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MULTIPLE FORMS OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE FROM HUMAN BLOOD PLATELETS

I. KINETIC AND ELECTROPHORETIC CHARACTERIZATION OF TWO MOLECULAR SPECIES

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SUMMARY

The soluble cyclic adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase of human blood platelets consists of two forms with distinct electrophoretic mobility in starch gel. The less anodic form (form I) has a high K_m ($5 \cdot 10^{-4}$ M) for cyclic AMP, is thermostable at 50 °C and is competitively inhibited by aminophylline. The more anodic form (form II) has a low K_m ($5 \cdot 10^{-5}$ M) for cyclic AMP, is thermolabile at 50 °C and is less inhibited by aminophylline. Both forms are strongly inhibited by dipyridamole and 6-mercaptopurine.

INTRODUCTION

The activity of the degradative enzyme cyclic 3′,5′-nucleotide phosphodiesterase is critical for the regulation of a variety of metabolic phenomena. The possibility that there might be more than one form of 3′,5′-nucleotide phosphodiesterase in a single cell has been first suggested from the kinetic data established by Brooker *et al.*[1]. There is now increasing evidence for the existence, in most tissues investigated, of two molecular forms of the enzyme, one with higher, the other with lower affinity for cyclic adenosine 3′,5′-monophosphate (cyclic AMP)[2–5].

Electrophoresis followed by specific staining of phosphodiesterase provides an interesting approach of the problem of molecular heterogeneity of this enzyme, not only in different tissues, but also within a single cell type[6–9]. Song and Cheung[10] have recently shown that human platelets possess a high cyclic AMP phosphodiesterase activity. The platelets thus represent the most convenient source of phosphodiesterase of human origin.

We provide here evidence for the presence in human blood platelets of two distinct molecular forms of phosphodiesterase, differing by their affinity for cyclic

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AMP, their electrophoretic mobility, their thermostability and their sensitivity towards inhibition by aminophylline.

MATERIAL AND METHODS

Chemical and reagents

Nucleotides were purchased from Sigma, Calbiochem and Boehringer, Mannheim. Cyclic-[8-^{3}H]AMP (21 Ci/mmole) was from C.E.A., Saclay, France. Snake venom *(Ophiophagus Hannah)* was obtained from Sigma; myokinase, pyruvate kinase and lactate dehydrogenase were products of Boehringer-Mannheim. Imidazole and aminophylline were purchased from Merck. Other drugs were gifts from Boehringer-Ingelheim (dipyridamole), Geigy (imipramine and opipramol) and Wellcome (6-mercaptopurine). Exchange resin AG1-X2 (200–400 mesh) was from Biorad, and hydrolyzed starch was from Connaught Medical Research Co.

Preparation of platelet extracts

Platelets were prepared from human blood donors according to the method of Caen *et al.*[11]. Careful microscopic checks showed that platelets were free from erythrocyte and leukocyte contamination. The pellet could be kept frozen at −20 °C for weeks without appreciable loss of phosphodiesterase activity. Extracts were prepared by homogenizing the sedimented platelets in a Potter–Elvehjem apparatus with 3 vol. of cold 50 mM Tris–HCl buffer, pH 7.4, containing 2 mM $MgSO_4$. After centrifugation at 33 000 × *g* for 30 min, the supernatant containing most of the activity was the source of enzyme for all the experiments described.

Phosphodiesterase assays

The enzyme activity was measured by two procedures. In Assay I the optical method of Cheung[12] was used. After incubation of the extract with cyclic AMP for 30 min at 37 °C the reaction was stopped by boiling for 3 min, and the amount of 5′-AMP produced was estimated enzymatically in a system containing phosphoenolpyruvate, ATP, NADH and an excess of rabbit muscle myokinase, pyruvate kinase and lactic dehydrogenase[12]. In the blank the mixture containing the platelet extract was boiled for 3 min prior to addition of cyclic AMP. In control experiments the recovery of 5′-AMP added to the first incubation step was 100%, thus indicating the absence of interfering reaction.

Assay II consisted in the radioactive method described by Thompson and Appleman[13]. In the first step the extract was incubated for 10 min at 37 °C with ^{3}H-labeled cyclic AMP to provide 100 000 cpm per reaction and various amounts of unlabeled cyclic AMP. The reaction was stopped by boiling for 3 min. In the second step 0.1 mg of snake venom *(Ophiophagus Hannah)* was added to convert the 5′-AMP formed into adenosine and phosphate. After addition of AG1-X2 resin and centrifugation the unreacted cyclic AMP remained adsorbed on the resin and the supernatant containing the non-adsorbed radioactive adenosine was counted. The blank, in which the platelet extract had been omitted, was processed similarly. In the first step of both assays the amount of platelet protein was 0.3–0.5 mg in a final volume of 0.5 ml. The incubation times allowed measurements of the initial rate of the reaction at any substrate concentration in both systems.

The protein content was estimated by the method of Lowry *et al.*[14] and the phosphodiesterase activity expressed in nmoles of cyclic AMP hydrolyzed per min and per mg protein.

Electrophoresis

Electrophoresis with specific staining for phosphodiesterase was performed in starch gel according to the method of Monn and Christiansen[6], in which the 5'-AMP formed is linked to the oxidation of fluorescing NADH into non-fluorescing NAD^+ through adenylate kinase, pyruvate kinase and lactate dehydrogenase. The electrophoresis was carried out at 4 °C for 5 h at 6 V/cm. The staining mixture was that originally described by Monn and Christiansen[6] with 4.5 mM imidazole added. After incubation at 37 °C for 20–30 min, dark bands on the fluorescing background were visible, representing cyclic AMP phosphodiesterase. Photographs were taken on Polaroid film type 107 at different intervals under long-wave ultraviolet light through a yellow filter. Controls were routinely done by omitting cyclic AMP in the staining mixture.

RESULTS

Kinetic studies

Determination of K_m for cyclic AMP. The affinity for cyclic AMP was studied by varying the substrate concentration between 4 mM and 0.2 μM. Identical results were obtained with both Assay I and Assay II, indicating the absence of interference of 5'-nucleotidase and adenylate deaminase in the reaction. The Lineweaver–Burk plot showed two distinct straight lines with different slopes, indicating the existence of two apparent K_m and V values (Fig. 1A). The high K_m was $5 \cdot 10^{-4} \pm 2 \cdot 10^{-4}$ M with a corresponding V of 50 nmoles/mg protein/min; the low K_m was $5 \cdot 10^{-5} \pm 2 \cdot 10^{-5}$ M with $V = 10$ nmoles/mg protein per min.

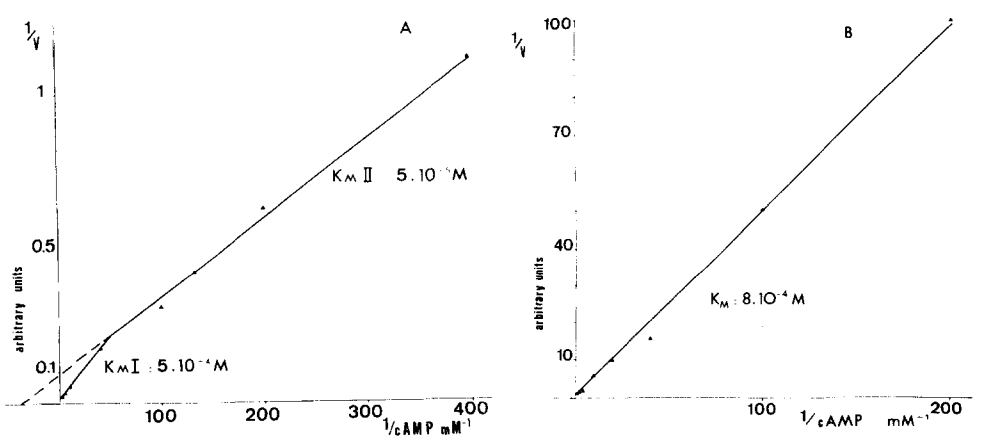

Fig. 1. Double-reciprocal kinetic plots of cyclic AMP hydrolysis by platelet phosphodiesterase. A, determination of low and high K_m values. Identical values were obtained both with the optical method[12] (range of cyclic AMP: 4 mM–2.5 μM) and with the radioactive assay using ^{3}H-labeled cyclic AMP[13] (range of cyclic AMP: 2 mM–0.2 μM). B, Lineweaver–Burk kinetic plot observed with platelet extracts pre-heated at 50 °C for 30 min and centrifuged.

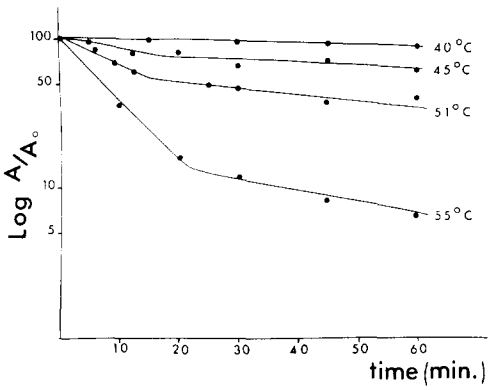

Fig. 2. Thermostability of platelet phosphodiesterase. After heating, the residual enzyme activity was assayed at 37 °C (see text).

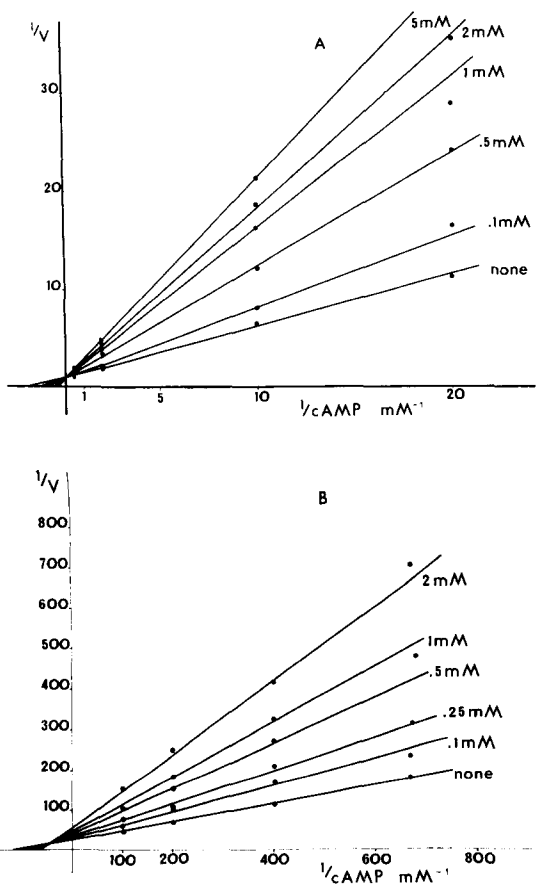

Fig. 3. Inhibition of platelet phosphodiesterase by aminophylline at (A) high concentration (2 mM–0.05 mM) and (B) low concentrations (10 μM–1.5 μM) of cyclic AMP.

Heat stability. The supernatant of platelet extracts containing 12–15 mg protein/ml was heated at 40, 45, 50 and 55 °C for different periods of times. As displayed in Fig. 2, a biphasic curve of thermal inactivation was obtained, indicating the presence of two components with different thermostability. When the substrate affinity was studied after heating the platelet extract at 50 °C for 30 min, only one apparent K_m for cyclic AMP was found ($8 \cdot 10^{-4}$ M) (Fig. 1B). This value corresponds to the high K_m found in nonheated extracts.

Effect of aminophylline. Methylxanthines, such as theophylline, are well-known inhibitors of cyclic AMP phosphodiesterase[15]. The type of inhibition of aminophylline, a soluble derivative of theophylline, was examined over a wide range of cyclic AMP concentration. On a Lineweaver–Burk plot it appears that at high concentrations of cyclic AMP the inhibition is strictly competitive (Fig. 3A). At low cyclic AMP concentrations the curves display the pattern of a mixed-type inhibition (Fig. 3B).

Electrophoretic studies

The electrophoretic pattern of platelet cyclic AMP phosphodiesterase at pH 7.7 consists of two distinct bands with unequal intensity (Fig. 4). The less anodic band

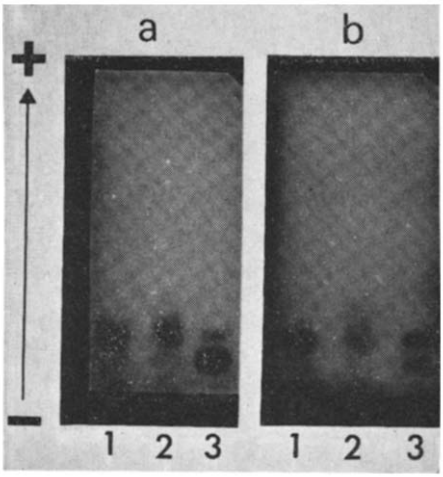

Fig. 4. Starch gel electrophoresis of rat liver (1), rat kidney (2) and human platelet (3) 1:4 extracts. After electrophoresis the gel was cut into two halves and stained for phosphodiesterase (see text) without aminophylline (a) and with 5 mM aminophylline in the reagent mixture (b), respectively.

(Band I) is stronger than the most anodic band (Band II) which becomes visible with some delay. In the absence of cyclic AMP no band was seen. This pattern is very peculiar to platelets since none of the investigated human tissues displayed the band I, with the only exception of leucocytes (Kaplan, J.-C., unpublished). In contrast, a band with a mobility identical to the Band II of platelets is predominant in soluble extracts of many human tissues, including liver, heart, lung, muscle, spleen, fat, kidney, pancreas, adrenals, pituitary, but not of brain[9].

The respective affinity for cyclic AMP of each electrophoretic form was investigated by varying the concentration of cyclic AMP in the developing mixture applied

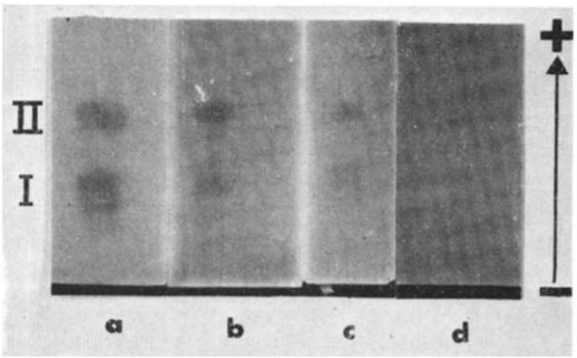

Fig. 5. Substrate affinity of the two electrophoretic fractions of platelet phosphodiesterase. Electrophoresis was performed as described under Material and Methods, except that various concentrations of cyclic AMP were incorporated in the reagent mixture (a, 2 mM; b, 1 mM; c, 0.5 mM; d, no cyclic AMP).

to the gel. As the concentration of cyclic AMP was decreased, the intensity of Band I, which is stronger at 2 mM cyclic AMP, became progressively weaker, while Band II was less affected (Fig. 5). These results suggest that Band I has a lower affinity for cyclic AMP than Band II.

The effect of heat denaturation upon the electrophoretic pattern was also investigated. In platelet extracts which had been heated at 50 °C for 30 min prior to electrophoresis, Band II was no longer visible (Fig. 6), thus confirming the difference already found in the kinetic study.

The effect of several phosphodiesterase inhibitors was studied by incorporating each effector in the staining mixture applied to the gel. 5 mM aminophylline inhibited partially Band I, but did not affect Band II (Fig. 4). Two tricyclic antidepressants, imipramine (1 mM) and opipramol (1 mM), known to affect only partially the platelet phosphodiesterase activity[9], had the same effect as aminophylline, *viz.* a partial inhibition of Band I without effect upon Band II. Strong inhibitors of the platelet phosphodiesterase, such as 6-mercaptopurine (1 mM) and dipyridamole (10 μM)[9], were found to inhibit completely both Band I and II.

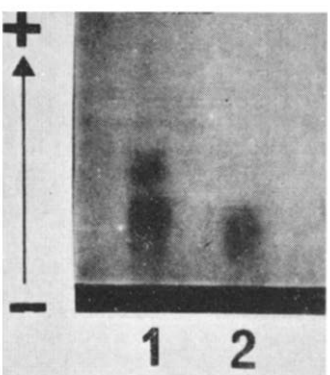

Fig. 6. Differential thermal sensitivity of the two electrophoretic forms. 1, nonheated platelet extracts; 2, platelet extracts heated at 50 °C for 30 min prior to electrophoresis.

DISCUSSION

The data presented here confirm the existence in human platelets of a phosphodiesterase exhibiting two K_m values for cyclic AMP differing by one order of magnitude. Our K_m values ($5 \cdot 10^{-4}$ M and $5 \cdot 10^{-5}$ M) are close to those reported by Song and Cheung[10]. Moreover, by combining electrophoretic investigations with kinetic studies (K_m determination for cyclic AMP, type of inhibition by aminophylline and thermo-inactivation) we have characterized each of these two species. The slow moving component (Band I) corresponds to the "high K_m" enzyme, which is also thermostable at 50 °C. This species is competitively inhibited by aminophylline. The fast moving component (Band II) corresponds to the "low K_m" enzyme, which is also thermolabile at 50 °C. An accurate analysis of the type of inhibition by aminophylline of the "low K_m" enzyme is difficult to establish by kinetic studies because of the interference of the "high K_m" enzyme. However, after electrophoresis, it is clear that the fast-moving low K_m component is less sensitive to aminophylline.

From our results, it is not yet possible to decide whether or not these two forms are in fact unrelated entities. Recent data have led to the conclusion that the two forms are differently sensitive to cyclic GMP activation[16], specific hormonal regulation[17,18] or even to different genetic modulation[19]. In contrast, that the two kinetic forms of the enzyme are interconvertible has been suggested in some systems[20,21]. Actually the multiplicity of molecular forms of cyclic nucleotide phosphodiesterase within a single tissue remains an open problem which may have a variety of non-exclusive solutions[5,22].

Work is now in progress to determine whether the two phosphodiesterase species we have found in human platelets represent entirely different enzymes or different oligomeric or conformational forms of the same protein.

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