Cyclic Adenosine 3':5'-Monophosphate Phosphodiesterase

DISTINCT FORMS IN HUMAN LYMPHOCYTES AND MONOCYTES

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Adenosine 3':5'-monophosphate (cyclic AMP) phosphodiesterase activity of normal human peripheral blood leukocyte suspensions containing 90% lymphocytes and 10% monocytes showed anomalous kinetic behavior indicative of multiple enzyme forms. Kinetic analyses of purified lymphocyte (99%) or monocyte preparations (95%) indicated that only one type of phosphodiesterase was present in each cell type. None of the preparations contained any detectable guanosine 3':5'-monophosphate (cyclic GMP) hydrolitic activity. The lymphocyte enzyme had an apparent \( K_m \approx 0.4 \mu M \) for cyclic AMP and \( V_{max} \approx 0.5 \) picomoles/min/10^6 cells. These kinetic parameters were confirmed by several cell purification techniques used alone and sequentially. Sedimentation velocity analyses indicated that the higher \( K_m \) monocyte enzyme had a molecular weight near 45,000 and that the lower \( K_m \) lymphocyte enzyme most likely had a molecular weight near 98,000. A variety of procedures led to a loss of the higher molecular weight, high affinity enzyme leaving only the enzyme of 45,000 daltons with a much lower substrate affinity. A long term, stable human lymphoblastoid cell line had cyclic AMP phosphodiesterase activity that was similar to the lymphocyte enzyme by both physical and kinetic criteria. Lymphocyte cyclic AMP phosphodiesterase appears to be a soluble enzyme whose pH and temperature optima and cationic requirements are similar to those of other mammalian phosphodiesterases. The distinct cyclic AMP phosphodiesterase forms of these cells may possibly represent the basic, active subunit of mammalian cyclic nucleotide phosphodiesterases. We hypothesize that the extremely high affinity cyclic AMP phosphodiesterase of normal lymphocytes plays an important role in the regulation of normal function in these cells, and also in the rapid proliferative responses characteristic of the stimulated lymphocyte.

The role of cyclic nucleotides in the regulation of the function of the immune system is of great current interest since there is increasing evidence that the maturation, differentiation, and response of this system is closely linked to cyclic nucleotide metabolism (1, 2). The exact role of cyclic AMP and cyclic GMP in lymphocyte maturation, antigen and/or mitogen recognition, proliferative response, and antibody synthesis is unclear, but both nucleotides are thought to be important regulatory agents. Relatively little information is available on the synthetic and hydrolytic enzymes that control intracellular cyclic nucleotide levels in these cells (3-11). The studies reported here detail an investigation of the properties of human peripheral blood lymphocyte and monocyte cyclic nucleotide-hydrolyzing enzymes and point toward the necessity for attaining a high degree of cell purity before investigating cyclic nucleotide metabolism in these immune cell populations.

EXPERIMENTAL PROCEDURE

Materials—Cyclic [8-'H]AMP (specific activity 16.3 or 23 Ci/millimole) and cyclic [G-32P]GMP (specific activity 4.1 Ci/millimole) were purchased from Schwarz/Mann and New England Nuclear. Cyclic [3H]AMP was purified by Dowex 1-X8 (200 to 400 mesh) anion exchange chromatography and stored at -20°C in acidic 50% ethanol. Snake (Ophiophagus hannah) venom, cyclic AMP, cyclic GMP (Sigma), Ficoll (Pharmacia); Agarose A-0.5m and Dowex 1-X8 (200 to 400 mesh) (Bio-Rad Laboratories); Hypaque-M, 90% (Winthrop Laboratories); enzyme grade sucrose (Merck & Co.), RPMI-1640 and MEM' (Grand Island Biological); carbonyl iron powder, Grade SS (GAR

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The abbreviations used are: MEM, Spinner modified minimal essential medium; Mes, 2-(N-Morpholino)ethanesulfonic acid; Hepes, N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid.
We have found that this nonspecific adenosine binding is minimized as previously described (22). As reported by Rutten et al. anion exchange resin and the measurements of the [8-SH]adenosine.

Reactions were terminated by boiling for 45 s. The addition of snake ficated for 1 min at 30°C. Incubations were routinely for 30 min at 30°C. microscopic examination after this procedure.

To remove monocytes from the final cell preparation, carboni on 15-18) was incubated with agitation at 37°C for 30 min with the defibrillated blood by adding 1 ml of Hanks' balanced salt solution (HBSS) containing 50 mg of tetracarbonyl iron, 220 mM CaCl₂, and 169 mmol/L NaCl, adjusted to pH 7.4 with 1 N KOH. After 5 min, the mixture was centrifuged at 200 x g for 10 min. The supernatant was removed by gentle swirling for 10 min. The defibrinated blood was observed occasionally. The cells were then washed two to five times with 280 mM NH₄Cl/30 mM Tris Cl (pH 7.2) with a 15 min incubation at 37°C for each wash to lyse remaining erythrocytes (13). They were washed again in MEM, and suspended as 6 x 10⁶ cells/mL in MEM. Total and differential cell counts were determined by hemacytometer and cytocentrifuge slide counts (14), and 500 total cells were counted routinely. Cell viability was assessed by trypan blue dye exclusion and was always ≥ 99%. Approximately 1 to 1.5 x 10⁶ cells were routinely obtained per mL of whole blood.

For some studies, cells were disrupted by freezing in MEM in a dry ice acetone bath at densities of 15 x 10⁶ cells/mL in glass culture tubes (12 x 75 mm). The frozen cell suspensions were thawed on ice and then sonicated for 10 s with a Biosonic IV at a setting of 40 (scale 0 to 100 arbitrary units). Whole cells were not evident by light microscopic examination. For some studies, cells were disrupted by freezing in MEM in a dry ice acetone bath at densities of 15 x 10⁶ cells/mL in glass culture tubes (12 x 75 mm). The frozen cell suspensions were thawed on ice and then sonicated for 10 s with a Biosonic IV at a setting of 40 (scale 0 to 100 arbitrary units). Whole cells were not evident by light microscopic examination.
cyclic AMP whether the monocytes were removed by the carbonyl iron procedure (Fig. 1, upper right) or by a glass bead column (Fig. 1, lower right). Removal of monocytes by both methods in succession gave similar results. Data from six "pure" lymphocyte populations with less than 2% monocyte and no apparent granulocyte contamination showed an average apparent $K_m = 0.47 \pm 0.20 \mu M$ for cyclic AMP and an average maximum velocity of 0.65 ± 0.3 picomole, of cyclic AMP hydrolyzed/min/10⁶ cells.

Glass adherent cells (monocytes) also showed a single enzyme form by kinetic analysis (Fig. 1, lower left). These cells, however, contained only the higher $K_m$ enzyme form previously observed in the mixed cell populations (Fig. 1 and Table I). The apparent $K_m$ of the monocyte cyclic AMP phosphodiesterase was 1.3 $\mu M$ for cyclic AMP with a maximum velocity of 2.2 picomoles of cyclic AMP hydrolyzed/min/10⁶ cells.

**General Properties of Lymphocyte Cyclic AMP Phosphodiesterase**—Like most mammalian cyclic nucleotide phosphodiesterases, magnesium was the preferred divalent cation (Table II). The $K_m$ for magnesium was 20 $\mu M$ with maximum velocity obtained at 10 mM magnesium concentrations. No inhibition was apparent even up to 50 mM magnesium. Manganese, barium, or cobalt could partially substitute for magnesium. In the absence of magnesium, calcium from 2 $\mu M$ to 100 $\mu M$ stimulated activity about one-third that of maximum magnesium catalyzed activity, the extent depending on the ratio of $Ca^{2+}:Mg^{2+}$ with 100:1 inhibiting 75%.

The pH optimum for lymphocyte cyclic AMP phosphodiesterase was near 8.0 and the activity was greater in Tris Cl buffer than in Mes, Glycylglycine, or phosphate/citrate (data not shown). The temperature optimum of this enzyme...
was 31°. Dithiothreitol (10 mM) in the incubation medium had no effect on the temperature optimum of this phosphodiesterase.

Cyclic AMP phosphodiesterase activities of a lymphocyte homogenate and the supematant of a 100,000 x g centrifugation for 90 min were nearly identical using 0.02 or 0.25 mM cyclic AMP (Fig. 2). No activity was detectable in the 105,000 x g pellet. Discontinuous sucrose gradient fractionation performed as described under "Experimental Procedure" also failed to show any membrane-bound enzyme. Dithiothreitol (10 mM) or 0.5 mM EDTA had no effect on either the homogenate or the 105,000 x g supematant activity when included in the enzyme incubation medium. The activity of 1 x 10^8 cell equivalent homogenate protein (100 μg) was linear throughout the 30 min incubation period.

Stability of Lymphocyte and Monocyte Cyclic AMP Phosphodiesterase Forms—Purified preparations of lymphocytes frozen in MEM retained their total activity and the very high affinity enzyme form after thawing and homogenization (Fig. 3, lower panel). However, if purified lymphocytes were frozen in 40 mM Tris Cl (pH 8.0), 30% glycerol, or 1% albumin, the enzyme activity showed only the lower affinity form (apparent K_m = 2 μM).

Mixed cell populations also lost the high affinity enzyme upon freezing (Fig. 3 upper panel). The maximum velocity of frozen-thawed homogenates (n = 3) decreased 50% after freezing (0.51 to 0.27 ± 0.03 pmol/min/10^6 cells), but the apparent K_m of the enzyme remained constant (0.46 ± 0.07 μM). These activity changes caused by freezing and thawing were independent of time needed to freeze the preparation (5 s to 1 h), the length of time the sample remained frozen (1 to 21 days), or the storage temperature (-20° or -70°). In addition, if purified lymphocytes were suspended in 0.32 M sucrose/40 mM Tris Cl (pH 8.0) instead of MEM, the cells tended to aggregate, especially at lower temperatures (e.g. 4°C).

Upon aggregation, only the higher K_m enzyme form was evident by kinetic analysis.

Purified lymphocyte homogenates also showed a dramatic change in kinetic behavior when stored at 4°C for 24 hours (Fig. 4) by shifting completely to an enzyme with much lower affinity and higher maximum velocity. This change in kinetic properties was not prevented by the addition of dithiothreitol (15 mM), benzamidine (30 mM), or sodium fluoride (10 mM) to the homogenization medium. However, if intact lymphocytes were incubated for 24 hours at 37°C in RPMI-1640 medium supplemented with 10% autologous serum and 10 mM Hepes buffer, the cyclic AMP phosphodiesterase kinetic parameters of these cells were identical to nonincubated cells homogenized without freezing.

Fractionation of Lymphocyte and Monocyte Cyclic AMP Phosphodiesterase—Linear sucrose density gradient sedimentation and agarose A-0.5m gel filtration were used to physically characterize cyclic AMP phosphodiesterases of both pure and mixed lymphocyte cell populations. Linear sucrose gradient fractionation of cell populations containing a mixture of 40% monocytes and 60% lymphocytes showed two enzyme forms, one of 3.6 S (molecular weight = 98,000) and a much smaller peak of 6.0 S (molecular weight ~ 45,000) (Fig. 5). When this...
Homogenate of a pure lymphocyte cell population displayed only the single high affinity enzyme form, which had an apparent $K_m$ of 1.1 nM for cyclic AMP and maximum velocities of 0.5 picomole of cyclic AMP hydrolyzed/min/10^6 cell equivalents. An agarose gel filtration analysis of this homogenate immediately and after the homogenate was “aged” for 24 hours at 4°C (Fig. 7). The high molecular weight enzyme present in the fresh preparation was not present in the aged preparation using 0.01 μM cyclic AMP substrate concentrations. The lower molecular weight enzyme showed a linear Lineweaver-Burk plot with an apparent $K_m$ of 1.1 μM for cyclic AMP (see inset) while the fresh homogenate showed anomalous kinetic behavior as described above.

Cyclic AMP Phosphodiesterase Activities of Long Term Lymphoblastoid Cell Lines—The long term lymphoblastoid cell line cell lines used for these studies were Epstein-Barr virus genome containing cells derived from normal subjects. These cells synthesize immunoglobulin and possess surface properties resembling those of normal “B” lymphocytes. The characteristics of cyclic AMP phosphodiesterase activity of these cells were similar to those of normal lymphocytes. Homogenates and 105,000 x g supernatant preparations both showed linear Lineweaver-Burk plots with apparent $K_m$ values of 0.2 μM for cyclic AMP and maximum velocities of 0.5 picomole of cyclic AMP hydrolyzed/min/10^6 cell equivalents. Sucrose density gradient sedimentation analysis of this phosphodiesterase activity showed a single 6.0 S peak of activity (Fig. 8). When analyzed using pH 6.0 buffered sucrose gradients, instead of the normal pH 8.0 procedure, a small peak of 8.1 S enzyme was also found. Benzamidine (30 mM) or dithiothreitol (15 mM) had no effect on this enzyme form or activity.

**DISCUSSION**

Human peripheral blood lymphocyte and monocyte cyclic AMP phosphodiesterases have properties that appear to be distinct among mammalian cyclic nucleotide phosphodiesterases. Homogenates of mixed preparations of lymphocytes and monocytes showed anomalous Lineweaver-Burk plots similar to those reported for rat thymic lymphocytes (7), human platelets (30), and other tissues (29). However, in contrast to previous reports, our studies have shown that the very high affinity enzyme form which is apparent by kinetic analyses of mixed cell preparations exists exclusively in lymphocytes and that the lower affinity enzyme form exists exclusively in monocytes. These findings have been verified using several cell purification techniques. Although more detailed kinetic data must be obtained to rule out negative cooperative mechanisms, the enzymes of either cell displayed apparent Michaelis-Menten kinetic behavior indicative of a single enzyme form. Monocyte cyclic AMP phosphodiesterase activity has a higher apparent $K_m$ than the lymphocyte enzyme, but its affinity is similar to the enzyme of other tissues often referred to as a “low $K_m$” cyclic AMP specific phosphodiesterase (29). On the other hand, the affinity of the lymphocyte cyclic AMP phosphodiesterase for cyclic AMP is among the highest yet reported (29).

Previous investigations of human lymphocyte cyclic AMP phosphodiesterase have reported multiple kinetic behavior (3-5) with $K_m$ and $V_{max}$ values for this enzyme 20- to 100-fold.
Lymphocyte Cyclic AMP Phosphodiesterase

LYMPHOCYTES 99 %

FIG. 6. Linear sucrose gradient fractionation of cyclic AMP phosphodiesterase from purified lymphocytes. S values are from ovalbumin markers run in parallel. Cell equivalents (45 x 10^6) of a 10,000 x g supernatant were applied to the gradient as 0.2 ml immediately upon preparation (O——O) or after "aging" for 24 hours at 4° (●—●).

FIG. 7. Agarose A-0.5m gel filtration of cyclic AMP phosphodiesterase from a mixed cell population (40% monocytes, 60% lymphocytes). The preparation was chromatographed immediately upon homogenization and centrifugation and part of it chromatographed 24 hours later after storage at 4°. Bovine serum albumin marker peaked at 31 ml.

higher than those reported here. In each previous study, in contrast to those reported here, heparin was used before and/or after lymphocyte isolation. In addition, the purity of the lymphocyte preparations was not clearly defined. Using the data of Prichard et al. (30), we calculated that human platelet cyclic AMP phosphodiesterase is approximately 10,000 times more active than the lymphocyte enzyme. Therefore, minor platelet contamination, which is often a problem when using heparinized blood, could pose a serious difficulty in such studies. In addition, the higher \( K_m \) previously reported for the lymphocyte enzyme is virtually identical to the platelet low \( K_m \) enzyme.

Studies of heterogeneous tissues or homogeneous cell populations have shown that the cyclic nucleotide phosphodiesterase enzyme system consists of multiple enzyme forms (29). The anomalous kinetic behavior that is almost always observed with crude cyclic nucleotide phosphodiesterase preparations is one criterion from which this thesis was derived. However, subsequent studies have verified this interpretation of the kinetic data by showing that enzyme forms from many tissues can be physically separated by a variety of techniques with differences in cellular localization, molecular weights, substrate specificities, kinetic constants, etc. (29). Lymphocytes and monocytes constitute the only known examples of total cellular cyclic AMP phosphodiesterase activity showing apparent Michaelis-Menten kinetic behavior, particularly of the high affinity enzyme, and thus by kinetic criteria consisting presumably of only one enzyme form. They also are the only known example of successfully resolving the anomalous kinetic plots by separating cell types, subsequently shown to contain each indicated enzyme. It seems possible, therefore, that the cyclic AMP phosphodiesterases of these cells may represent a prototype cyclic AMP phosphodiesterase form. The cyclic nucleotide phosphodiesterases of other mammalian tissues may constitute an arrangement of these basic active subunits such that the needs of the tissue for cyclic nucleotide hydrolysis are met.

Perhaps this concept is related to the very low cyclic GMP hydrolytic activity in these cells. Cyclic GMP phosphodiesterases of most tissues are thought to be higher molecular weight enzymes than low \( K_m \) cyclic AMP phosphodiesterases (31) and often correlate with high \( K_m \) cyclic AMP phosphodiesterase activities, enzyme activities also undetectable in these cells. To regulate cellular cyclic GMP content after synthesis, low molecular weight enzyme subunits might be aggregated upon stimulation by lymphocyte regulatory agents to form cyclic GMP phosphodiesterases.

The monocyte cyclic AMP phosphodiesterase appears to be one enzyme form by sedimentation velocity and gel filtration criteria. It has a molecular weight of 45,000 (3.6 S) and is the stable enzyme form. It should be emphasized that this molecular weight is extremely low when compared to data available on crude enzyme preparations (29).
Despite kinetic data also indicating a single enzyme form in lymphocytes, the techniques of sucrose gradient or gel filtration did not resolve one form. The fractionation studies did show that the higher affinity lymphocyte enzyme contains at least 50% of 6.0 S (98,000 molecular weight) enzyme in addition to the lower molecular weight species of 3.6 S (45,000 molecular weight), perhaps representing a monomer-dimer relationship. However, lymphoblastoid cell line cells, which were shown to contain a kinetically similar phosphodiesterase consisted essentially of a single 6.0 S phosphodiesterase. In addition, physical manipulations of lymphocytes and simply "aging" of the homogenate, changed the lymphocyte enzyme to a lower affinity, low molecular weight (3.6 S) enzyme form, virtually identical to the monocyte enzyme. The high affinity lymphocyte enzyme might, therefore, consist in vivo entirely of the less stable 98,000 molecular weight species (or larger polymers), but this requires additional proof. Our preliminary studies do not indicate, however, that the lower molecular weight species resulted from sulfhydryl oxidation, limited chymotrypsin-like proteolysis, or minimal protein phosphatase activities.

As mentioned above, the lymphocyte enzyme might exist in an equilibrium between the two molecular weight species. However, this does not seem likely since: (a) if the lower molecular weight enzyme had been present in the original homogenate it would have been apparent by kinetic analysis unless the two enzyme forms had identical molecular weight but different affinities, (b) the maximum velocity of the same preparation increased despite a decrease in the apparent $K_m$ and (c) commonly used enzyme-stabilizing agents (e.g. dithiothreitol, benzamidine, sodium fluoride, and sucrose) either added to the homogenization medium or included in the sucrose gradients, did not affect the multiple peaks seen in lymphocyte preparations.

An attractive hypothesis to explain this finding is that a factor(s) normally present in the lymphocyte environment can regulate the amount of high or low molecular weight forms present. This factor(s) might be removed during cell purification of the cells to cause a destabilization of the higher molecular weight enzyme. This would be particularly evident in the preparations where multiple procedures were used to ensure cell purity. Pledger et al. (32) have shown that the concentration of fetal calf serum can alter the amounts and kinetic properties of cyclic nucleotide phosphodiesterases present in cultured fibroblasts. Also, our preliminary experiments indicate that cultured lymphocytes have the high affinity enzyme activity following incubation in serum free medium, whereas 24-hour lymphocyte cultures in media containing 30% autologous serum still possess the high affinity cyclic AMP phosphodiesterase. In addition, the lymphoblastoid cells which showed only the high molecular weight, high affinity enzyme, were exposed to serum-free media for a much shorter period of time before enzyme fractionation than were the lymphocytes. Since lymphocytes consist of subpopulations of cells with different characteristics (e.g. B and T cells), it would seem possible that one subpopulation of lymphocytes may contribute to the stability and regulation of the phosphodiesterase activity of another subpopulation.

In addition to its unique size and kinetic behavior, lymphocyte cyclic AMP phosphodiesterase has several other interesting properties. A portion of most mammalian high affinity, cyclic AMP-specific phosphodiesterase resides in the plasma membrane of their respective cells (29, 30). Using both differential centrifugation and discontinuous sucrose density gradient fractionation techniques, we found no indication of membrane-bound enzyme in lymphocytes. Since lymphocytes contain only a small amount of cytoplasm, it seems possible that this phosphodiesterase is of nuclear origin. Coulson and Kennedy (6) using cytochemical techniques, have previously suggested that phosphodiesterase activity may be associated with the nuclear membrane of these cells. Since some membrane bound cyclic AMP phosphodiesterases respond to insulin (34–36), growth hormone (24), and thyroid hormone (37), and since lymphocytes are thought to contain specific receptors for these hormones (38–40), further investigations are needed to explore in detail the cellular location and hormone regulation of lymphocyte cyclic AMP phosphodiesterase.

The temperature optimum, pH optimum, and divalent cation requirements of lymphocyte cyclic AMP phosphodiesterase are similar to other mammalian systems. Calcium above 1 mm was inhibitory to magnesium-catalyzed cyclic AMP hydrolysis, lending some support to the hypothesis that the mitogenic effects of calcium on thymic lymphocytes may be the result of phosphodiesterase inhibition resulting in increased cellular cyclic AMP content (41).

In summary, human peripheral blood lymphocytes and monocytes contain unique cyclic AMP hydrolytic enzymes. Multiple kinetic forms are not apparent in these cells and lymphocytes contain one of the highest affinity enzymes yet reported. We have speculated that the high affinity of this cyclic AMP phosphodiesterase and its singular molecular composition may be linked to the rapid proliferative functions of these cells. These studies also indicate the need to establish cell purity by several criteria in order to study cyclic nucleotide metabolism in these cells.

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REFERENCES

Lymphocyte Cyclic AMP Phosphodiesterase

Cyclic adenosine 3':5'-monophosphate phosphodiesterase. Distinct forms in human lymphocytes and monocytes.

W J Thompson, C P Ross, W J Pledger, S J Strada, R L Banner and E M Hersh