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MULTIPLE HIGH-AFFINITY cAMP-PHOSPHODIESTERASES IN HUMAN T-LYMPHOCYTES

STEVEN A. ROBICSEK,* D. KAY BLANCHARD,† JULIE Y. DJEU,†
JOSEPH J. KRZANOWSKI,* ANDOR SZENTIVANYI‡ and JAMES B. POLSON*§

Departments of *Pharmacology and Therapeutics, †Medical Microbiology and Immunology, and ‡Internal Medicine, College of Medicine, University of South Florida, Tampa, FL 33612-4799, U.S.A.

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Abstract—Cyclic nucleotide phosphodiesterases (PDEs) are the only enzymes that inactivate intracellular cyclic AMP (cAMP). Because the functions of T-lymphocytes are modulated by cAMP levels, the isozymes of PDE in these cells are potential targets for new drugs designed to modify the body's immunity through selective alteration of T-lymphocyte PDE activity. Cyclic GMP and 3(2*H*)-pyridazinone-4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-5-methyl-monohydrochloride (CI-930) selectively inhibit the catalytic activity of one of the two high affinity cAMP-PDE isozyme families known to occur in mammals, whereas *d,l*-1,4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone (Ro 20-1724) selectively inhibits the other. The objectives of this investigation were: (1) to determine whether human T-lymphocytes contain one or both of these pharmacologically distinguishable high-affinity cAMP-PDEs, and (2) to determine the effects of selective inhibitors of these PDEs on lymphocyte blastogenesis. High-affinity cAMP-PDE was found in both the soluble and particulate fractions of T-lymphocyte sonicates. Cyclic GMP and CI-930 inhibited PDE in the particulate fraction better than in the soluble fraction, but the converse was found for Ro 20-1724. CI-930 or Ro 20-1724, used alone, attenuated T-lymphocyte blastogenesis, but neither suppressed it completely. In combination, the same PDE inhibitors caused greater suppression of blastogenesis than either produced alone. The results indicate that human T-lymphocytes contain both CI-930- and Ro 20-1724-inhibitable isozymes. Either of the isozymes can modulate human T-lymphocyte blastogenesis, but inhibition of both isozymes produces synergistic antiblastogenic effects.

Intracellular cyclic AMP (cAMP) is an essential regulator of lymphocyte blastogenesis [1]. The only enzymes known to catalyze the inactivation of this second messenger are the phosphodiesterases (PDEs‖). In the past two decades, several laboratories have demonstrated and partially characterized high-affinity cAMP-PDE activity in human lymphocytes (reviewed by Epstein and Hachisu [2]).

Blastogenesis has been shown to be attenuated by agents that elevate cAMP in lymphocytes by the inhibition of PDE [2–4]. However, these findings were made using heterogeneous populations of lymphocytes from peripheral blood, and it was uncertain which lymphocyte subsets were directly affected by the cAMP-elevating agents. Recent improvements in the purification of peripheral blood T-lymphocytes now provide more homogeneous preparations of this lymphocyte subpopulation for study than were available in the past [5, 6]. Also, the availability of selective pharmacological inhibitors of PDE isozymes has provided a means of distinguishing different high-affinity cAMP-hydrolyzing isozymes and their effects [7–9].

The purpose of this investigation was to study cAMP- (or type IV-) PDEs [10] in highly purified human T-lymphocytes using known selective inhibitors (cGMP, CI-930, and Ro 20-1724) of two different high-affinity cAMP-PDE isozyme families [7, 9]. In addition, we compared the antiblastogenic effects of two of the selective PDE inhibitors (CI-930 and Ro 20-1724) with the antiblastogenic effects of a non-selective PDE inhibitor, papaverine [11], on T-lymphocytes. This investigation differs from previously reported investigations by focusing on the human T-lymphocyte subpopulation and by including selective inhibitors for both of the known high-affinity cAMP-PDE isozyme families.

§ To whom correspondence should be sent at the following address: Department of Pharmacology and Therapeutics, College of Medicine, University of South Florida, 12901 Bruce B. Downs Boulevard, Tampa, FL 33612-4799.

‖ Abbreviations: bis-tris, bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane; BSA, bovine serum albumin; CD, cluster designation; CGI-PDE, cGMP-inhibitable PDE (also inhibitable by CI-930); CI-930, 3(2*H*)-pyridazinone-4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-5-methyl-monohydrochloride); complete medium, supplemented medium containing 5% heat-inactivated human AB serum and 50 μM β-mercaptoethanol; $F_{x,y}$, calculated *F* value, where x and y are degrees of freedom, between and within groups, respectively; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, sodium salt; I3, monoclonal antibody (mouse IgG2/a) reacting with a nonpolymorphic class II (HLA-DR) Ia antigen; P2, monoclonal antibody (mouse IgG1/K) reacting with glycoprotein IIb–IIIa complex (CD41a) found on human platelets; PDE, 3',5'-cyclic nucleotide phosphodiesterase (EC 3.1.4.17); Percoll, colloidal polyvinylpyrrolidone coated silica; PHA, phytohemagglutinin; Ro 20-1724, *d,l*-1,4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone; RoI-PDE, Ro 20-1724-inhibitable PDE; supplemented medium, RMPI-1640 containing 2 mM L-glutamine, 100 units/mL penicillin-streptomycin; t_x, calculated Student's *t* value, where x is the degree of freedom; and [^{3}H]TdR, [*methyl*-^{3}H]thymidine.

METHODS

Chemicals and reagents. Tris–HCl, bis-tris, cAMP, cGMP, BSA (Fraction V, A 3294), EDTA, alkaline phosphatase (bovine intestinal mucosa, type VII-N, P-2276), pepstatin A (P-4265), aprotinin (A-6279), β-mercaptoethanol (β-ME), Percoll (colloidal polyvinylpyrrolidone coated silica, P-1644), Histopaque-1077 (Ficoll and sodium diatrizoate), trypan blue stain, L-glutamine, penicillin-streptomycin, and HEPES were purchased from the Sigma Chemical Co. (St. Louis, MO). Piperazine dihydrochloride was obtained from Alpha Products (Danvers, MA). Calcium chloride and magnesium chloride were purchased from Mallinckrodt Inc. (St. Louis, MO). Sodium chloride was obtained from Fisher Scientific (Fair Lawn, NJ), and AG 1-X8 (200–400 mesh) resin from Bio-Rad Laboratories (Richmond, CA). Human serum (type AB) was purchased from the Flow Laboratories Inc. (McLean, VA) and was heat-inactivated by incubation at 56° for 30 min. Aliquots were stored frozen at −80° until used. Purified phytohemagglutinin (PHA) (HA-16) was purchased from Wellcome Diagnostics (Research Triangle Park, NC). [^{3}H]cAMP (29 Ci/mmol) was purchased from NEN Research Products (Boston, MA) and [*methyl*-^{3}H]thymidine ([^{3}H]TdR, 2 Ci/mmol) from ICN Biomedicals, Inc. (Irvine, CA). Prosil-28 organosilane coating compound was obtained from Thomas Scientific (Swedesboro, NY).

Buffers and media. Sterile phosphate-buffered saline solution (6.7 mM, pH 7.4) (PBS) was from Whittaker M.A. Bioproducts (Walkersville, MD). Sonication buffer was 20 mM bis-tris (pH 6.8), 1 mM EDTA, 50 kallikrein units of aprotinin/mL, 1 mg pepstatin/mL, 20 μg leupeptin/mL, 50 mM benzamidine and 3.75 mM β-ME except where indicated. Assay buffer was 100 mM Tris–HCl, 20 mM $MgCl_2$, 20 μM $CaCl_2$, 0.2% BSA, and 7.5 mM β-ME.

RPMI-1640 (Gibco Laboratories, Grand Island, NY) was the medium used for all cell cultures and cell storage. RPMI-1640 was supplemented with 2 mM *l*-glutamine, 100 units penicillin/mL, 100 μg streptomycin/mL, and 5 mM HEPES (supplemented medium). Five percent human AB serum was added to the supplemented medium for some procedures (complete medium).

PDE inhibitors. Ro 20-1724 (*d,l*-1,4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone) and CI-930 [3(2*H*) - pyridazinone - 4,5 - dihydro-6-[4-(1*H*-imidazol - 1 - yl)phenyl] - 5 - methyl - monohydrochloride] were gifts from Hoffmann-LaRoche, Inc. (Nutley, NJ) and Warner-Lambert/Parke-Davis (Ann Arbor, MI), respectively. Papaverine was purchased from the Sigma Chemical Co. All inhibitor solutions were freshly dissolved in either assay buffer (for the phosphodiesterase assay) or RPMI-1640 medium (for the blastogenesis experiments).

Cell enumeration. Cells were counted throughout purification procedures using a hemocytometer and a 1:1 (v:v) addition of 0.4% trypan blue stain. A minimum of 250 cells were counted for each cell enumeration.

Purification of lymphocytes. Buffy coat units prepared from human peripheral venous blood were obtained from the Southwest Florida Blood Bank (Tampa, FL). Donors were healthy adult (above 18 years of age) male and female volunteers. Mononuclear cells were prepared by the depletion of erythrocytes and polymorphonuclear leukocytes by Ficoll–Hypaque centrifugation. Adherent cells were subsequently depleted by plating on plastic tissue culture flasks for 1 hr at 37°, 5% CO_2 (10^8 cells/75 cm^2) in complete medium followed by incubation on nylon wool columns for 30 min at 37°, previously equilibrated with complete medium. The nonadherent cells were purified further by discontinuous Percoll density gradient centrifugation [5, 6]. For this procedure, cells were placed on a four-step discontinuous gradient using 42.5%, 45.0%, 47.5% and 67.5% Percoll and centrifuged for 30 min at 550 *g* at room temperature. The cells banding between 47.5 and 67.5% Percoll were collected. Approximately 10–15% of the beginning leukocyte population was recovered and referred to as "purified T-lymphocytes."

To minimize contamination by platelets, B-lymphocytes and activated T-lymphocytes of preparations used to characterize resting T-lymphocyte PDE, "highly purified" preparations were obtained from purified T-lymphocyte preparations by the depletion of cells expressing glycoprotein (gp)IIb–IIIa complex and class II (HLA-DR) Ia cell surface antigens. For this purpose, murine IgG monoclonal antibodies against (1) human platelet gpIIb–IIIa complex (P2; Amac, Inc. Westbrook, ME) and (2) HLA-DR (I3; Coulter Immunology, Hileah, FL) were employed. Two micrograms of P2 and 10 μg I3 were added per 10^8 cells in PBS containing 1% AB serum (2×10^8 cells/mL) in siliconized borosilicate tubes, and the mixtures were incubated for 30 min on ice. The cells were washed twice in 50-mL volumes of PBS. Magnetic goat anti-mouse IgG (Advanced Magnetics Inc., Cambridge, MA) was added to the cell suspension (1.25 mg/10^8 lymphocytes) and incubated at room temperature for 30 min. Positive cells (i.e. those that bound magnetic-antibody complex) were removed by placing the suspension next to a magnet for 5 min. The negative cell population was then collected for use.

Cytofluorometric analysis. One hundred microliter aliquots (1×10^6 cells) were added to various monoclonal antibody pairs tested. Cluster designations (CD) were analyzed using antibodies from the following sources: from Coulter Immunology (Hileah, FL), CD2 (anti-T11), CD4 (anti-T4), CD8 (anti-T8), and CD20 (anti-B1); from Becton-Dickinson (Mountain View, CA), CD3 (anti-Leu4), CD19 (anti-Leu1213), Simultest™ LeucoGATE™ [CD45 (anti-leucocyte-fluorescein isothiocyanate, FITC)] and CD14 (anti-LeuM3 phycoerythrin).

After the addition of fluorescent-labeled antibodies, the samples were incubated in siliconized test tubes on ice for 30 min. Then the cells were washed with PBS and resuspended in a 1% paraformaldehyde–PBS solution. The samples were then stored in the refrigerator and protected from light until analysis, which occurred usually within 24 hr. Analysis was performed using a FACScan flow cytometer (Becton-Dickinson).

Cell disruption. Cell preparations were suspended to provide a concentration of 1×10^8 cells/mL in sonication buffer at 4° and sonicated on ice with a

Branson Cell Disrupter model 200 (Danbury, CT) fitted with a microprobe. Sonication was performed three times for 10 sec at the No. 2 setting. This provided the minimal energy necessary to obtain 99% disruption of the cells as verified by light microscopy.

Soluble and particulate fractions. The cell sonicate was centrifuged at 23,600 *g* for 30 min at 4°. The supernatant fluid was collected and the particulate fraction resuspended and washed twice in 2 volumes of sonication buffer.

PDE assay. Cells were purified and assayed on the same day the blood was donated (total elapsed time from phlebotomy to assay was approximately 10 hr) or stored overnight in complete medium at 4° and assayed the following day. A modification of the assay described by Thompson and Appleman [12] was used. Briefly, [^{3}H]cAMP was incubated with PDE at 37° in 20-μL volumes (10 μL cell sonicate plus 10 μL assay buffer, final pH 8.0) which contained 0.22 units of alkaline phosphatase. The reactions were initiated by the addition of PDE and stopped by the addition of 0.5-mL volumes of a 1:3 (v:v) slurry of AG 1-X8 anion exchange resin, a mixture of equal volumes of water and isopropanol, which bound the unreacted nucleotide but not the dephosphorylated nucleoside. The samples were centrifuged at 550 *g* for 15 min and the radiolabeled nucleosides in the supernatant fluid were counted using liquid scintillation spectrometry. Three to five enzyme dilutions were prepared in duplicate and assayed to determine each velocity. Reactions were run for 10 min and linearity of velocity with respect to enzyme concentration was verified.

Kinetic analysis and Hill coefficient determination. Cyclic AMP-PDE activity was assayed at seventeen substrate concentrations ranging from 0.052 to 17 μM cAMP. All velocities used for this analysis were derived from duplicate assays of three to five dilutions of enzyme. The maximum amount of cAMP hydrolyzed at any enzyme dilution was less than 20% of the initial cAMP concentration. Analysis of Michaelis–Menten kinetics and determination of the Hill coefficient for cAMP hydrolytic activity were accomplished using Enzfitter® non-linear regression analysis (Elsevier–Biosoft, Cambridge, UK). Velocities determined at four to eight consecutive substrate concentrations were used in the calculation of each apparent Michaelis–Menten constant (K_m) and maximum velocity (V_{max}). All velocity measurements were used for the determination of the Hill coefficient.

Lymphocyte blastogenic assay. Measurements of cell proliferation were made by determining the incorporation of [^{3}H]TdR into cellular DNA. Purified T-lymphocytes were suspended to provide 2×10^6 cells/mL in complete medium containing 50 μM β-ME and distributed in 96-well flat bottom microtiter plates in 100-μL volumes. The cells were preincubated in the presence of various inhibitor concentrations for 30 min and then cultured for 36 hr in 5% CO_2 at 37° with or without 0.63 μg PHA/mL, in total volumes of 200 μL. At the end of this incubation period, 0.5 μCi of [^{3}H]TdR was added to each well, and the cells were incubated for an additional 18-hr period. Cells were then collected onto glass fiber filters and counted by liquid scintillation spectrometry. To determine the concentrations of inhibitors that attenuated proliferation by 50%, concentration–response curves were fitted (GraphPAD Inplot, GraphPAD Software, San Diego, CA) to the four parameter logistic equation (sigmoid curve) described below:

$$Y = A + (B - A)/[1 + (10^C/10^X)^D]$$

where X is the log concentration of drug, Y is the response, A and B are the bottom and top plateaus, respectively, C is the log concentration of drug at the inflection point, and D is the maximum slope.

Statistical analysis. The data are expressed as the arithmetic mean ± SEM except for 50% inhibitory concentrations for blastogenesis for which data are expressed as geometric means ± 95% confidence limits [13]. Comparisons of the means of two groups were performed by a two-tailed Student's *t*-test for paired observations. For three groups, equality of variances was tested [14] and the data were analyzed by one-way analysis of variance followed by the Duncan multiple range test [15] for comparisons of means. A probability (P) value of less than 0.05 was considered significant.

Table 1. Characterization of purified and highly purified T-lymphocyte populations

Antigen cluster designation	% Cell population	
	Purified T-lymphocytes	Highly purified T-lymphocytes
CD45	ND*	97 ± 1
CD2	98 ± 1	98 ± 1
CD3	88 ± 5	94 ± 2
CD4	54 ± 10	59 ± 6
CD8	45 ± 6	39 ± 3
CD14	2 ± 2	2 ± 0
CD19/20	2 ± 2	0 ± 0
HLA-DR	ND	2 ± 0

Monoclonal antibodies conjugated with FITC were used to determine antigen cluster determinants of cell subsets by FACS analysis. Each value is the mean ± SEM of preparations from three individuals.
* ND = not determined.

RESULTS

Cell population. Purified and highly purified preparations of T-lymphocytes were analyzed by flow cytofluorometry to enumerate the cells that bound monoclonal antibodies directed against defined cell surface antigens. Table 1 shows the percentages of positive cells for CD2 (T-cells and subsets of natural killer cells), CD3 (T cells), CD4 (T helper/inducer cells), CD8 (T suppressor/cytotoxic cells and subsets of natural killer cells), CD19/20 (B cells), CD14 (monocytes, macrophages, and granulocytes), CD45 (leukocyte common antigen), and HLA-DR (Class II major histocompatibility complex). The results indicate that both preparations contained 98% lymphocytes of

Table 2. PDE activities in the soluble and particulate fractions of purified T-lymphocytes

	PDE activity* (fmol cAMP hydrolyzed/min/10^6 cells)			
	One-day procedure		Two-day procedure	
0.2 μM cAMP				
Soluble	270 ± 52	(79%)†	230 ± 41	(81%)†
Particulate	72 ± 1.2	(21%)	66 ± 9.1	(19%)
1.0 μM cAMP				
Soluble	990 ± 211	(83%)	770 ± 44	(80%)
Particulate	210 ± 20	(17%)	190 ± 4	(20%)

* cAMP (0.2 or 1.0 μM) was used as substrate (mean ± SEM from three individuals).
† Percent of total activity is indicated in parentheses.

which 88 and 94% were T-lymphocytes in the purified and highly purified preparations, respectively. Both helper/inducer and suppressor/cytotoxic T-lymphocytes were present in normal ratios and contamination by other peripheral blood cells (erythrocytes, polymorphonuclear leukocytes, macrophages and B-lymphocytes) was near or below the limits of detection. All preparations contained more than 99% viable cells as determined by trypan blue exclusion.

Platelets were enumerated by phase contrast microscopy and the results verified periodically by immunofluorescence microscopy using FITC-labeled anti-gpIIb–IIIa. Purified cell preparations contained no more than 3 platelets per 100 lymphocytes (0.4 ± 0.1 for 49 preparations), while no platelets were detected in any of the highly purified preparations (limit of detection was 1 platelet:3000 lymphocytes).

Phosphodiesterase activity in soluble and particulate fractions. Soluble and particulate PDE activities measured in extracts from purified T-lymphocytes the same day or following overnight storage of cells were compared (Table 2). The soluble cAMP-PDE activity was approximately four times greater than particulate in extracts from purified lymphocytes. The soluble activity remained in the supernatant fraction after an additional centrifugation of 100,000 *g* for 1 hr (data not shown). Because storage of intact cells produced no significant change in overall activity, which was consistent with the previous report by Thompson *et al.* [16], the cells were stored overnight and used in experiments the next day unless otherwise indicated.

Kinetic studies of PDE were performed on highly purified T-lymphocytes. Linear transformation of these data [17, 18] revealed two apparently linear regions as illustrated in Fig. 1. The apparent Michaelis constants (K_m) and maximum velocities (V_{max}) were determined and are shown in Table 3. Both high-affinity ($K_m < 1$ μM) and lower-affinity activities were detected in both the soluble and particulate fractions. The maximum velocities associated with the lower affinity activities were eleven (soluble) and five (particulate) times greater than those associated with the high-affinity activities. To determine whether allosteric interactions could explain the nonlinear kinetics, Hill coefficients were calculated. These were 0.88 ± 0.017 and 0.87 ± 0.027

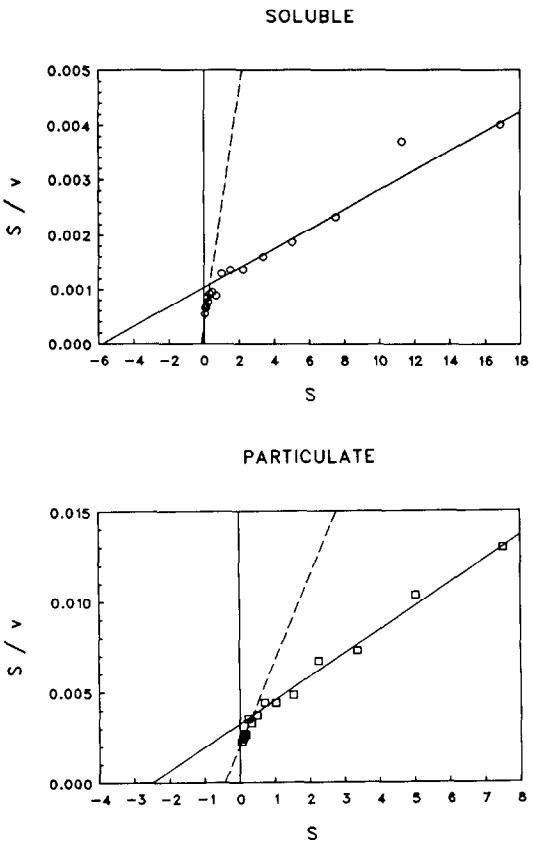

Fig. 1. Representative Hanes–Wilkinson plots of particulate and soluble cAMP-PDE activity in highly purified T-lymphocytes. Substrate concentration (S) was plotted against substrate concentration/velocity (S/V) according to Hanes [17].

for soluble and particular fractions, respectively (mean ± SEM from three individuals).

The concentration dependence of PDE inhibition by CI-930 and Ro 20-1724 was determined using extracts from highly purified T-lymphocytes and is illustrated in Fig. 2. CI-930 produced approximately 90% inhibition of particulate activity at its highest concentration (52 μM), and its IC_{50} was determined

Table 3. Apparent K_m and V_{max} values for cAMP-PDE in the soluble and particulate fractions obtained from highly purified T-lymphocytes

	Apparent K_m (μM)	Apparent V_{max} (fmol cAMP hydrolyzed/min/10^6 cells)
Soluble	0.18 ± 0.01*†	380 ± 43*
	4.9 ± 0.4‡	4100 ± 810
Particulate	0.38 ± 0.03§	170 ± 13
	3.4 ± 0.6‖	790 ± 30

* Mean ± SEM from three individuals.
† Significantly different from ‡ ($t_2 = 11.4$; $P < 0.01$).
§ Significantly different from ‖ ($t_2 = 4.8$; $P < 0.05$).

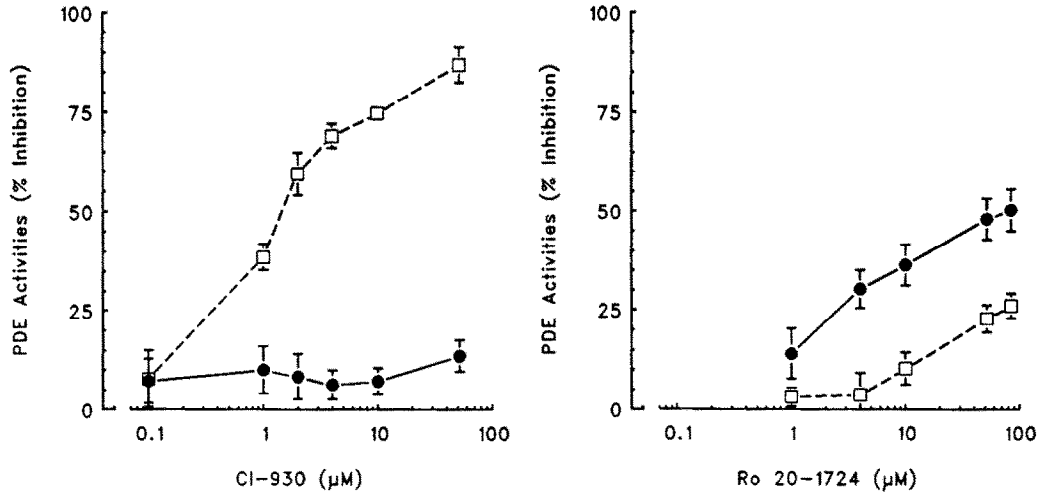

Fig. 2. Inhibition of soluble and particulate PDE activities of highly purified T-lymphocytes by CI-930 or Ro 20-1724. Assays were performed using 0.2 μM cAMP substrate as described in Methods for soluble (●—●) and particulate (□---□) fractions. Data are expressed as percent inhibition compared to control values of cAMP-PDE in the soluble (220 ± 20 fmol cAMP hydrolyzed/min/10^6 T-lymphocytes) and particulate (51 ± 14 fmol cAMP hydrolyzed/min/10^6 T-lymphocytes) fractions (means ± SEM from four individuals). All points on the particulate curves were significantly different ($P < 0.05$) from soluble except for 0.1 μM CI-930 and 1 μM Ro 20-1724.

to be between 1 and 2 μM. In contrast, CI-930 was ineffective against soluble activity. Cyclic GMP (1 μM) also produced inhibition of the particulate (48 ± 3.7% inhibition) but not the soluble (2.4 ± 1.6% inhibition) using 1.0 μM cAMP as substrate (mean ± SEM from three individuals, data not shown). In contrast, Ro 20-1724 was clearly a better inhibitor of soluble than particulate PDE, but it did not produce 100% inhibition at the highest concentration studied (85 μM).

Effects of PDE inhibitors on blastogenesis. To assess the relative importance of the CI-930- and Ro 20-1724-sensitive isozymes in the regulation of blastogenesis, the concentration-dependent effects of these inhibitors on PHA-induced blastogenesis were determined. The concentration of PHA used for this purpose (0.63 μg/mL) was slightly less than the amount that produced maximal blastogenesis in concentration–response experiments (not shown). Both PDE inhibitors attenuated the incorporation of [^{3}H]TdR into T-lymphocytes (Fig. 3), but relatively high concentrations were required for 50% inhibition. The combination of equimolar amounts of the two agents (Table 4) caused 50% inhibition of [^{3}H]TdR incorporation at a total concentration of 8.9 μM (4.5–17) compared to the calculated concentration of 56 μM (27–110) assuming zero interaction between the drugs [19]. This difference was significant ($t_6 = 4.931$; $P < 0.01$), indicating a synergistic effect on T-lymphocyte proliferation. Papaverine, a non-selective PDE inhibitor, caused total suppression of blastogenesis at 33 μM concentrations, and produced 50% suppression of blastogenesis at 2.9 μM. The anti-proliferative effects of these PDE inhibitors were confirmed by periodic cell counts. Cell viability at the end of the incubations was greater than 90%.

DISCUSSION

T-lymphocytes coordinate the body's immune

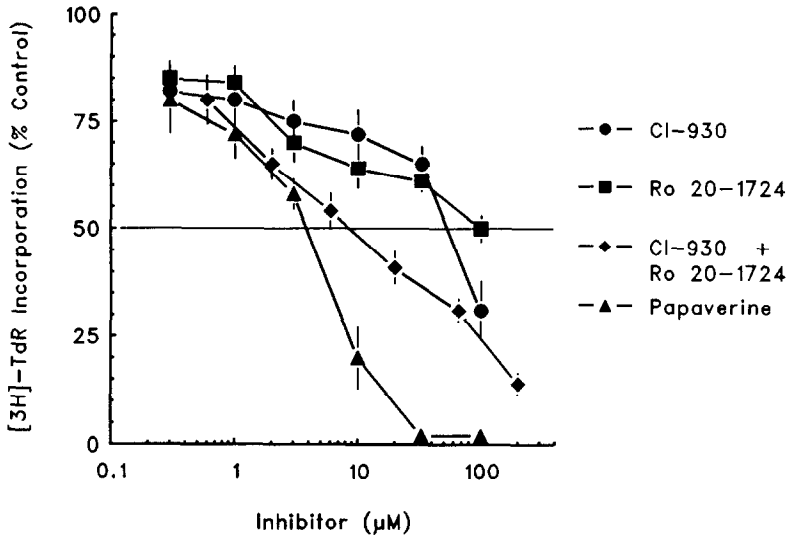

Fig. 3. Attenuation of PHA-induced T-lymphocyte blastogenesis by PDE inhibitors. Proliferation of T-lymphocyte cultures was assessed by the incorporation of [^{3}H]TdR into the cells. The effects of the selective PDE inhibitors CI-930 and Ro-20-1724 and an equimolar concentration of the two inhibitors (CI-930 + Ro 20-1724) are illustrated (from seven individuals). The effect of papaverine on proliferation is also shown (from four individuals). Inhibitors were added to T-lymphocyte cultures 30 min prior to stimulation with 0.63 μg PHA/mL. The effect of each concentration of inhibitor on proliferation was assayed in triplicate. The data are means ± SEM. Incorporation of [^{3}H]TdR in the absence of inhibitors (100%) was 27,100 ± 8880 cpm.

Table 4. Concentrations of PDE inhibitors producing 50% inhibition of PHA-induced T-lymphocyte blastogenesis

PDE Inhibitors	IC_{50} (μM)
CI-930*	47 (20–110)†
Ro 20-1724*	98 (42–230)
CI-930 + Ro 20-1724*	8.9‡ (4.5–17)§
Papaverine‖	2.9 (1.3–6.4)

* From seven individuals.
† Geometric means (95% confidence limits).
‡ Total concentration (i.e. 4.4 μM of each inhibitor).
§ Significantly different from the calculated IC_{50} value [56 μM (27–110)] assuming zero interaction between CI-930 and Ro 20-1724 ($t_6 = 4.931$; $P < 0.01$).
‖ From four individuals.

responses to invading organisms and tumor cells by secreting a wide variety of cytotropic hormones [20]. Activation of the cAMP pathway modifies distinct T-lymphocyte effector functions [21] which can result in an inhibition of the immunologic and inflammatory functions of leukocytes [22]. Therefore, the intracellular enzymes that regulate T-lymphocyte intracellular cAMP levels are potential targets for new drugs that may be developed for the purpose of modulation of immune function. This investigation was directed toward the study of high-affinity cAMP-PDE isozymes that can be inhibited by selective pharmacological agents (i.e. cyclic GMP, CI-930, and Ro 20-1724). Additionally, we studied the ability of these isozymes to modulate the immune responsiveness of T-lymphocytes as determined by the effects of the selective inhibitors on blastogenesis.

The soluble PDE extracted from cell sonicates exhibited high-affinity toward cAMP and linear transformations of the Michaelis–Menten equation revealed nonlinear kinetics. These findings appeared similar to previous data reported by Thompson *et al.* [16], Epstein and Hachisu [2], and others who studied unfractionated populations of lymphocytes. They are also similar to the kinetics reported by Epstein *et al.* [4] in sonicates of a human lymphoblastic T-cell line which exhibited apparent K_m values of 0.1 and 1.2 μM. Like the soluble PDE, particulate activity in our studies displayed high affinity for cAMP and showed nonlinear kinetics.

The nonlinear kinetic plots may be explained by either a single enzyme with negatively cooperative binding sites, a single enzyme with multiple noninteracting substrate sites or multiple isozymes with different substrate affinities [23]. Determination of Hill coefficients did not support the view that negative cooperativity was significant in either soluble or particulate fractions. Although protease inhibitors were used, the possibility that the nonlinearity was due to limited proteolysis of a single isozyme to form multiple forms of PDE in both fractions cannot be excluded entirely. However, the pharmacological discrimination between PDE activities in soluble and particulate fractions by cGMP, CI-930 and Ro 20-1724 is probably not due to a proteolytic artifact since isozyme families inhibited by CI-930 or Ro 20-1724 are considered to be distinct gene products [11].

Previous reports on lymphocytes showed little or

no PDE activity in the particulate fraction except in activated or transformed cells [2]. However, we observed significant particulate PDE activity in both purified and highly purified T-lymphocytes. In light of these previous reports, our data raised the question of whether the T-lymphocytes in our study were activated. We believe they were not activated for the following reasons: (1) the $CD3^+$ cells did not bind antibodies against HLA Class II major histocompatability complex which is expressed by T-lymphocytes during activation, (2) the cells did not appear blastoid (enlarged) upon microscopic examination, (3) the cells did not increase in number (undergo blastogenesis) when incubated for 3 days (37°, 5% CO_2, complete medium) unless mitogenic agents such as PHA or concanavalin A were added, and (4) the cells did not release interleukin-2 (IL-2) into the media nor did they express IL-2 receptors, as measured by antibodies against CD25 (Tac) (data not shown). Therefore, the reason our finding of particulate PDE enzyme differs from previous reports apparently is not due to cell activation. It seems possible that T-lymphocytes contain relatively more particulate PDE than other mononuclear leukocytes which may be present in less extensively purified cell preparations. Alternatively, the difference may be explained by different enzyme extraction procedures [24].

High-affinity cAMP-PDEs have been subdivided into two isozyme families [11, 25]. One family of isozymes has been designated cGMP-inhibited low K_m PDE (CGI-PDE) because it is inhibited by low concentrations of cGMP. It is also selectively inhibited by micromolar concentrations of CI-930 and several other pharmacological agents, but not by Ro 20-1724 [9, 26, 27]. The other high-affinity cAMP-PDE isozyme family is not inhibited by low concentrations of cGMP or CI-930, but is selectively inhibited by Ro 20-1724. This latter family of isozymes has been referred to as Ro 20-1724-inhibited low K_m cAMP-PDE (RoI-PDE). It is probably identical to the cAMP-specific family of isozymes defined by Beavo and Reifsnyder [11].

The CGI-PDE was detected mainly in the particulate fraction as evidenced by inhibition (48%) of the PDE activity by 1 μM cGMP and by micromolar concentrations of CI-930 (IC_{50}: 1–2 μM). Ro 20-1724 inhibited mainly soluble PDE activity, but some inhibition of particulate PDE was also observed suggesting that at least some of this isozyme is membrane bound in the intact cell. The data did not clearly indicate the maximum inhibition produced by Ro 20-1724, but 50% inhibition by 85 μM concentrations appeared to be near maximal in the soluble extracts from highly purified T-lymphocytes. This suggests that a substantial amount (approximately 40%) of the soluble PDE was not inhibited by either CI-930 or Ro 20-1724. This finding supports our previous report [28] showing a peak of soluble PDE activity, resolved by high pressure liquid chromatography, that was not affected by either of these two inhibitors. Whether this activity represents another isozyme or a degradation product has not been determined.

Novogrodsky *et al.* [3] reported that mitogen-induced proliferation of lymphocytes could be inhibited by the non-selective PDE inhibitor, 1-methyl-3-isobutylxanthine. More recently, Epstein and Hachisu [2] described the attenuation of PHA-induced proliferation by a variety of non-selective PDE inhibitors in addition to the selective inhibitor, Ro 20-1724. Our data showed that PHA-induced proliferation could be attenuated by either CI-930 or Ro 20-1724 used alone. However, complete suppression of proliferation was not achieved without using inhibitor levels higher than 100 μM which could produce non-selective PDE inhibition [9] and affect cell viability. Papaverine, which is known to produce non-selective inhibition of PDEs with an IC_{50} in the range of 1 to 3 μM [2, 29] attenuated proliferation 50% at 2.9 μM and suppressed it completely at 33 μM levels. These results indicate that partial attenuation of T-lymphocyte proliferation can be achieved by inhibition of either CGI-PDE or RoI-PDE alone. They also demonstrate that inhibition of multiple PDE isozymes causes synergistic antiproliferative effects and may be essential to achieve complete suppression of blastogenesis.

Cyclic AMP levels were not measured, but it seems likely that the antiblastogenic effects of PDE inhibitors were mediated by cAMP since agents that elevate cAMP consistently suppress proliferation [3, 30]. The observed effects of the PDE inhibitors leading to the synergistic attenuation of lymphocyte blastogenesis are not readily understood but are suggestive of multiple roles of cAMP controlled, in part, by distinct PDE isozymes. This could be due to either the regulation of distinct pools of cAMP synthesized by discrete pools of adenylate cyclase [31], the selective activation of different cAMP-dependent protein kinase isozymes [32], and/or the alteration of intracellular cyclic nucleotide movement and distribution [33].

Phosphodiesterase inhibitors were added to cells 30 min before PHA to facilitate steady-state intracellular levels of the inhibitors throughout blastogenesis. It is notable that PDE activity increases during blastogenesis [4] which raises the question of which isozymes change. We have observed (unpublished data) a 3.5-fold increase in both soluble and particulate activities 4 days after the addition of PHA to purified human T-lymphocytes. The proportion of activity inhibitable by CI-930 and Ro 20-1724 was similar to unstimulated cells.

This is the first study to characterize the PDE isozymes in an immunologically defined population of non-activated human peripheral blood T-lymphocytes. It is also the first study to demonstrate the effect of inhibiting both CI-930- and Ro 20-1724-sensitive isozymes on T-lymphocyte proliferation. The data presented support the following conclusions: (1) human peripheral blood T-lymphocytes contain CGI-PDE, RoI-PDE, and possibly other PDE isozyme(s) not inhibited by either CI-930 or Ro 20-1724, (2) CGI-PDE is more tightly bound to particulate material in cell sonicates than is RoI-PDE, (3) both CGI-PDE and RoI-PDE are capable of modulating T-lymphocyte blastogenesis, and (4) inhibition of both CGI-PDE and RoI-PDE produces a synergistic suppression of blastogenesis.

These findings suggest that new drugs targeting

either CGI-PDE or RoI-PDE alone may cause partial but not complete attenuation of T-lymphocyte function in humans. They further suggest that selective PDE inhibitors used for other therapeutic objectives such as to produce cardiotonic, antithrombotic, antidepressant, or smooth muscle relaxant effects, could cause limited attenuation of T-lymphocyte function if either CGI-PDE or RoI-PDE is inhibited. In addition, the immunosuppression caused by such drugs would be expected to be enhanced by the inhibition of both isozymes. This could occur by the simultaneous presence of chemical substances capable of inhibiting each T-lymphocyte PDE isozyme selectively or by the effects of one of the selective T-lymphocyte inhibitors in the presence of non-selective PDE inhibitors such as papaverine.

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