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HIGH PRESSURE LIQUID CHROMATOGRAPHY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM PURIFIED HUMAN T-LYMPHOCYTES

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SUMMARY: The cyclic nucleotide phosphodiesterase (EC 3.1.4.17) in extracts of purified human peripheral blood T-lymphocytes was examined by ion exchange high pressure liquid chromatography. Four peaks of activity were isolated. The first peak of activity selectively hydrolyzed cyclic GMP. The following 3 peaks of activity (Ia, IIa and IIIa) were selective for cyclic AMP. The selective low K_m cyclic AMP-phosphodiesterase inhibitor, Ro 20-1724 (d,l-1,4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone), did not inhibit the activity in Ia whereas it did inhibit the activity in IIa and IIIa (IC_{50} = 17 μM). The authors conclude that ion exchange high pressure liquid chromatography described in this communication is a useful method for the isolation of different forms of cyclic nucleotide phosphodiesterase activity from human T-lymphocytes.

Recent evidence implicates cyclic nucleotides in the regulation of lymphocyte growth and differentiation (1,2,3,4). Cellular levels of cyclic nucleotides can be modulated by biosynthesis and by changing the rates of breakdown by PDE (5,6,7). Investigations of different molecular form(s) of PDE in lymphocytes have employed purification of the enzyme by electrophoresis (8), density gradient centrifugation (5,7,9,10), gel filtration (7,11), isoelectric focusing (9,11) and conventional ion exchange chromatography (9,12). HPLC offers the advantages of speed, high sample recovery, and in some cases, higher resolution, compared to other methods of protein purification. The purpose of the research reported in this communication was to determine whether HPLC could be used to isolate different peaks of PDE activity from human lymphocyte extracts.

MATERIALS and METHODS

Chemicals and media. Tris, Bis-tris, cAMP, cGMP, BSA (fraction V), EDTA, alkaline phosphatase (bovine intestinal mucosa, type VII-N), pepstatin A (P 4265), aprotinin (A 6279), 2-mercaptoethanol, Percoll (colloidal polyvinylpyrrolidone coated silica), Histopaque-1077 (ficoll and

Abbreviations: PDE, cyclic nucleotide phosphodiesterase; HPLC, high pressure liquid chromatography; cAMP, 3'5'-cyclic AMP; cGMP, 3'5'-cyclic GMP; Bis-Tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; BSA, bovine serum albumin.

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sodium diatrizoate), trypan blue dye, penicillin-streptomycin, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, sodium salt (HEPES) were purchased from Sigma Chemical Company (St. Louis, MO). Piperazine dihydrochloride was from Alpha Products (Danvers, MA). AG 1-X8 (200-400 mesh) resin was from Bio-Rad Laboratories (Richmond, CA). RPMI 1640 was from Gibco Laboratories (Grand Island, N.Y.). Human serum (type AB) was from Flow Laboratories Inc. (McLean, VA). [^{3}H]cAMP (36.1 Ci/mmole) and [^{3}H]cGMP (10.2 Ci/mmole) were purchased from NEN Research Products (Boston, MA). Other chemicals were purchased from Mallinckrodt Inc. (St. Louis, MO).

Buffers. Phosphate buffered saline solution was from Whittaker M.A. Bioproducts (Walkersville, MD). Homogenization buffer: 20 mM Bis-Tris (pH 6.8), 1mM EDTA, 50 Kallikrein units of aprotinin/ml, and 1 mg pepstatin/ml. Assay buffer: 75 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 10 μM $CaCl_2$, 0.1% BSA, and 3.75 mM 2-mercaptoethanol.

PDE inhibitors. Ro 20-1724 (d,l-1,4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone) was donated by Hoffman-LaRoche, Inc. (Nutley, NJ) and CI-930 (3(2H)-pyridazinone, 4,5-dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-5-methyl-monohydrochloride) was a gift from Warner-Lambert/Parke-Davis (Ann Arbor, MI).

Purification of lymphocytes. Buffy coat units prepared from peripheral blood were obtained from the Southwest Florida Blood Bank (Tampa, FL). Mononuclear cells were purified by ficoll density-gradient centrifugation (13) and macrophages were removed by adherence to plastic tissue culture flasks (1 hr at 37° C, 5% CO_2) in RPMI 1640 medium containing 5% human serum. B-lymphocytes and remaining macrophages were removed by adherence to nylon wool columns (30 min at 37°) equilibrated with RPMI 1640 containing 5% AB serum. T-lymphocytes were further purified by a discontinuous percoll-density gradient centrifugation (14). The resulting preparation contained at least 98% lymphocytes of which approximately 88% were T-lymphocytes (CD3 positive; Leu 4, Beckton Dickinson, Mountain View, CA) and less than 2% B-lymphocytes (CD20 positive; B1, Coulter Immunology, Hialeah, FL) as determined by fluorescence activated cell sorter (FACScan, Becton Dickinson). The viability of the cells was verified by trypan blue dye exclusion and was greater than 99%. The preparation contained no more than 2 platelets per 250 lymphocytes.

Enzyme preparation. T cell preparations were resuspended in homogenization buffer at 4° and sonicated on ice 3 times for 10 sec with a Branson Cell Disrupter model 200 (Danbury,CT) fitted with a microprobe. The setting used (#2) provided the minimal energy necessary to disrupt the cells. The sonicate was centrifuged at 23,000 x g for 30 min and the supernatant fraction was filtered through a Costar μ Star filter (0.22 micron pore size). The filtered extract was then injected onto the ion exchange column of the HPLC system.

HPLC system. Two Waters (Milford, MA) model 6000A solvent delivery systems controlled by a model 660 programmer were used to deliver buffers to a Waters DEAE 5PW Protein Pak column to produce preprogrammed gradients. Volumes of eluate ranging from 200 to 500 μl were collected in culture tubes containing buffer to provide final concentrations of 20 mM Tris HCl (pH 8.0), 10μM $MgCl_2$, 10 mM $CaCl_2$ and 0.1% BSA. The collected fractions were assayed immediately.

PDE assay. A modification of the assay described by Thompson and Appleman (15) was used. Briefly, [^{3}H]cAMP or [^{3}H]cGMP was incubated with PDE at 37° in 20 μl volumes of assay buffer which contained enough alkaline phosphatase to rapidly convert all of the 5'-nucleotide product to the nucleoside. The reaction was started by addition of PDE and stopped by addition of 0.5 ml volumes of a 1:3 (v:v) slurry of Bio-Rad AG 1-X8 resin which bound the nucleotides but not the nucleosides. Radiolabeled nucleosides in the supernatant fluid were counted using liquid scintillation spectrometry. The assays were linear with respect to enzyme concentration and with respect to time up to 60 min. Reactions were run for either 10 or 40 min.

Statistical Analysis. The data were analyzed by one-way analysis of variance followed by the Duncan multiple range test (16) for comparisons between means. A probability (p) value of less than 0.05 was considered significant.

RESULTS

A variety of buffers and gradients were tested to separate PDE using HPLC. Buffer systems with pH between 7.0 and 8.8 failed to support resolution of PDE peaks. Buffers ranging in pH below 4.7 dramatically reduced recoveries of PDE activity. Our studies indicate that pH in the range from 5.9 and 6.5 was optimal. Ionic strength gradients corresponding to 5 to 10 mmoles NaCl/liter at 1 ml/min flow rates were optimal for separating cAMP-PDE activities.

Low levels of cGMP hydrolyzing activity were present in lymphocyte sonicates. This activity did not separate from cAMP-PDE when HPLC was carried out with high pH buffers, but it eluted ahead of the cAMP hydrolyzing activity when buffers below pH 7 were utilized. Figure 1 shows a representative HPLC separation of cGMP-PDE activity from two peaks of cAMP-PDE.

The two peaks of cAMP hydrolyzing activity shown in Figure 1 were seen in HPLC eluates when 1 μM cAMP was used to assay the collected fractions. When 0.2 μM levels of cAMP were used, a third peak of cAMP hydrolyzing activity was apparent which eluted ahead of the other two cAMP-PDE peaks as illustrated in Figure 2. We designated the cAMP hydrolyzing peaks as Ia, IIa, and IIIa corresponding to their order of elution. All three cAMP-PDE peaks eluted with longer retention times than the cGMP-PDE peak in corresponding chromatograms.

The amount of cAMP-PDE activity recovered from HPLC is presented in Table 1. Total activity recovered was 77% or 85% depending on whether it was measured with 0.2 or 1.0 μM

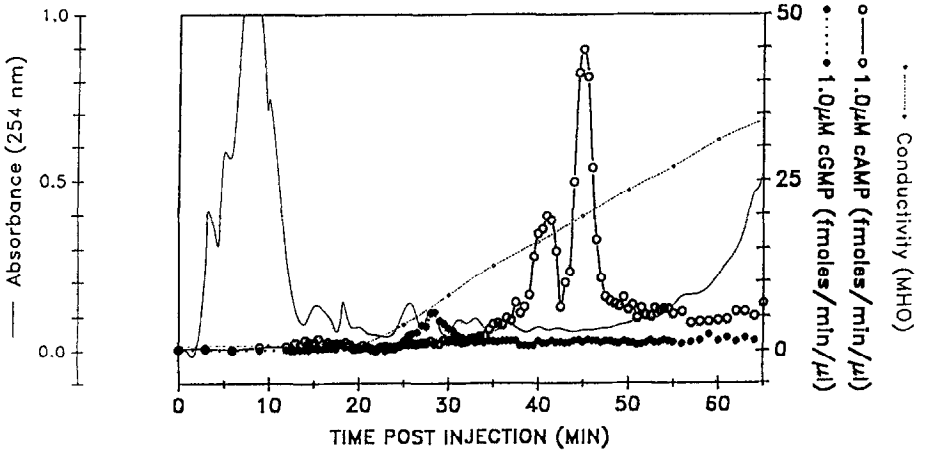

Figure 1. *Chromatogram demonstrating cGMP- and cAMP-PDE activities.* A typical profile of PDE activity following the injection of lymphocyte sonicate representing 2 x 10^8 cells is illustrated. The mobile phase consisted of a 20 mM piperazine buffer pH 5.9 with a flow rate of 1 ml/min. 5 min after sample injection, a linear NaCl gradient was started to elute PDE activity from the column. Eluent was collected in 250 μl volumes. PDE activity was assayed using 1.0 μM concentrations of cGMP and cAMP.

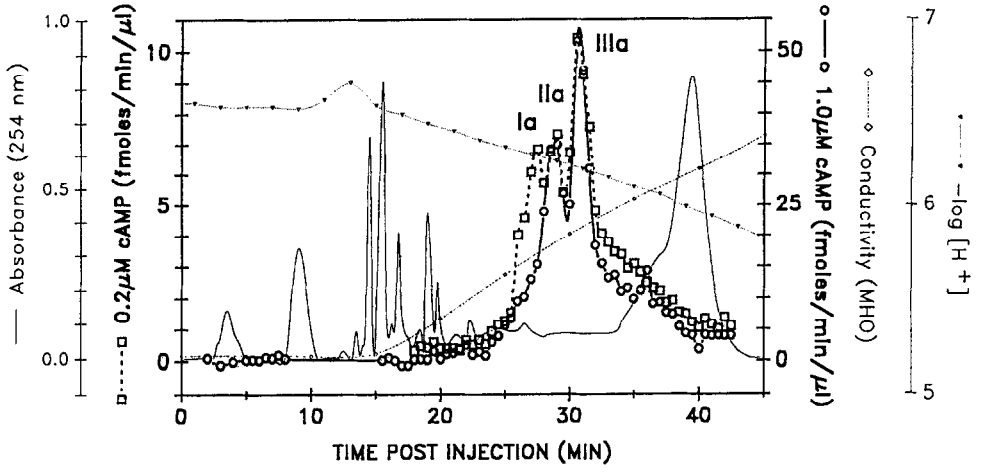

Figure 2. *Chromatogram of cAMP-PDE activities.* The initial mobile phase was 20 mM Bis-Tris, pH 6.8, with a 1 ml/min flow rate. A linear gradient was initiated 10 min after sample injection and consisted of increasing NaCl and hydrogen ion concentrations. PDE activity was determined on 250 μl volumes of eluant using 0.2 μM and 1.0 μM concentrations of cAMP as substrate.

levels of cAMP as substrate. The first cAMP-PDE peak (Ia) contained 15% of the activity in the original sample (before HPLC) measured with 0.2 μM cAMP or 5% measured with 1 μM levels. The majority of the cAMP hydrolyzing activity was recovered in peaks IIa and IIIa.

The ratio of the rate of cAMP hydrolysis at 1.0 μM compared to the rate at 0.2 μM cAMP was significantly lower ($F_{2,31}$ = 35; $p < 0.01$) for peak Ia compared to peaks IIa and IIIa and is illustrated in Table 2. This finding suggests that Ia contains a lower K_m enzyme.

Figure 3 shows that Ro 20-1724, a selective inhibitor of low K_m cAMP-PDE, inhibited peaks IIa and IIIa with an IC_{50} of about 17 μM, but did not inhibit Ia in the same concentration range. Inhibition of PDE in peaks IIa and IIIa was significantly higher compared to peak Ia ($F_{2,6}$ = 5.56; $p < 0.05$). CI 930, another selective low K_m PDE inhibitor, did not inhibit any of the three peaks of activity in concentrations up to 100 μM (data not presented).

Table 1. *Recoveries of cAMP-PDE from DEAE HPLC*

	SUBSTRATE USED	
	0.2 μM cAMP	1.0 μM cAMP
Peak Ia	13.7 ± 3.5	5.1 ± 2.3
Peak IIa	22.0 ± 5.2	34.9 ± 1.5
Peak IIIa	41.3 ± 8.7	45.1 ± 10.4
Total	77.1 ± 3.9	85.2 ± 4.7

Activity in each peak is expressed as percent of the activity injected into the HPLC system. Total recovery was calculated as the sum of recoveries in peaks Ia, IIa and IIIa. Values represent mean ± S.E.M. (n = 3 HPLC separations).

Table 2. *Ratio of the hydrolytic rates of 1.0 μM to 0.2 μM cAMP by HPLC peaks Ia, IIa and IIIa*

	ACTIVITY RATIO (1.0 μM cAMP / 0.2 μM cAMP)
Peak Ia	2.73 ± 0.17
Peak IIa	4.52 ± 0.46 **
Peak IIIa	4.83 ± 0.19 **

Activity contained in each of the three cAMP-PDE peaks is expressed as the ratio of velocities obtained with 1.0 μM and 0.2 μM cAMP as substrate. Values represent mean ± S.E.M. (n = 10 HPLC separations). ** Significantly different from peak Ia.

DISCUSSION

The purpose of this research was to determine whether HPLC can be used to isolate peaks of PDE activity from human lymphocytes. Four peaks of activity were separated by this method in less than 40 min following injection of the sample into the HPLC system. Total elapsed time between sonication of the cells and collection of the separated PDE fractions was less than 90 min. Progress reports on this research have been presented previously in abstract form (17,18,19).

Low levels of cGMP-PDE were found in lymphocyte extracts. Similar findings have been reported by other investigators (11,20), but Thompson *et al.* (7) found no cGMP hydrolytic activity in human lymphocytes purified from defibrinated blood. Takemoto and coworkers (12) demonstrated cGMP hydrolytic activity in cell preparations that contained platelets, but when the

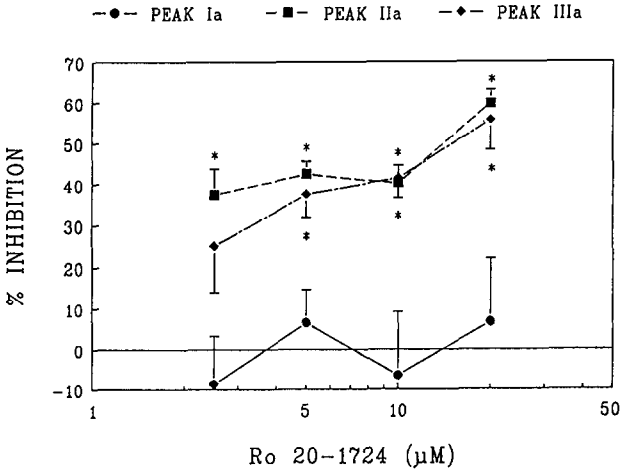

Figure 3. *Inhibition of PDE by Ro 20-1724.* Inhibition of peaks Ia, IIa and IIIa from 3 HPLC separations is shown (mean ± S.E.M.). * Significantly inhibited compared to Ia.

platelets were removed by defibrination, cGMP-PDE was not detected. The preparations used in our study contained less than 1% platelets. This low contamination suggests that the cGMP-PDE in these preparations did not come from platelets, but the possibility cannot be ruled out conclusively by our data. The study by Takemoto *et al.* (12) using conventional DEAE chromatography showed the cGMP hydrolyzing activity from non-defibrinated lymphocytes eluting ahead of cAMP-PDE. Our results using ion exchange HPLC show a similar elution pattern.

Three peaks of cAMP-PDE were observed following HPLC. It seems unlikely that any of these peaks was derived from platelets because of the small number of platelets in the cell preparations, and because the predominant PDE in platelets contains significant cGMP hydrolytic activity (21) which was not found in the cAMP-PDE HPLC fractions.

Several articles published during the 1970's reported both high and low affinity cAMP-PDE activity in human lymphocytes, but evidence presented by Thompson *et al.* (7) and Epstein and Hachisu (9) indicated the presence of only high affinity PDE that can exist in monomeric and oligomeric forms. The investigation reported in this communication differs from the studies cited above in two ways. First, it employed HPLC which isolated the PDE in a relatively short time minimizing the effects of ageing that can alter the enzyme (7). Second, the lymphocyte preparation was more extensively purified to increase the proportion of T-lymphocytes (88%) and reduce B-lymphocytes (< 2%). B-lymphocytes have been shown to contain 62 times more PDE activity than T-lymphocytes (22). Therefore the more homogeneous cell population used in this investigation may be essential to reveal forms of PDE contained in T-helper/inducer and suppressor/cytotoxic lymphocytes.

Peak Ia differed from IIa and IIIa in 3 ways. First, it eluted from the HPLC ion-exchange column ahead of the other two peaks. Second, its rate of hydrolysis of 1.0 μM relative to 0.2 μM cAMP suggests it has a lower K_m than the other two peaks. That is, the levels of cAMP used appear to be nearer the concentration needed to saturate the catalytic site of the Ia enzyme. Third, Ia was not inhibited by Ro 20-1724 in the same concentrations that produced 50% inhibition of IIa and IIIa. This last finding suggests a possible similarity to the 2 subclasses of low K_m, cAMP specific phosphodiesterase that have recently been described in cardiac muscle (23) and hepatocytes (24). One subclass is inhibited by Ro 20-1724 and the other is not. Studies are continuing in our laboratory to further investigate the forms of human lymphocyte PDE reported in this communication.

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