J. Enzyme Inhibition, 1987, Vol. 1, pp. 267–274
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INHIBITION OF HUMAN LYMPHOCYTE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES BY THE CHLORINATED ADENOSINE ANALOG DTA-35

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(Received August 22, 1986)

Human (E^+)-T-lymphocytes have multiple cyclic nucleotide phosphodiesterase activities, some of which are markedly inhibited by the chlorinated adenosine derivative 9-(3′,5′-dichloro-2′,3′,5′-trideoxy-β-*D-threo*-pentofuranosyl)adenine (DTA-35).

KEY WORDS: Phosphodiesterase, cyclic nucleotides, chlorinated adenosine analog.

INTRODUCTION

Variations in the concentrations and turnover rates of cyclic AMP and cyclic GMP are associated with physiological processes such as cell division.[1-3] The levels of those two nucleotides are dependent on adenylate and guanylate cyclase activities for their synthesis and on a group of phosphodiesterases for their degradation. A number of purine derivatives such as theophylline and 3-isobutyl-1-methylxanthine are inhibitors of both lectin-induced lymphocyte mitogenesis and cyclic nucleotide phosphodiesterase activities.[4,5] The nucleoside analog 9-(3′,5′-dichloro-2′,3′, 5′-trideoxy-β-*D-threo* pentofuranosyl)adenine (DTA-35), has been reported to be an inhibitor of lectin-induced mitogenesis.[6] DTA-35 is now also shown to be an inhibitor of cyclic nucleotide phosphodiesterase activities from human lymphocytes.

METHODS

Materials

[^{3}H]-cAMP and [^{3}H]-cGMP (Dupont-NEN). Nucleotides, nucleosides and *Crotalus admanteus* venom (Sigma). Polyethyleneimine (PEI) thin layer plates from Brinkman Instruments. Scintillation fluor (3a20) – Research Products Incorporated (Elk Grove, IL).

The nucleoside, 9-(3′,5′-dichloro-2′,3′,5′-trideoxy-β-*D-threo*-pentofuranosyl)-adenine (DTA-35), was a gift from H.P.C. Hogenkamp. It was prepared from 2′-deoxyadenosine using thionyl chloride and hexamethylphosphoramide.[7] The structure of DTA-35 is shown in Figure 1. The chlorination at C-3 of the deoxyribose ring results in an inversion of configuration at that carbon producing a deoxy xylose

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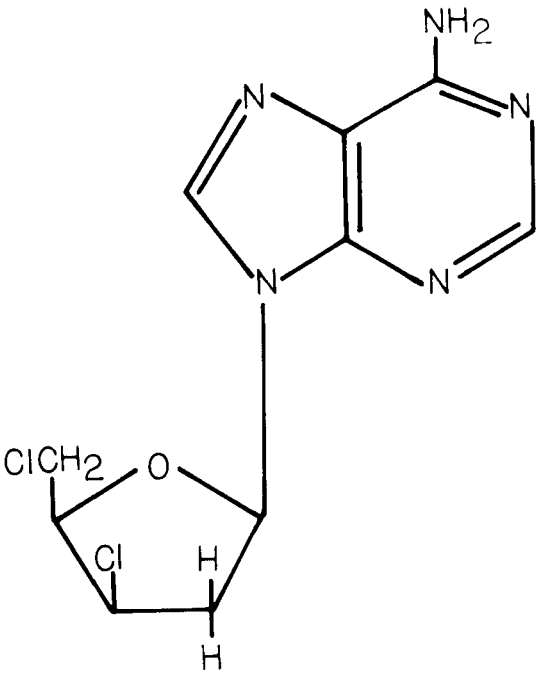

FIGURE 1 Structure of DTA-35. Note the inversion at C-3′ compared to the starting material, 2′-deoxyadenosine.

derivative. Crystalline DTA-35 was dissolved in 0.1 mM acetic acid immediately before use ($E = 14.4 \times 10^3 M^{-1} cm^{-1}$ at 260 nm).

Phosphodiesterase Preparations

A subpopulation of human lymphocytes which form rosettes with neuraminidase-treated sheep erythrocytes, (E^+)-T-cells, was isolated from heparinized blood of a normal donor (typically 4×10^8 cells were recovered per 500 ml).[8] Numbers of cell types were determined as described by Hurwitz *et al.*[9] Cells were suspended (2×10^8 ml) in 50 mM Tris-Cl pH 8.0, 7 mM β-mercaptoethanol, 0.2 mM phenylmethyl sulfonylfluoride. After swelling for 30 minutes at 20°C, cells were ruptured by 100 strokes of a Dounce homogenizer (B-pestle). Protein was determined by the method of Lowry *et al.*[10]

Cyclic nucleotide phosphodiesterase activities were assayed by a combination of the assays of Thompson and Appleman[11] and Hurwitz and Edstrom.[12] Incubation I: Assay mixtures contained 40 mM Tris-Cl pH 8.0, 2 mM $MgCl_2$, 20 μM $CaCl_2$, 300,000 cpm of [^{3}H]-cyclic nucleotides at appropriate concentrations, and enzyme samples in a final volume of 100 μl. After 30 minutes at 30°C, reactions were terminated by heating the tubes in boiling water for two minutes. Incubation II: After the assay mixtures had cooled, 25 μl of venom 5′-nucleotidase (1 mg/ml in water) were added to each tube. The mixtures were then incubated for 15 min at 30°C, cooled to 0°C, and 10 μl of the assay mixtures were spotted in duplicate and dried at an origin 1 cm from the bottom of a 1 × 10 cm section of a PEI plate. Substrate and products

were separated by ascending chromatography using 0.125 M sodium acetate buffer, pH 4.0. Markers were visualized under UV light and appropriate areas were cut out and counted directly in the toluene based scintillation fluor. For the cAMP phosphodiesterase activities, both the adenosine and inosine areas were counted to account for 5′-nucleotidase and adenosine deaminase activities present in the enzyme preparation. Data analysis was performed by direct curve fitting.[13]

RESULTS

A major concern in studying enzymes from lymphocytes is the problem of obtaining a homogeneous preparation of cells from blood. In order to obtain lymphocyte phosphodiesterases free from the platelet enzymes, a highly specific cell isolation method was required. We found that a previously described method of removing the platelets from lymphocyte preparations did not reduce the number of platelets to an insignificant number.[14] By isolating only those lymphocytes which formed a complex with sheep erythrocytes, we were able to obtain a preparation free of platelets.

The kinetic parameters of the cAMP and cGMP phosphodiesterase activities were examined by assay at substrate concentrations ranging up to 110 μM. All assays were performed under conditions where product formation was proportional to the time of assay and the amount of enzyme present. Analysis of the data indicated complex kinetics presumably due to the presence of multiple enzyme activities. The insets in Figures 2 and 3 show the kinetic data presented in the traditional reciprocal format. The kinetic constants derived from these Lineweaver-Burk plots are given in the first column of Table I; in the second column are the constants determined by curve fitting based on the sum of two standard Michaelis–Menten equations.

The upper v *vs.* [S] curves in Figures 2 and 3 show the rate of hydrolysis as a function of substrate concentration for cAMP (Figure 2) and cGMP (Figure 3). Standard statistical tests showed that the two activity model (solid lines) yielded a better fit than did the single activity model (dashed lines). From these results and the work of Wedner *et al.*[14] there is a clear indication that the cyclic nucleotide phosphodiesterases of human lymphocytes can be grouped into two types: those with a high

TABLE I

Kinetic Parameters for the Two Activity Model Using Direct Graphical Analysis and Computer Generated Curve Fitting

	Graphical Analysis	Curve fitting	
		− DTA-35	+ 200 μM DTA-35
cAMP			
K_m^1	2.47	1.19	1.84
V_{max}^1	38.2	20.4	9.50
K_m^2	15.7	30.5	172
V_{max}^2	75.2	58.8	100
cGMP			
K_m^1	3.70	2.78	4.97
V_{max}^1	52.6	30.1	42.4
K_m^2	31.3	42.3	33.6
V_{max}^2	139	102	54.0

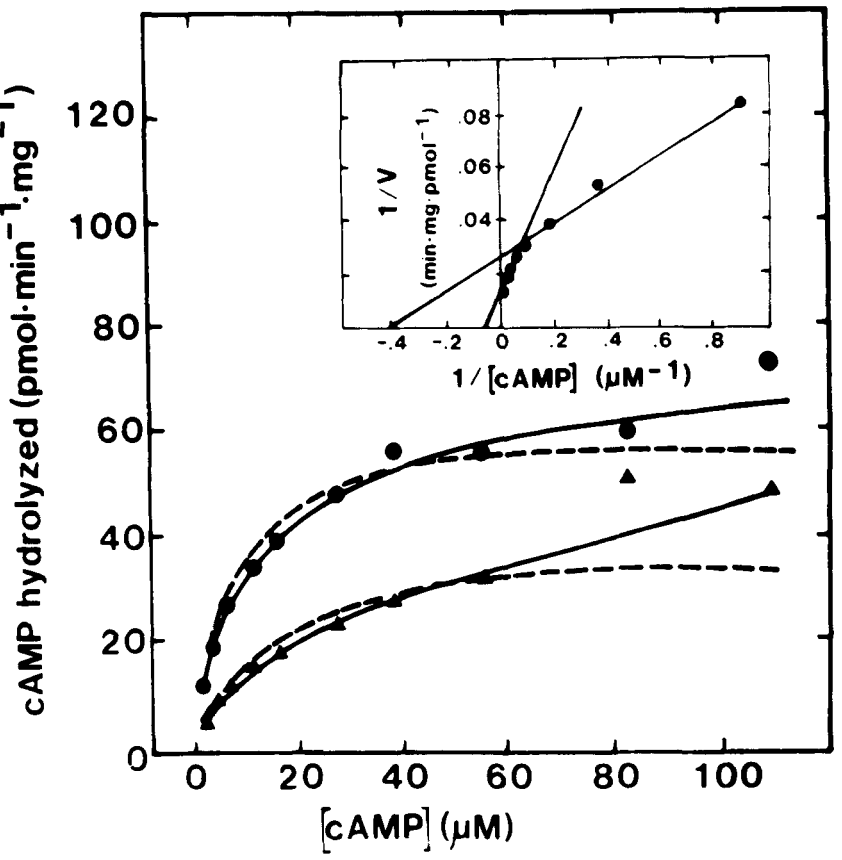

FIGURE 2 Effect of DTA-35 on the substrate saturation curve for (E^+)-T-lymphocyte cAMP phosphodiesterases (140 μg protein). The points on the upper curves (●) were determined in the absence of inhibitor while the lower points (▲) were obtained by assaying the enzyme in the presence of 200 μM DTA-35. The solid lines are for computer determined best fit for the two activity model while the dashed lines assume a single enzyme. The weighted sum of the squares of the deviations (WSSD) of the data from the line for the two activity model was 0.014 compared to 0.12 for the single activity equation. In the presence of DTA-35, the WSSD values were 0.0084 and 0.36 respectively. The inset is the data for the uninhibited enzymes in double reciprocal format. Assays were performed as described in Methods.

affinity toward their substrates (K_m = 1 to 5 μM) and those with a lower affinity (K_m = 10 to 100 μM).

The effects of DTA-35 on cyclic nucleotide phosphodiesterase activities from human (E^+)-T-lymphocytes were examined using two concentrations of both cAMP and cGMP as substrates. In Figure 4 it can be seen that 200 μM DTA-35 inhibited 70% of the cAMP phosphodiesterase activities at 5 μM substrate and 50% at 100 μM substrate. For the hydrolysis of cGMP, it was only at the higher substrate concentration that an inhibition of 50% was achieved.

We determined apparent kinetic constants for both cAMP and cGMP phosphodiesterase activities in the presence of the inhibitor. The lower curves in Figures 2 and 3 show the data and the fitted (solid) line for the inhibited activity based on a two activity model which again provided the better fit. The third column in Table I gives

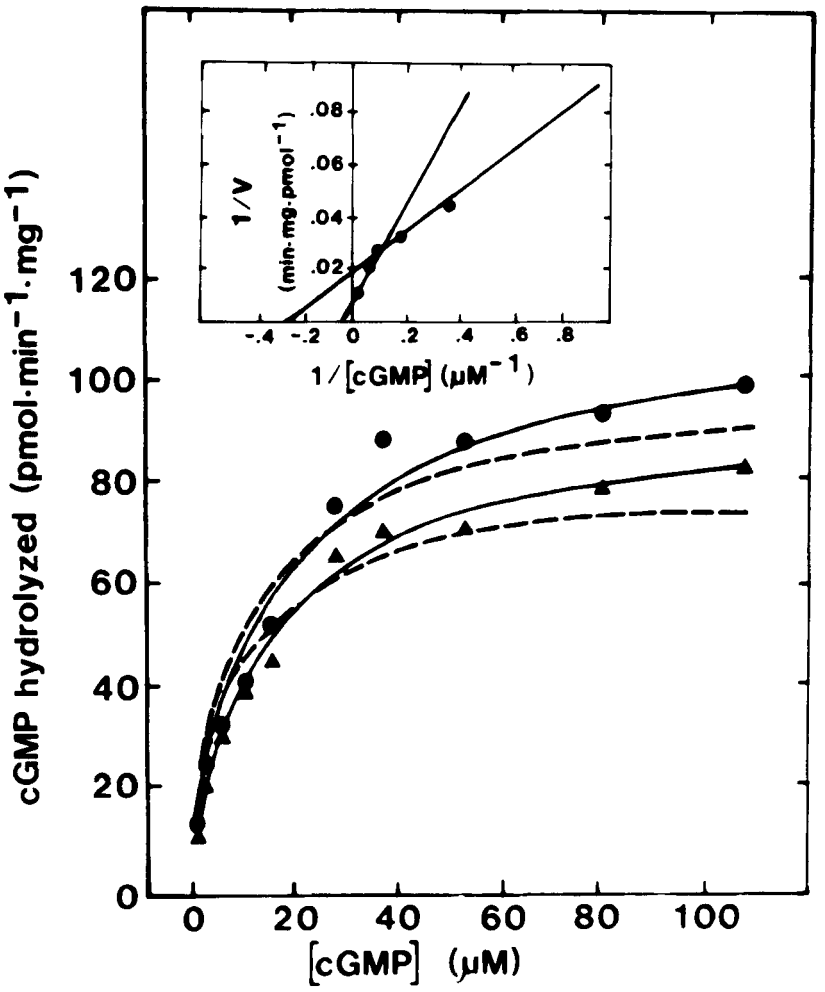

FIGURE 3 Effect of DTA-35 on the substrate saturation curve for (E^+)-T-lymphocyte cGMP phosphodiesterases (140 μg protein). The upper curves (●) were determined without inhibitor while the lower points (▲) were obtained by assaying in the presence of 200 μM DTA-35. For further details, see legend to Figure 2. The WSSD value for the two activity model was 0.073 compared to 0.17 for the single enzyme line. In the presence of DTA-35 the comparable values were 0.025 and 0.044.

the kinetic constants for the two different substrates using the two enzyme model in the presence of 200 μM DTA-35. It is clear from these results that DTA-35 effects complex changes with the various phosphodiesterase activities. As DTA-35 changes both the K_m and V_{max} it is clearly not a simple competitive inhibitor but rather one of the noncompetitive or mixed types.

DISCUSSION

Cyclic nucleotide phosphodiesterase activities have been reported in a variety of tissues and cell types including lymphocytes, monocytes and platelets.[14] These three

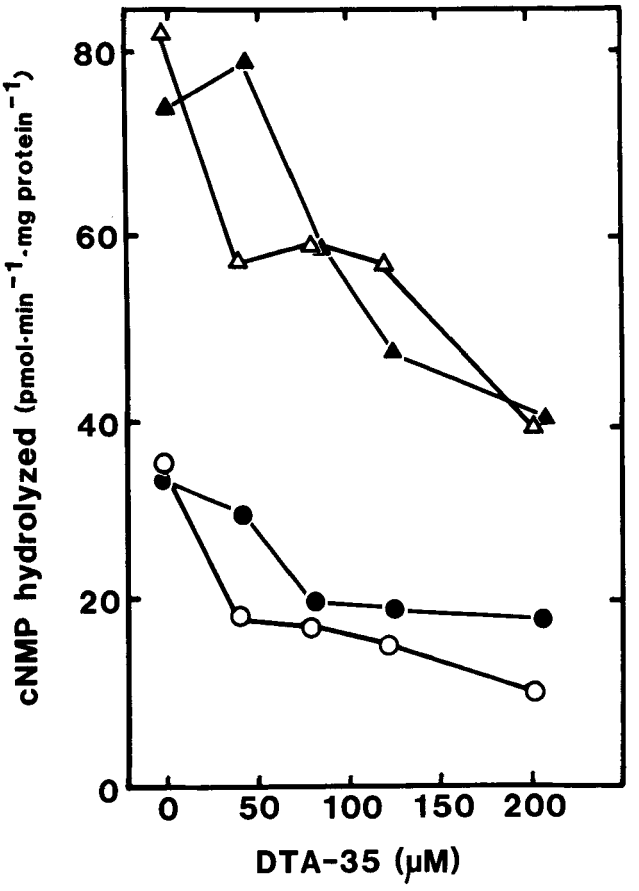

FIGURE 4 Inhibition of cyclic nucleotide phosphodiesterase by DTA-35. (E^+)-T-lymphocyte homogenate (28 μg protein) was assayed for phosphodiesterase with cAMP (○, △) and cGMP (●, ▲) as substrates as described in Methods. Cyclic nucleotide concentrations were 5 μM (○, ●) or 100 μM (△, ▲) with the DTA-35 concentration ranging up to 200 μM.

cell types are all present in Ficol-Isopaque preparations of mononuclear cells from blood.[15] In order to study the effects of DTA-35 on human peripheral blood lymphocyte cyclic nucleotide phosphodiesterase, the subpopulation of lymphocytes which forms rosettes with sheep erythrocytes was chosen. This subpopulation, (E^+)-T-lymphocytes, provides an ideal system with which to study lymphocytic phosphodiesterases because the cells can be isolated virtually free from other cell types.

Stimulation of human peripheral blood lymphocytes by *Lens culinaris* hemagglutinin has been previously reported to be inhibited 50% by 75 μM DTA-25 and to be completely inhibited by 200 μM DTA-35[6]. Two hundred micromolar DTA-35 inhibits approximately 50 to 70% of the total cyclic nucleotide phosphodiesterase activities assayed at 5 μM cAMP, 100 μM cAMP or 100 μM cGMP and it inhibits about 30% of the activities assayed at 5 μM cGMP. Multiple forms of cyclic nucleotide phosphodiesterase have been reported in human lymphocytes.[14] The incomplete inhibition of enzyme activity at concentrations of DTA-35 which completely block *Lens culinaris*

hemagglutinin-induced mitogenesis may be due to selective inhibition of only some of the forms of cyclic nucleotide phosphodiesterase. While lectin-induced mitogenesis has been shown to be accompanied by a rise in cGMP levels[2,3] a portion of the complex of phosphodiesterase activities may well be required for completion of the initiation of mitosis and it is at that level that such mitogenic inhibitors as DTA-35 may play a role.

The K_m values calculated for the enzyme preparation (Table I) are comparable to those reported by others for the hydrolysis of cAMP by lymphocyte homogenates.[14] The use of a two enzyme model here assumes two classes of enzyme where each class could contain several enzymes with similar K_m values. DTA-35 had a major effect on the activities which hydrolyze cAMP with low affinities (high K_m); it raised that K_m by a factor of about six. There was substantially less effect on the "low" K_m activities where only the V_{max} seems to be changed. The V_{max} was reduced by about 50%. For the cGMP hydrolyzing activities, the K_m of the high affinity form is rasied two-fold while the V_{max} for the low affinity form is halved. These observations indicate that DTA-35 is not a simple competitive inhibitor of cyclic nucleotide phosphodiesterases. Erneux *et al.*[16] have described a "paradoxical" stimulation of rat liver cAMP phosphordiesterase by 3-isobutyl-1-methylxanthine, generally a phosphodiesterase inhibitor.

Although we have made some preliminary evaluations of the activity of DTA-35 as an inhibitor of phosphodiesterases, homogeneous enzyme preparations must be isolated in order to complete the study of these interesting properties of DTA-35. We have shown that radiolabeled DTA-35 is freely able to enter the lymphocyte but it is not metabolized once inside (unpublished results). The absence of hydroxyl groups at positions 2′, 3′ or 5′ of the pentose ring of course precluded phosphorylation. Thus it is apparently the free nucleoside analog which acts as the inhibitor and not a metabolite. Furthermore in those same studies, there was no evidence of covalent attachment of the radiolabeled derivative to the enzyme. This nucleoside analog may be a useful new probe for the investigation of the role of cyclic nucleotides in cellular processes.

Acknowledgements

This work was supported by grants from the University of Minnesota Graduate School and the Minnesota Leukemia Task Force. We would like to thank Harry P.C. Hogenkamp for the gift of the DTA-35 and Marilyn H. Meinke for helpful discussions while preparing the manuscript.

References

1. T. Wang, J.R. Sheppard and J.E. Foker, *Science* **201**, 155 (1978).
2. J.W. Hadden, E.M. Hadden, M.K. Haddox and N.G. Goldberg, *Proc. Natn. Acad. Sci. USA* **69**, 3024 (1972).
3. J.W. Hadden, E.M. Hadden, J.R. Sadlik and R.G. Coffey, *Proc. Natn. Acad. Sci. USA* **73**, 1717 (1976).
4. R. Hirschhorn, J. Grossman and G. Weissmann, *Proc. Soc. Exp. Biol. Med.* **133**, 1361 (1970).
5. T. Wang and J. Foker, *Fed. Proc.* **36**, 687 (1977).
6. R.D. Edstrom, C.A. Prody and H.P.C. Hogenkamp, *Biochem. Biophys. Res. Commun.* **77**, 1552 (1977).
7. H.P.C. Hogenkamp, *Biochem.* **13**, 2736 (1974).
8. S.M. Wahl, D.L. Rosenstreich and J.J. Oppenheim, in *In Vitro Methods in Cell Mediated and Tumor Immunity*, B. Bloom and J. David (eds.), Acad. Press, NY, pp 231 (1976).
9. R.L. Hurwitz, D. Schreinemachers and J.H. Kersey, *Exp. Hematology* **7**, 81 (1979).

10. O.H. Lowry, N.J. Rosebrough, L. Farr and R.J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
11. W.J. Thompson and M.M. Appleman, *Biochemistry* **10**, 311 (1971).
12. M.Y. Hurwitz and R.D. Edstrom, *Anal. Biochem.* **84**, 246 (1978).
13. R.G. Duggleby, *Anal. Biochem.* **110**, 9 (1981).
14. H.J. Wedner, B.Y. Chan, C.S. Parker and C.W. Parker, *J. Immunol.* **123**, 725 (1977).
15. A. Boyum, *Scand. J. Clin. Lab. Invest.*, **21**, (Supp. 97), 1 (1968).
16. C. Erneux, F. Miot, J. Boeynaems and J.E. Dumont, *FEBS Lett.* **142**, 251 (1982).