CONCISE REPORT

Diadenosine 5′,5‴-p^1,p^4-Tetraphosphate Deficiency in Blood Platelets of the Chédiak-Higashi Syndrome

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Diadenosine tetraphosphate (AP_4A) is an unusual nucleotide found in a variety of cells, including platelets. It has been suggested that platelet AP_4A is stored in the dense granules and is metabolically inactive. We have studied the AP_4A content of blood platelets in two patients and three cattle with Chédiak-Higashi syndrome (CHS), a hereditary platelet defect with dense granule deficiency. Acid-soluble extractions of whole blood and platelets were neutralized. The adenosine triphosphate (ATP) level was measured by luminescence technique. To measure the AP_4A content, the neutralized extract was treated with phosphomonoesterase for removal of ATP. The AP_4A content was then measured by coupling the phosphodiesterase and luciferase reaction. The AP_4A content was 0.43 nmol/mg protein for normal human platelets and 0.004 nmol/mg protein for CHS platelets. The ATP/AP_4A ratio was 67 for normal and 3,023 for CHS platelets. The whole blood AP_4A was reduced by 89% in CHS patients who had only a slight decrease in ATP level (26% reduction). Similarly, bovine platelets with CHS showed a marked decrease of AP_4A content and a moderate reduction of the ATP level. The platelet ATP/AP_4A ratio was 351 and 3,133 for normal and CHS cattle, respectively. Results demonstrate a marked reduction of AP_4A in CHS platelets and suggest that AP_4A may be a useful marker for the measurement of dense granule content in platelets.

DIADENOSINE 5′,5‴-p^1,p^4-tetraphosphate (AP_4A) was discovered in 1966 as a product of the back-reaction of amino acid activation.[1,2] This compound has since been shown to be a ubiquitous component of living cells.[3] Normal human platelets contain AP_4A at a concentration of 0.42 nmol/mg protein, with the ratio of adenosine triphosphate (ATP) to AP_4A at approximately 100.[4] Some evidence suggests that platelet AP_4A may be stored in the dense granules. For example, incubation of platelets with ^3H-adenosine resulted in no significant incorporation of radioactivity into AP_4A.[4] Treatment of platelets with thrombin caused the complete release of AP_4A along with the release of the storage pool of other nucleotides.[4,5] However, the precise subcellular localization of AP_4A in platelets has not been directly determined.

The Chédiak-Higashi syndrome (CHS) is a rare autosomal recessive congenital disorder characterized by a partial oculocutaneous albinism, an increased susceptibility to infection,[6,7] and a bleeding tendency with the functional defect of the platelets[8,9] showing a marked decrease of the storage pool of adenine nucleotides and serotonin.[10–12] This syndrome also occurs in animal species such as mice, mink, cats, and cattle.[13–16] It has recently been reported that the AP_4A content in platelets is markedly decreased in animals with CHS.[17] We report herein the results of the studies on the AP_4A content of platelets from two human patients and three cattle with CHS.

MATERIAL AND METHODS

Human blood was drawn from two CHS patients and four healthy volunteers and mixed with 0.1 vol of 1.5% EDTA solution. Informed consent was obtained prior to blood donation. Characterization of CHS patients and cattle has been described previously.[9,16] Hemoglobin content of the blood was determined in a Coulter Counter (model S Plus, Coulter Electronics, Hialeah, Fla). To 0.1 mL of the whole blood was added 0.9 mL of phosphate-buffered saline (PBS) and then 1 mL of cold 7% trichloroacetic acid (TCA) solution. The samples were vortexed and kept on ice for 30 minutes with intermittent mixing. Platelet-poor plasma (PPP) was separated from the whole blood (1 mL) by centrifugation at 12,000 g for two minutes in a microfuge (Fisher model 59, Fisher Scientific Co, Pittsburgh, Pa) and mixed with an equal volume of 7% TCA. The remainder of the whole blood was centrifuged at 220 g for 15 minutes to obtain platelet-rich plasma (PRP). Platelets were pelleted by centrifugation at 800 g for 15 minutes, washed twice with PBS containing 0.15% EDTA, resuspended in the same buffer, mixed with an equal volume of 7% TCA and incubated on ice for 30 minutes with intermittent mixing. Protein content of the washed platelet preparation was measured by the method of Lowry et al.[18] Bovine blood was obtained from three CHS animals and five healthy animals. Bovine platelets were separated, washed, and then homogenized in a 5% cold TCA solution.

The TCA precipitates were centrifuged at 3,000 g for 30 minutes at 4 °C. The supernatant was neutralized by extraction of TCA twice with 0.5 mol/L tri-*n*-octylamine in Freon.[19] After vigorous mixing (by continuous vortexing for one minute), the reaction mixture was allowed to stand at room temperature for ten minutes to separate the aqueous (top layer) and amine-Freon phase. The aqueous phase containing nucleotides was used for determination of ATP and AP_4A content. Recovery of the trace amounts of ^3H nucleotides added to whole blood and platelet preparations was greater than 99%. The measurement of ATP was performed by a luminescence technique[20] using the commercial reagent and procedure according to the manufacturer's instructions (Packard).

To assay AP_4A, the neutralized extract (100 μL) was first incubated with 3 units of purified phosphomonoesterase (specific activity, 1,000 U/mg protein, Calbiochem-Behring) for 30 minutes at 37 °C. Between 98% to 99% of the ATP in the extract was degraded by this incubation step, following which the sample was kept in a 90 °C water bath for five minutes. The AP_4A content was then determined by coupling the phosphodiesterase and luciferase reactions using a luminometer (model 6100 Picolite, Packard, Downers Grove, Ill).[20] An aliquot of the sample (10 μL) was mixed

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Table 1. ATP and AP_4A Contents in Whole Blood and Platelets From Subjects With Chédiak-Higashi Syndrome

Sample	Source	ATP	AP_4A	ATP/AP_4A
Human whole blood	Control (n = 4)	5.21 ± 0.29; µmol/g Hb	3.15 ± 1.12 nmol/g Hb	1,818 ± 625
	Chédiak-Higashi Syndrome (n = 2)	3.84; µmol/g Hb	0.35 nmol/g Hb	10,971
Human platelets	Control (n = 4)	28.5 ± 3.3 nmol/mg protein	0.43 ± 0.06 nmol/mg protein	67.0 ± 7.8
	Chédiak-Higashi Syndrome (n = 2)	13.3 nmol/mg protein	0.0044 nmol/mg protein	3,023
Bovine platelets	Control (n = 5)	23.6 ± 10.1 nmol/10^9 platelets	0.078 ± 0.035 nmol/10^9 platelets	351 ± 158
	Chédiak-Higashi Syndrome (n = 3)	16.3 ± 4.0 nmol/10^9 platelets	0.006 ± 0.0036 nmol/10^9 platelets	3,133 ± 1,026

with 40 µL of luciferin-luciferase reagent (Packard) and the counts were monitored every 30 seconds (integration mode). After stabilization of the luminescence background due to residual ATP, 0.01 unit of phosphodiesterase (specific activity, 52 U/mg protein, Calbiochem-Behring, San Diego, Calif) was added and the 30-second counting was continued until reaching a maximum luminescence, usually completed in two minutes. The net change in luminescence due to the addition of phosphodiesterase was used to calculate the AP_4A content based on internal standards.

RESULTS

Results of ATP and AP_4A measurements in whole blood and platelet preparations are summarized in Table 1. The mean AP_4A content in control human whole blood was 3.15 nmol/g hemoglobin (Hb) (range 1.86 to 4.39 nmol/g Hb) and 0.35 nmol/g Hb for two CHS patients (0.39 and 0.31 nmol/g HB). There was an 89% reduction of blood AP_4A in these patients. In comparison, the ATP content in whole blood was only slightly decreased (26%) in the same patients (Table 1). The PPP from healthy volunteers contained less than 0.2% of the AP_4A and 0.01% of the ATP that were observed in the whole blood.

The average AP_4A content in human platelets was 0.43 nmol/mg protein, (range 0.353 to 0.485 nmol/mg protein) for four controls and 0.004 nmol/mg protein for two CHS patients (0.0037 and 0.005 nmol/mg protein). The ATP content in platelets was 28.5 and 13 nmol/mg protein for controls and patients, respectively. Thus, the ATP content in CHS platelets was reduced by 53% when compared to controls, whereas the decrease in AP_4A content was 99%. A similar pattern of a marked decrease in AP_4A content (92% reduction) and a modest reduction of ATP (31%) was observed in bovine platelets from three CHS cattle.

DISCUSSION

We demonstrate a marked reduction of AP_4A content in human and bovine platelets from subjects with CHS, which has the characteristics of a deficiency in the platelet dense granules.[10-12] The modest degree of reduction of ATP (31% to 53%) in the affected platelets, as shown herein, is consistent with a deficiency in the platelet dense granules in CHS because approximately 40% of the ATP in normal platelets is stored in these granules.[21] Our results of AP_4A measurement are in general agreement with the value observed by Flodgaard and Klenow in normal human platelets[4] and with those reported by Flodgaard et al in bovine CHS platelets.[17] The marked reduction (92% to 99%) of AP_4A in CHS platelets, as demonstrated in this study, together with previous results from other studies of the release and metabolism of AP_4A[4,5] strongly support a subcellular localization of AP_4A in the platelet dense granules. It appears that AP_4A may be a useful marker for the measurement of dense granule content in blood platelets.

All the AP_4A in blood appears to be virtually cell bound since PPP contains only a trace amount of AP_4A. It should be mentioned, however, that 90% of ^{32}P-labeled AP_4A added to normal PPP is destroyed during a ten-minute incubation at 37 °C (P.C.Z., unpublished observations, November 1984). Whole blood AP_4A in CHS patients is reduced by 89%, whereas in normal controls platelet AP_4A accounts for up to 86% of the whole blood AP_4A content (62% and 86%, respectively). The latter results suggest that platelets are the major source of blood AP_4A. In preliminary experiments we found that human granulocytes from two normal volunteers contained 0.75 nmol/10^9 cells of AP_4A (equivalent to 1% of the whole blood AP_4A) and 974 nmol/10^9 cells of ATP, respectively.

The physiologic role of AP_4A in the blood platelet has not yet been defined. AP_4A has been shown to stimulate the initiation of DNA synthesis in permeabilized resting tissue culture cells[22] and to bind with a subunit of DNA polymerase alpha.[23,24] Although the platelets show a low rate of protein synthesis,[25,26] normal human platelets contain only mitochondria and a trace amount of RNA, but no nucleus and no nuclear DNA.[27] Harrison et al[28] and more recently Luthje and Ogilvie[29] reported that AP_4A inhibited adenosine diphosphate (ADP)-induced platelet aggregation. We confirmed and expanded these observations.[30] In addition, Luthje and Ogilvie[5] have shown that platelets also contain diadenosine $5',5'''$-p^1-p^3 triphosphate (AP_3A), which is degraded to ADP by an enzyme in plasma and produces platelet aggregation.[29] It may be that AP_3A and AP_4A play some roles in the induction and dissolution of platelet aggregates in vivo.

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