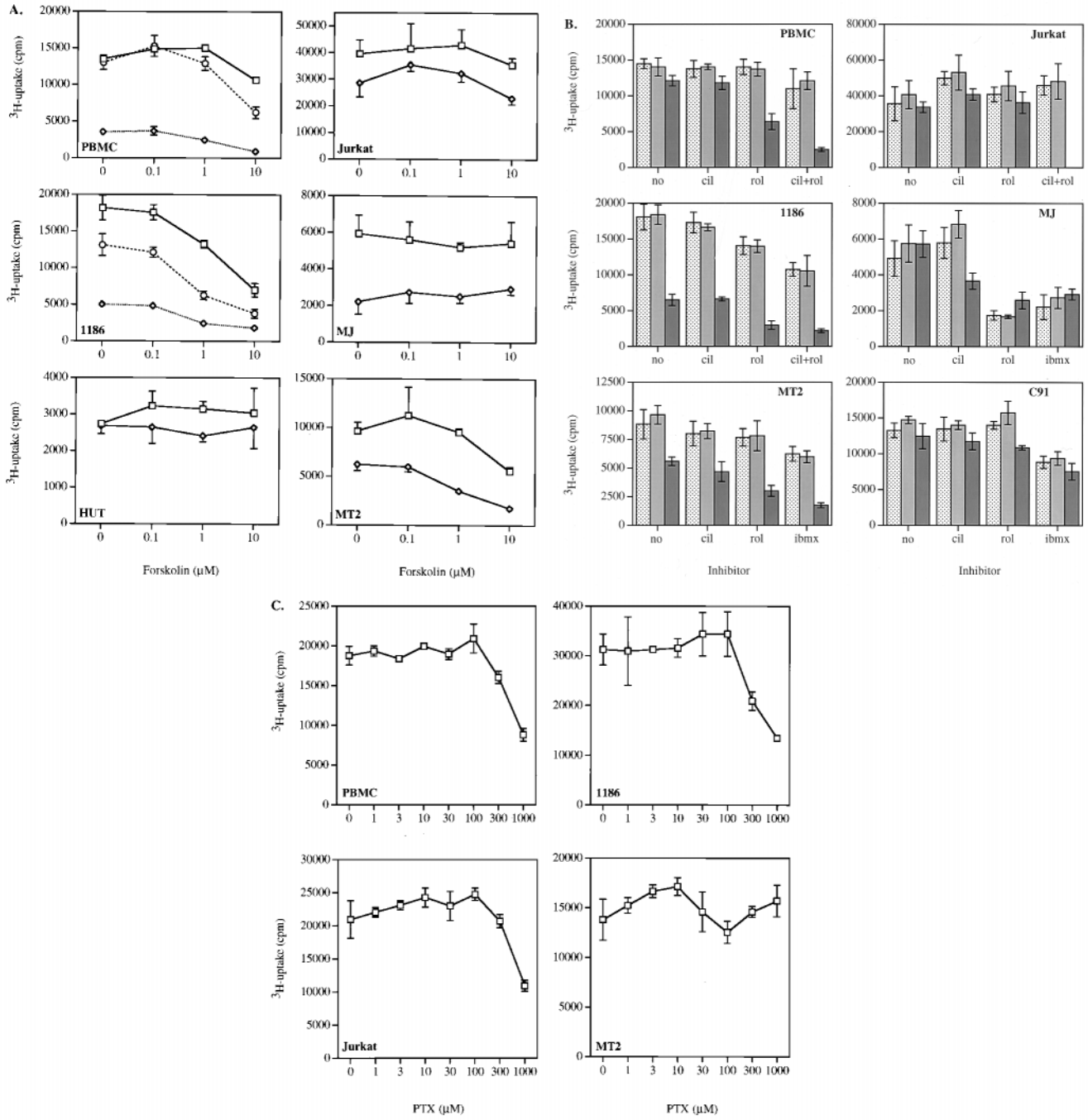

FIG. 7. Effects of forskolin, cilostamide, rolipram, and IBMX on cell proliferation. Cells (5000/well) were cultured in 96-well microtiter plates (Falcon) in 100 μL of RPMI 1640 medium with 10% FBS (20 U/mL of IL-2 was included for 1186 and PBMC-PHA) for 66 hr with (Fig. 7A) 0, 0.1, 1, and 10 μM forskolin in the absence (□) or presence (◇) of 200 μM IBMX or (○) 50 μM rolipram; (Fig. 7B) with 0 (dotted bar), 0.1 (light shaded bar), and 10 μM (dark shaded bar) forskolin in the absence or presence of 10 μM cilostamide, 50 μM rolipram, or 200 μM IBMX as indicated; (Fig. 7C) with the indicated concentrations of pentoxifylline. Concentration of vehicle (DMSO) was the same in all wells. Independent triplicate incubations were set up for all conditions. One μCi/well of [^{3}H]thymidine (NEN Dupont) was added for 6 hr, and cultures were incubated another 24 hr. Then cells were harvested with a micro cell harvester (Skatron), and [^{3}H]thymidine incorporation was determined on a Wallac liquid scintillation counter. Means ± SD (N = 3) for the triplicate incubations are shown. All experiments were repeated with similar results.

HTLV-I$^+$ T-cells. The significance of PDE3A mRNA expression in HTLV-I$^+$ T-cells is not known.

The second portion of this report describes our attempts to define the mechanism(s) by which PDEs are regulated in HTLV-I$^+$ and other lymphoid cells. Neither constitutive expression of the HTLV-I regulatory protein Tax in Taxl-1 cells [58] nor induction of Tax in Jpx9 Jurkat cells [57] resulted in up-regulation of PDE4. Therefore, Tax is presumably not directly responsible for the higher proportion of PDE4 activity in HTLV-I$^+$ T-cells. Although the JAK/

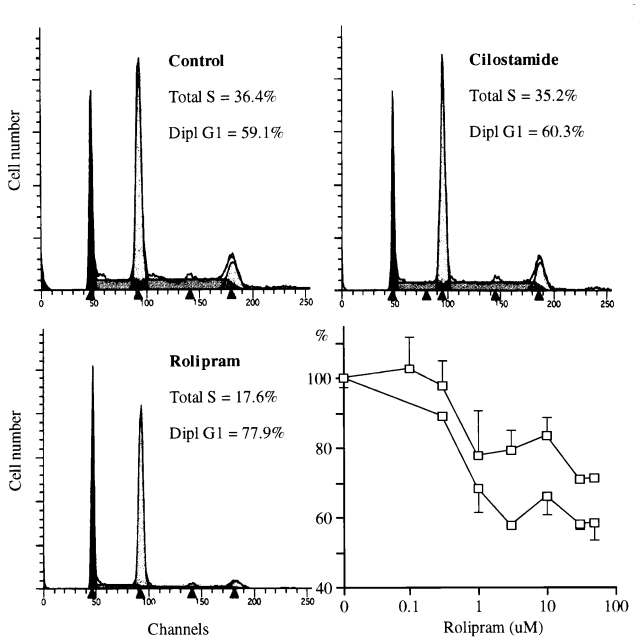

FIG. 8. Effects of rolipram on cell cycle phase distribution of MJ cells. MJ cells were incubated without or with 10 μM cilostamide or 50 μM rolipram for 24 hr; DNA content was analyzed by FACS flow cytometry as described in Materials and Methods. Lower right panel: MJ cells were incubated in triplicate with the indicated concentrations of rolipram, and [^{3}H]thymidine incorporation was measured as described in the legend to Fig. 7. Results of two experiments are presented. Means ± SD (N = 3) for the triplicate incubations in each experiment are shown.

STAT system is activated in some HTLV-I$^+$ cells, the JAK inhibitor AG-490 did not alter PDE activities in or proliferation of these cells. Since recent evidence suggests that, in adipocytes [33, 34] and FDCP2 hematopoietic cells [31, *], PDE3 and PDE4 can be activated by PI3-K and PKB- and MAPK-dependent signals, respectively, selective inhibitors of PI3-K, MAPKK, PKC, and src kinases were tested for their effects on PDE activities in HTLV-I$^+$ cells. Of these, only herbimycin A affected PDE activity. Herbimycin A markedly decreased PDE activity within 8 hr in HTLV-I$^+$ MJ cells, Jurkat cells, and PBMC-PHA, but produced much smaller, if any, effects in some other HTLV-I$^+$ T-cells, including HUT-102 and MT-2 cells. Herbimycin A did not directly inhibit PDE activity in cell lysates.

In cultured rat theca and mouse TM3 Leydig cells, herbimycin A has been reported to reduce PDE4 activity by ~70% within 8 hr [36]. In TM3 cells expressing a temperature-sensitive src kinase, PDE activity was increased at the permissive temperature [36], suggesting a role for src kinase in regulation of PDE4 activity. PP1, another src family kinase inhibitor, however, did not mimic the effect of herbimycin A on PDE activities in HTLV-I$^+$ cells. In addition, other tyrosine kinase inhibitors, including genistein, which has been reported to alter cAMP and PDE activities in intact cells [59–61], and tyrphostin and the erbstatin analog directly inhibited PDE activities in cell lysates. Thus, herbimycin A and other tyrosine kinase inhibitors might alter PDEs and cell proliferation by mechanisms other than inhibition of tyrosine kinases. In T-cells, inhibition of IP3 production by herbimycin A is usually ascribed to inhibition of tyrosine kinases that activate PLC-1, but might also be related to inhibition of formation of inositol phospholipids [65].

The third portion of this report deals with effects of PDE inhibitors. Since inhibition of PDEs, resulting in elevated cAMP, has been shown to inhibit proliferation in several cell types, including leukemic cells, the effects of cAMP-elevating drugs such as forskolin and PDE inhibitors on cell growth of HTLV-I$^+$ cells and other cells were investigated. For example, specific PDE3 and PDE4 inhibitors have been reported to inhibit proliferation in MBP-specific TCC [12], antigen-specific Th1 and Th2 TCC [64], peripheral human lymphocytes [8, 13], cultured rat [66] and porcine [67] aortic smooth muscle cells, rat mesangial cells [68], and murine melanoma and human mammary carcinoma cells [69]. Although PDE3 inhibitors were not as effective as PDE4 inhibitors in blocking proliferation of peripheral lymphocytes [8, 13], cilostamide did inhibit proliferation of some MBP-specific TCC [12]. In some TCC, the inhibitory effects of rolipram on proliferation were associated with inhibition of IL-2 production.† The nonspecific PDE inhibitor IBMX as well as forskolin and cAMP analogs inhibited proliferation of malignant glioma cells [70]. In a lymphoblastoid cell line from a patient with acute B-lymphocytic leukemia, inhibition of PDE1 or PDE4 induced apoptosis [53]. Vesnarinone, a PDE3 inhibitor, inhibited growth and induced apoptosis in rat myeloid leukemia cells [71], and theophylline, a nonspecific PDE inhibitor, had a synergistic effect with chlorambucil in inducing apoptosis in malignant cells from patients with chronic B-lymphocytic leukemia [72].

The different HTLV-I$^+$ cells exhibited different responses with respect to effects of different agents on cell growth. One striking effect was that of rolipram in MJ cells, a HTLV-I$^+$ cell line that exhibits extremely high PDE4 activity. Both rolipram and the nonspecific PDE inhibitor IBMX strongly inhibited growth in the absence of forskolin. FACS analysis showed the arrest to take place in the G_1 phase of the cell cycle. Several other cell lines responded to rolipram, but some did not. No cell line responded to the PDE3 inhibitor cilostamide. The nonspecific PDE inhibitor pentoxifylline, presently used to treat peripheral vascular disease in human patients, inhibited growth of HTLV-I$^+$ cell lines (except MT-2 cells), but only at concentrations far above therapeutic levels. Only one cell line, 1186, responded to forskolin. The different responses of the different cell lines could reflect phenotypic differences in

* Ahmad F, Cong L-N, Stenson Holst L, Wang L-M, Rahn-Landstrom T, Pierce JH, Quon MJ, Degerman E and Manganiello VC, Manuscript submitted for publication.

† Roland Martin, Laboratory of Immunopharmacology, NINDS, NIH, personal communication. Cited with permission.

drug uptake/metabolism, in regulation, organization, or compartmentalization of the adenylyl cyclase/PDE systems, or could indicate that disruptions in cAMP signaling are not sufficient to inhibit proliferation. Since HTLV-I-infected cell lines show different sensitivities to PDE inhibitors with respect to proliferation, it is possible that effects of PDE inhibitors on growth of malignant cells from individual ATLL patients could be tested *in vitro*. In cases where high sensitivity was found, treatment with the PDE inhibitor could be tried.

The potential use of PDE inhibitors in HTLV-I associated myelopathy (TSP/HAM) is supported by recently reported inhibitory effects of rolipram [73] and pentoxifylline [74] on expression of the surface adhesion molecules (LFA-1 and VLA-4) [74], TNF-α production [73, 74], and spontaneous proliferation of PBMC [73, 74] from HAM patients and some HTLV-I$^+$ T-cell lines [73]. Although in our studies concentrations of pentoxifylline required to inhibit proliferation of PBMC, Jurkat, and MJ cells were much higher than those achieved by therapeutic doses, lower concentrations of pentoxifylline inhibit TNF-α and IL-12 production by human PBMC [75], and administration of pentoxifylline to clinical subjects does alter cytokine production and proliferation of PBMC isolated from these subjects [76]. Cytokine modulation also may account for effects of PDE inhibitors on the HIV retrovirus. Rolipram and pentoxifylline inhibited HIV replication (which is stimulated by TNF-α) and TNF-α production in acutely HIV-infected PBMC and U1 promonocyte cells; rolipram was much more potent than pentoxifylline [77].

By their immunosuppressive effects, PDE4 inhibitors seem to hold considerable potential as anti-inflammatory drugs in a number of diseases. Rolipram [78–81] and pentoxifylline [82] effectively suppressed experimental allergic encephalomyelitis (EAE) in two animal models of multiple sclerosis (MS), namely in Lewis rats [79, 82] and monkeys [80]. The effect may be related to their effects on the production of TNF-α and interferon (IFN)-γ by autoreactive CD4$^+$ Th1 T-cells, thought to be important in the pathogenesis of EAE and MS [79, 80]. Rolipram also has been shown to suppress collagen-induced arthritis in rats [78], an animal model of rheumatoid arthritis. In non-obese diabetic (NOD) mice, rolipram and, less potently, pentoxifylline reduced the severity of insulitis and prevented diabetes [83]. PDE4 inhibitors and combined PDE3/PDE4 inhibitors also are being investigated for use in asthma [54, 84, 85].

The possible therapeutic benefits of PDE inhibitors in patients (perhaps specific patients) with lymphoproliferative malignancies, virus-induced or caused by other agents, remain to be examined.

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