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CYCLIC 3',5'-AMP PHOSPHODIESTERASE OF HUMAN BLOOD LYMPHOCYTES

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SUMMARY

We have specified the main kinetic parameters of human blood lymphocyte cyclic 3',5'-AMP phosphodiesterase using a crude extract (34 000 × *g* supernatant).

1. The pH optimum was found to be about 8.0 and the activity is classically inhibited by methyl-xanthines. Mg^{2+} fulfills the cation requirement. Integrity of SH groups is essential.

2. Ca^{2+} at low concentration ($1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ M) has a stimulatory effect in the presence of Mg^{2+} and a (Ca^{2+} + Mg^{2+})-dependent phosphodiesterase is assumed to occur.

3. Lymphocyte cyclic 3',5'-AMP phosphodiesterase is neither subject to inhibition nor to stimulation by cyclic 3',5'-GMP.

4. Activity–substrate concentration relationship revealed a low (K_m = 20 μM) and a high (K_m = 0.72 μM) affinity for cyclic AMP, suggesting the existence of several molecular forms of the enzyme.

5. We failed to observe cyclic 3',5'-IMP production from cyclic 3',5'-AMP in a lymphocyte homogenate, thus proving the unique degradative conversion of cyclic AMP into 5'-AMP.

6. None of the blastogenic or interferon inducers investigated (phytohaemagglutinins from *Phaseolus vulgaris* and *Pisum sativum*, streptolysin O, lipopolysaccharides from *Salmonella typhi* endotoxin) has been found to be a cyclic AMP phosphodiesterase effector at physiologically active concentrations. Such a property is discussed in connection with present researches on the role of cyclic 3',5'-nucleotides in specific and non specific immunity processes.

INTRODUCTION

Convincing evidence is accumulating for multiple roles of cyclic AMP in the regulation of cell metabolism and increasing progress is expected to elucidate its mo-

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'* tetraacetic acid; PCMB, *p*-chloromercuribenzoate.

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lecular targets[12]. Synthesized from ATP by adenylate cyclase, cyclic AMP is degraded through the conversion into AMP by a cyclic 3',5'-nucleotide phosphodiesterase.

The assumption that cyclic AMP could be involved in the process of lymphocyte transformation[3], is accredited by several authors[4–10] but contested by others[11–13]. Special emphasis has been focused on adenylate cyclase stimulation by specific antigens and polynucleotides[14,15] and phytohaemagglutinins from *Phaseolus vulgaris*[4,16].

Puzzling results reported in a preliminary note[17], showing that cyclic AMP phosphodiesterase was stimulated by both phytohaemagglutinins and anti-lymphocyte serum have not been borne out in the present study. Detailed examination of this point led us to conclude that stimulation was not an intrinsic property of these inducers but the consequence of cation contaminants (mainly Ca^{2+}), introduced concomitantly at effective concentrations. Other substances such as streptolysin O and endotoxin from *Salmonella typhi*, accredited as interferon inducers[18,19] also failed to affect human lymphocyte cyclic AMP phosphodiesterase activity.

MATERIALS AND METHODS

Lymphocyte purification

Small lymphocytes were obtained from human heparinized veinous blood (200 ml) by filtration on nylon fiber columns, followed by accelerated sedimentation with Dextran and ammonium chloride osmotic lysis, according to the method of Debray-Sachs and Dormont[20].

The cell button was washed by resuspension 2–3 times in a saline hypotonic buffer (10 mM Tris–HCl, 0.15 M NaCl, pH 7.4, and 0.1 mM EDTA) and centrifuged at 1000 × *g* for 5 min at 4 °C.

Contamination of platelets was, therefore, reduced to 1–3 for one lymphocyte while about 0–5% polymorphonuclear leukocytes were present.

Cyclic 3',5'-AMP phosphodiesterase preparation

200–250 μl of ice cold 10 mM Tris–HCl, 0.15 M NaCl, pH 7.4, buffer were added to the lymphocyte pellet. Thirty strokes in a glass Potter–Elvehjem type homogenizer with motor-driven Teflon pestle (1000 rev./min) at ice bath temperature gave 90–100% cell lysis. This crude lymphocyte homogenate was centrifuged in a M.S.E. highspeed centrifuge at 34 000 × *g* for 30 min. The supernatant referred to as post-microsomal[21], was routinely used as a source of cyclic 3',5'-AMP phosphodiesterase and crude homogenate was only occasionally employed (see Results and Discussion, Sections 1 and 2).

Protein concentration was estimated by the method of Lowry *et al.*[26] with bovine γ-globulin (Fraction II) as a standard.

Cyclic 3',5'-AMP phosphodiesterase assay

Enzymatic activity was measured as the rate of splitting of cyclic [^{3}H]AMP in a standard reaction medium (final volume, 100 μl) containing 10–20 μM cyclic 3',5'-[^{3}H]-AMP (spec. act. 0.42 mCi/mM), 5 mM $MgCl_2$, 40 mM Tris–HCl, pH 8.0, enzyme at 1.0 mg/ml protein and other substances as stated in the figures and tables. Incubations were run at 30 °C for 30 min and were stopped by immersion in boiling water for 1 min.

After centrifugation (4000 × *g*, 15 min), 50 μl of the clear supernatant were co-chromatographied with reference nucleotides on paper (Carl Schleicher and Schüll) with 95% ethanol–1 M ammonium acetate, pH 7.5 (75:30, v/v) as descending solvent. Cyclic AMP spots (R_F = 0.31–0.35) observed under ultraviolet light were cut and transferred into counting vials with 1 ml distilled water for elution and 10 ml scintillation fluid (200 g naphthalene, 60 g dimethyl-POPOP, 12 g PPO, 100 ml methanol, 2 l dioxane).

Non-adjacent channel ratio counting method was used and the efficiency ranged between 6 and 8%. No correction was made on the basis of quantitative nucleotide recovery. Blank tubes with buffer or heat-denatured enzyme were used as assays.

Cyclic 3′,5′-AMP phosphodiesterase activity will be expressed as nmoles cyclic AMP hydrolysed per 30 min under the conditions outlined above.

Reagents and products used

Cyclic 3′,5′-[8-^{14}C]AMP (spec. act. 40–50 mCi/mM) was purchased from C.E.A. (France) and cyclic 3′,5′-[8-^{3}H]AMP (spec. act. 5 Ci/mM) from Schwartz Bioresearch. Radiochemical purity was tested by thin-layer chromatography and found to be 99.5 and 90% respectively. (^{3}H-labelled contaminants were: AMP, 7% and adenosine, 3%). Phytohaemagglutinins P and M from *Phaseolus vulgaris* were obtained from Difco. Phytohaemagglutinins from *Pisum sativum* was a gift from Dr G. Betail; details for preparation and purification are given in refs 22 and 23. Streptolysin O was from the Pasteur Institute. Crude (lipopolysaccharide I), detoxified (lipopolysaccharide II), and nucleic acid-free (lipopolysaccharide III) lipopolysaccharides extracted from *Salmonella typhi* were kindly supplied by Dr O. Creach[24]. Nucleotides and dithiothreitol were from Calbiochem. Disodium EDTA, sodium EGTA and bovine γ-globulin (Fraction II) were from Sigma.

RESULTS AND DISCUSSION

Degradation products from cyclic 3′,5′-AMP hydrolysis

To date, only cyclic 3′,5′-AMP and -GMP have been found to occur *in vivo*[2] and cyclic 3′,5′-nucleotide phosphodiesterase is the unique enzyme known to be responsible for their degradation.

Incidentally, we discovered in lymphocyte homogenates both potent adenosine deaminase (EC 3.5.4.4) and adenine deaminase (EC 3.5.4.2) and since at least adenosine deaminase from *Aspergillus oryzae* was reported to possess a broad specificity including cyclic AMP[25], we investigated the possibility of such a deamination in lymphocytes.

In a typical experiment cyclic 3′,5′-[^{14}C]AMP was incubated in the presence of lymphocyte homogenate and the supernatant was submitted to two-dimensional chromatography on cellulose. Autoradiography revealed that the radioactivity was distributed between cyclic AMP, AMP, adenosine and inosine, and possibly IMP.

Cyclic 3′,5′-IMP could not be identified and this fact confirms previous results given by Dousa and Rychlik[27,28] for rat kidney and Gulyassy[29] for epithelial cells of toad bladder.

Since AMP deaminase seems to be present in our crude preparations, this result is in agreement with those of Zielke and Suelter[30] who found that cyclic AMP could

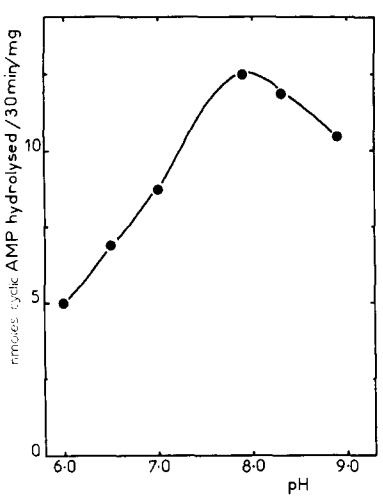
not be a substrate for AMP deaminase from rabbit muscle. Gulyassy and Oken[31] recently emphasized the necessity to be careful about the methodology when crude homogenates are used as a source of cyclic AMP phosphodiesterase. In our non-purified preparations adenosine and IMP were formed at approximately the same rate from AMP. One can expect that 5'-nucleotidase (EC 3.1.3.5) remains associated with the membrane fraction in a 34 000 × *g* pellet, whereas AMP deaminase should be a soluble enzyme present in 34 000 × *g* supernatant. Consequently, cyclic AMP phosphodiesterase assays measuring end-product formation[32] instead of cyclic AMP disappearance are liable to underestimate cyclic AMP phosphodiesterase activity. This is the reason why we adopted the latter method.

Time-course and protein dependence

Cyclic AMP hydrolysis in both lymphocyte homogenate and 34 000 × *g* supernatant is linear with respect to time (up to 30 min) and protein concentration (up to approx. 1.0 mg/ml).

pH dependence

The pH optimum was found to be between 8.0 and 8.2 in 25 mM Tris–maleate buffer with activity reduced 60% at pH 6.0 and 15% at pH 8.9 (Fig. 1).



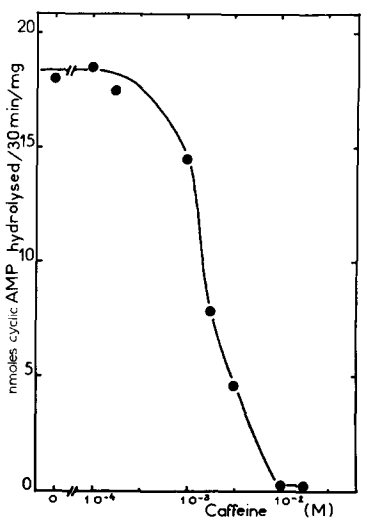


Fig. 1. pH optimum of lymphocyte cyclic AMP phosphodiesterase. Substrate concentration: 17.90 μM. 25 mM Tris–maleate buffer was adjusted to the desired pH with NaOH.

Fig. 2. Inhibition by caffeine. Conditions are as described in Material and Methods with varying concentrations of caffeine; substrate concentration: 18.45 μM.

Inhibition by methylxanthines

Cyclic 3',5'-AMP phosphodiesterase is inhibited by caffeine between $2 \cdot 10^{-4}$ and $1 \cdot 10^{-2}$ M (Fig. 2) and 50% inhibition occurs at about 2.10^{-3} M. At this concentration, theophylline inhibits slightly more than caffeine.

Intracellular localization

Cyclic AMP phosphodiesterase has been found to be exclusively located in 34 000 × *g* supernatant. Attempts to release any latent activity from 34 000 × *g* pellet by preincubation in the cold 30 min with Triton X-100 (final concentration 0.2%) have been unsuccessful.

Human blood lymphocyte cyclic AMP phosphodiesterase localization in the post-microsomal supernatant[21] differs from that of rat thymus lymphocyte cyclic AMP phosphodiesterase which has been studied in a particulate fraction sedimenting at 20 000 × *g* by Frank and MacManus[33]. The reason for such a discrepancy between cells which are physiologically alike is unknown. Intracellular distribution of cyclic AMP phosphodiesterase seems to be very dependent upon tissues, since particulate as well as soluble location may be proved in a same cell[34].

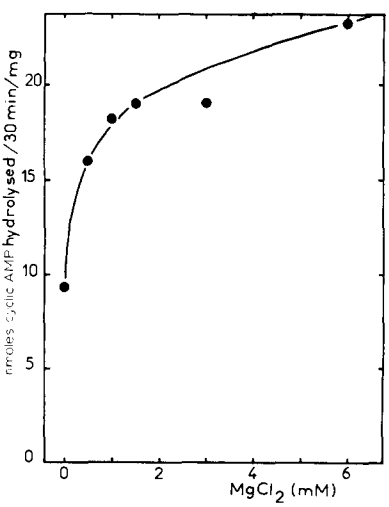
Cheung and Salganicoff[35] succeeded in releasing masked activity from brain homogenates by use of detergent treatment. The negative result obtained with our 34 000 × *g* pellet is an indication that no activity remains trapped in intact subcellular organelles.

Requirement for divalent cations

Cyclic AMP phosphodiesterase basal activity obtained in the absence of added ions could be increased up to approx. 150% by adding Mg^{2+} at different concentrations (Fig. 3).

The apparent K_m calculated from this curve by substracting basal activity, is about $7 \cdot 10^{-4}$ M and reciprocal plots give a straight line.

1.10^{-4} M EDTA inhibits basal activity by about 70% and Mn^{2+} (5 mM) is 78% as effective as Mg^{2+} (5 mM). Mg^{2+} could not be replaced by Hg^{2+} nor Ca^{2+}. In the presence of $MgCl_2$ (5 mM), concentrations of $HgCl_2$ as low as 1 to 10 μM are inhibitory.



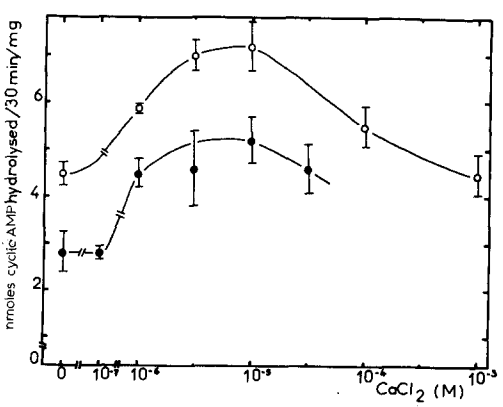


Fig. 3. Influence of Mg^{2+} concentration. Conditions are as described in Material and Methods with varying concentrations of $MgCl_2$; substrate concentration: 18.45 μM.

Fig. 4. Stimulatory effect of Ca^{2+} on lymphocyte cyclic AMP phosphodiesterase. Standard reaction medium (see Material and Methods) containing $MgCl_2$ 1.0 mM (●—●) or 2.5 mM (○—○) was incubated in the presence of 1.10^{-6} M EGTA and varying concentrations of $CaCl_2$. Results are the mean of a duplicate ± S.E.

Effect of Ca^{2+}

When low concentrations of Ca^{2+} ($1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ M) are added to the standard reaction medium where Mg^{2+} is already present, cyclic AMP phosphodiesterase activity is significatively enhanced (Fig. 4).

$1 \cdot 10^{-5}$ M is the optimum concentration and results in a 1.7-fold increase, whereas at $1 \cdot 10^{-3}$ M no stimulatory effect of Ca^{2+} could be detected.

Enhancement of lymphocyte cyclic AMP phosphodiesterase by low concentrations of Ca^{2+} confirmed the previous reports by Kakiuchi and Yamazaki[36], who found such a property for rat brain cyclic AMP phosphodiesterase. These authors succeeded in separating, by gel filtration on Sepharose 6B, two peaks of cyclic AMP phosphodiesterase[39], one exhibiting a 2-fold increase in activity in the presence of $4 \cdot 10^{-5}$ M Ca^{2+}, called (Mg^{2+} + Ca^{2+})-dependent phosphodiesterase, the other being unaffected by Ca^{2+} and named Mg^{2+}-dependent phosphodiesterase. It should be noted that the stimulatory effect of Ca^{2+} on lymphocyte cyclic AMP phosphodiesterase takes place between a narrow range of concentration ($5 \cdot 10^{-7}$–$1 \cdot 10^{-5}$ M) since at a higher level ($1 \cdot 10^{-3}$ M) the basal activity returns.

Action of parachloromercuribenzoate

Lymphocyte cyclic AMP phosphodiesterase is inhibited about 73% by 50 μM PCMB and this inhibition could be reversed by 1 mM dithiothreitol (Table I).

The requirement of intact sulfhydryl groups for cyclic AMP phosphodiesterase activity is in agreement with many authors[40].

TABLE I

INHIBITION OF CYCLIC AMP PHOSPHODIESTERASE ACTIVITY BY PCMB AND ITS REVERSION BY DITHIOTHREITOL

Experimental conditions are as described in Materials and Methods with a substrate concentration of 17.90 μM. Tube a is a control where PCMB is omitted. Tubes b and c contain 50 μM PCMB. All tubes are incubated for 20 min (inhibition step). Then Tube c receives dithiothreitol (final concentration: 1 mM) whereas an equivalent volume of buffer is added to Tubes a and b. Reactions are run for 10 min more and stopped (reversion step). Phosphodiesterase activity is given in nmoles cyclic AMP hydrolysed per mg protein at the end of the 30 min incubation.

Tube	*Phosphodiesterase activity*	*% Control*
a	11.65	100
b	3.22	27.6
c	7.26	62.3

Stability during storage

Cyclic AMP phosphodiesterase exhibits good stability during storage at −20 °C for several weeks. 1 mM dithiothreitol has no effect on activity prior to storage. A slight increase (12%) in activity could be recorded for several frozen preparations. This is prevented when 1 mM dithiothreitol is mixed and stored with enzyme.

Some chemical events seem to occur when cyclic AMP phosphodiesterase preparations are stored at 20 °C. Cheung[40] has suggested that enzyme sulfhydryl groups are oxidized by oxygen into disulfide bounds. This process is inhibited by dithiothreitol and could indicate the possibility for association of subunit into a quaternary struc-

ture responsible for enhancement of activity. This view has been recently confirmed by Amer[41] who demonstrated low K_m-phosphodiesterase conversion into high K_m-phosphodiesterase of rabbit tissues either during storage at room temperature or by increasing the pH.

Effect of cyclic 3',5'-GMP

Cyclic 3',5'-AMP phosphodiesterase activity when measured at two substrate concentrations (1 μM and 10 μM) was found to be unaltered by cyclic 3',5'-GMP tested in the range $1 \cdot 10^{-8}$–$1 \cdot 10^{-4}$ M as shown in Table II.

TABLE II

CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN THE PRESENCE OF CYCLIC 3',5'-GMP

Experimental conditions are described in Material and Methods with a protein concentration of 1.08 mg/ml. Cyclic AMP was present either at 1 μM or 10 μM and varying concentrations of cyclic GMP were added as indicated. Incubation time was 10 min and activity is expressed in nmoles cyclic AMP hydrolysed per 10 min per mg protein.

Cyclic AMP concentration (μM)	*Cyclic GMP concentration (M)*							
	0	$1 \cdot 10^{-8}$	$1 \cdot 10^{-7}$	$5.5 \cdot 10^{-7}$	$1 \cdot 10^{-6}$	$5.5 \cdot 10^{-6}$	1.10^{-5}	1.10^{-4}
1	0.301	0.302	0.301	0.301	0.301	0.301	0.301	0.302
10	3.330	3.323	3.323	3.332	3.310	3.323	3.31	3.32

It has been proved that cyclic AMP phosphodiesterase from frog erythrocyte[42], beef heart[43], rat brain[44,47], rabbit skeletal muscle[45] and enzyme of a high molecular weight from rat heart, skeletal muscle, adipose tissue, kidney and brain[46] are subject to competitive inhibition by cyclic GMP. On the contrary Beavo *et al.*[34] have recently shown the stimulatory effect of cyclic GMP on the hydrolysis of cyclic AMP in the same rat tissues, depending upon the organ and for one organ upon the particulate or soluble preparation.

Data concerning the eventual cyclic GMP hydrolysis by lymphocyte phosphodiesterase is missing but our results tend to indicate the strict specificity of the enzyme toward cyclic AMP. Human blood lymphocyte cyclic AMP phosphodiesterase behaviour toward cyclic GMP is another distinctive feature from rat thymocyte cyclic AMP phosphodiesterase which is competitively inhibited by cyclic GMP (10^{-7}–10^{-4} M) at 1 μM substrate concentration and stimulated at 10 μM[33].

Velocity–substrate concentration relationship

When lymphocyte cyclic AMP phosphodiesterase activity of 34 000 $\times$ *g* supernatant is studied at varying substrate concentrations ($1 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ M) a high and a low affinity could be discerned as a result from atypical Lineweaver–Burk plots (Fig. 5). Apparent K_m values were found to be 0.72 μM and 20 μM, respectively.

Cyclic 3',5'-AMP phosphodiesterases with multiple affinities have been described by several authors[33,34,45–48] and negative cooperativity[49] has been put forward to explain the unusual shape of the reciprocal curve. But total activity could be attributed to simultaneous action of two isoenzymes (or two aggregation states of the same enzyme), each displaying a distinct K_m and V. Resolution of rat tissues phospho-

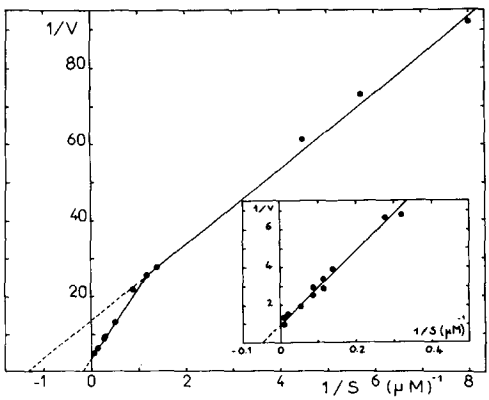


Fig. 5. Lineweaver–Burk plot of lymphocyte cyclic AMP phosphodiesterase activity. Reaction medium (100 μl) contained: cyclic 3′,5′-[^{3}H]AMP (10 000 cpm), cyclic 3′,5′-AMP (0.1–100 μM), $MgCl_2$ (5 mM), Tris–HCl (40 mM, pH 8.0). Protein concentration (34 000 × g supernatant) was 0.5 mg/ml and the incubation time was reduced to 10 min in order to measure the initial velocities. The insert is a magnification of the left portion of the graph. v is expressed as nmoles cyclic AMP hydrolysed per 10 min per mg protein.

diesterases into several molecular weight species by agarose filtration[46,47] and cyclic AMP phosphodiesterase sensitivity from epithelial cells of the toad bladder toward heat denaturation[48], strongly suggests the oligomeric structure of the enzyme.

Although further purification is needed, possible dissociation of lymphocyte cyclic AMP phosphodiesterase into active subunits has been suggested by the presence of sulfhydryl groups and behaviour during storage.

Whether or not Mg^{2+}- and (Ca^{2+} + Mg^{2+})-dependent phosphodiesterase activities could be identified with either low or high K_m activities should be a very attractive point to elucidate since Ca^{2+} may participate as a structural link between subunits, or be involved in the process of the dissociation.

Effect of some blastogenic and interferon inducers

Activity of lymphocyte cyclic AMP phosphodiesterase was found to virtually increase with phytohaemagglutinin concentration. The dose response curves exhibit a maximum at 1.5 mg/ml for phytohaemagglutinin P and above 3.0 mg/ml for phytohaemagglutinin from *Pisum sativum*.

Ability to be stimulated by phytohaemagglutinins completely disappears when 1 mM EGTA is added (and 5 mM Mg^{2+}). When we referred to the data given by Smith *et al.*[4] on the electrolytes present in phytohaemagglutinin P powder, it can be calculated that when phytohaemagglutinin P is used at 1 mg/ml in our assays, the concentration of Ca^{2+} introduced is about $5 \cdot 10^{-5}$–$9 \cdot 10^{-5}$ M which has been found to be stimulatory.

Ca^{2+} contamination of phytohaemagglutinins would conspiciously explain the analogy of the bell shaped dose-response curves, the same order of maximum magnitude observed and the fact that exceedingly high concentrations of phytohaemagglutinins were needed to elicit an effect.

Cyclic AMP phosphodiesterase activity was revealed to be unaffected by streptolysin O when concentrations ranged from 0.25 to 1.7 mg/ml for two different

lymphocyte batches. Endotoxins, lipopolysaccharides I, II and III, have been tested between 0.1 and 1.0 mg/ml without any change in cyclic AMP phosphodiesterase activity. At 2.0 mg/ml a slight inhibition (10%) can be registered for these three preparations.

CONCLUSION

Although cyclic 3',5'-AMP phosphodiesterase has been identified in all mammalian tissues[1,2], very little data is available relative to white blood cells[33]. Bourne *et al.*[50] mentioned the presence of phosphodiesterase in sonicates of human leukocytes but the contribution of each type of cell, namely lymphocytes, to the whole activity has not been assessed.

We specified human blood lymphocyte cyclic AMP phosphodiesterase properties which are very similar to those reported for other mammalian tissues[1,2], *i.e.* pH optimum about 8.0, inhibition by methylxanthines, divalent cation requirement, sulfhydryl groups and multiple affinities. Distinctive features are cellular localization (cytoplasm) and behaviour with respect to cyclic 3',5'-GMP and Ca^{2+}.

Our contribution to the comprehension of a possible role of cyclic AMP in the triggering of lymphocyte response by blastogenic and interferon inducers (phytohaemagglutinin, anti-lymphocyte serum, streptolysin O endotoxins) lies in the fact that if none of them has a significant action on lymphocyte phosphodiesterase this enzyme is not directly involved. In all likelihood, adenylate cyclase activity alterations must account for rapid changes in intracellular levels of cyclic AMP after nonspecific or antigenic stimulation. The privileged localization of this enzyme as part of plasmatic membrane[51], which is postulated to be the first site of action of the inducers[52], should be a main argument for the extension of the "second messenger" concept to the process of lymphocyte transformation and immune responses.

Eventual implication of cyclic 3',5'-AMP phosphodiesterase at any stage of the process is not precluded but could ensue from the consequent modification of the intracellular pool of effector metabolites (such as ions or nucleotides). This type of modulation is probably late and therefore insufficient to be responsible for the transient rise of cyclic AMP level observed by Smith *et al.*[4] after exposure to phytohaemagglutinins. But it could explain the following persistent decrease.

In addition, occurence in lymphocyte 34 000 $\times$ *g* supernatant of cyclic AMP protein binding activity (R. Barbier, unpublished) and perhaps of a heat-stable protein activator[53] may foreshadow the complexity of the interactions between the components of the intracellular cyclic AMP regulation.

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