BBA 75461

Mg^{2+}-DEPENDENT, $(Na^+ + K^+)$-STIMULATED ATPase OF HUMAN PLATELETS

PROPERTIES AND INHIBITION BY ADP

J. L. MOAKE, K. AHMED, N. R. BACHUR AND D. E. GUTFREUND

Biochemistry Section, Laboratory of Pharmacology, National Cancer Institute, Baltimore Cancer Research Center, 3100 Wyman Park Drive, Baltimore, Md. 21211 (U.S.A.)

(Received January 19th, 1970)

SUMMARY

The preparation and properties of a Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase from platelets are described. The enzyme system requires Mg^{2+} for activity, is stimulated maximally by the presence of Na^+ and K^+ in a ratio of 11.5/1, has a pH optimum 7.30–7.40, and is half-maximally inhibited by ouabain at a concentration of 0.15 μM. The K_m for ATP is approx. 0.4 mM. Enzyme activity is localized in both the "membrane" and "membrane-granule" subcellular fractions.

Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity is inhibited by ADP (which induces platelet aggregation) and by P_i. The inhibition by ADP is apparently not competitive with respect to ATP. Mg^{2+}-dependent, K^+-stimulated *p*-nitrophenyl phosphatase activity (a model for the externally-oriented K^+-phosphatase portion of Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase) is also inhibited by ADP. GDP, which does not induce aggregation of platelets, has no effect on the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity.

It is postulated that ADP interacts with the external platelet membrane surface to inhibit Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity with a resultant decrease in cation transport and membrane potential. This phenomenon may play a role in platelet aggregation.

INTRODUCTION

Membrane charges may determine the ability of platelets to aggregate in the presence of ADP, Ca^{2+}, and plasma protein(s)[1,2]. It is generally believed that an energy-dependent, coupled Na^+-K^+ exchange across cell membranes generates the transmembrane potential[3–5], and it is likely that a similar mechanism operates in platelets. The enzymic basis for Na^+-K^+ exchange is thought to be a Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase specifically oriented across cell membranes, and it is possible that a change in the activity of this ATPase system is related to platelet surface reactivity. We have, therefore, studied the properties of platelet Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase and have investigated the effect of the aggregating agent, ADP, on this enzyme activity.

MATERIALS AND METHODS

Chemicals

All solutions were prepared in distilled, ion-free water and checked for Na^+ and K^+ contamination by flame photometry. Mannitol was deionized by passage through a column of Dowex AG 501-X8, 20–50 mesh mixed-bed ion-exchange resin obtained from Calbiochem, Los Angeles, Calif. ATP, ADP, GDP, EDTA (versene) and *p*-nitrophenyl phosphate were obtained from the Sigma Chemical Co., St. Louis, Mo., and were converted to the Tris form as detailed previously[6,7].

Preparation of platelet lysate

Blood was drawn from normal donors into polyethylene bags containing 75 ml of anticoagulant solution (8 g/100 ml citric acid, 2.2 g/100 ml sodium citrate, and 2.45 g/100 ml dextrose). Glassware was siliconized and materials were kept at approx. 4° during the preparative procedure.

1 unit of platelet-rich plasma, obtained from 500 ml of whole blood, was centrifuged at 450 × *g* for 4 min to remove residual erythrocytes and leukocytes. The supernatant was centrifuged at 10800 × *g* for 10 min to sediment the platelets, which were washed in a medium of 0.25 M mannitol, 20 mM Tris, and 1 mM EDTA (pH 7.4 at 4°) and resuspended in 8 ml of mannitol–Tris–EDTA medium per unit of platelet-rich plasma. 72 ml of deionized water were added and the suspension was stirred on ice for 30 min, frozen in a dry ice–ethanol mixture and thawed. Microscopic examination revealed that almost all of the platelets were ruptured by this procedure. The lysed platelet suspension was centrifuged at 78000 × *g* for 30 min and the pellet obtained was suspended in 20 ml of mannitol–Tris–EDTA medium per unit of platelet-rich plasma and stored in 2-ml aliquots at −10°. The aliquots retained ATPase activity for several weeks. Only once-thawed preparations were used for enzyme analyses.

Assay of ATPase activity

Unless otherwise stated, the incubation mixture for the ATPase reaction consisted of 115 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 20 mM Tris–HCl buffer (pH 7.4 at 37°), and 3 mM ATP (Tris salt) in a final volume of 2.0 ml. Mg^{2+}-dependent, ($Na^+ + K^+$)-stimulated ATPase activity was measured as ΔP_i, the phosphate released in the presence of $Mg^{2+} + Na^+ + K^+$ *minus* that released in the presence of $Mg^{2+} + Na^+$. P_i released in the presence of $Mg^{2+} + Na^+$ (*i.e.* when KCl was omitted) was taken as a measure of Mg^{2+}-dependent ATPase activity. Reactions were carried out in polypropylene flasks at 37° for 30 min, and the quantity of platelet lysate added was always adjusted so that less than 10% substrate ATP was hydrolyzed. Reactions were initiated by the addition of platelet lysate and terminated with trichloroacetic acid (final concn. 5% (w/v)).

P_i released from ATP was measured by the method of FISKE AND SUBBAROW[8]. Protein was estimated by the method of LOWRY *et al.*[9].

In experiments where the effect of P_i on ATPase activity was studied, terminally-labeled [^{32}P]ATP (approx. 500000 disint./min) was added to the reaction mixture in addition to the usual amounts of ATP. ATPase activity in this case was measured according to the procedure used by GIBBS *et al.*[10].

The data reported in this study are based on many different platelet preparations. Specific activity figures for ATPase and *p*-nitrophenyl phosphatase activities varied by as much as 50% among different enzyme preparations; however, there were no differences in basic properties or in the effects of added inhibitors. With one lysate preparation, results did not vary more than 5% in different experiments. Unless stated otherwise, each table represents a typical experiment using one preparation, and has been verified by at least six observations with different preparations.

Assay of K^+-stimulated p-nitrophenyl phosphatase

The Mg^{2+}-activated, K^+-stimulated *p*-nitrophenyl phosphatase activity was measured by the method of AHMED AND JUDAH[11]. The reaction mixture consisted of 3–4 mM $MgCl_2$, 100 mM Tris–HCl buffer (pH 7.4 at 37°), 3 mM *p*-nitrophenyl phosphate as the Tris salt and 10 mM KCl in a final volume of 2 ml. Mg^{2+}-dependent phosphatase activity was determined by omitting KCl from the reaction mixture. Mg^{2+}-dependent K^+-stimulated phosphatase activity was estimated as ΔP_i by subtracting the activity in the presence of Mg^{2+} alone from that in the presence of $Mg^{2+} + K^+$.

Study of platelet subcellular components

Platelet lysates were centrifuged on continuous 30–60% sucrose density gradients according to the method of MARCUS *et al.*[12]. Two major fractions were obtained from the gradient: an upper, lighter region containing mostly membranes, and a lower, heavier band containing membranes, granules, mitochondria, and mitochondrial fragments (studied by electron microscopy). The fractions were analyzed for cytochrome *c* oxidase and acid phosphatase activities as described by MARCUS *et al.*[12], for glucose-6-phosphatase by the method of NORDLIE AND ARION[13], and for the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase by the procedure outlined above.

RESULTS

Properties of the platelet Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase

As with enzyme systems from other sources, Mg^{2+} was essential for activity. The platelet enzyme preparation exhibited considerable "basic" Mg^{2+}-dependent ATPase activity (in the presence of $Mg^{2+} + Na^+$). When both Na^+ and K^+ were present

TABLE I

PRESENCE OF Mg^{2+}-DEPENDENT, $(Na^+ + K^+)$-STIMULATED ATPase IN LYSED WHOLE PLATELET PREPARATIONS

Mg^{2+} concentration in the experiment shown was 3.0 mM. ΔP_i represents Mg^{2+}-dependent, $Na^+ + K^+$-stimulated ATPase activity as discussed in METHODS AND MATERIALS.

Cation concn.	*P_i split (μmoles P_i per mg protein per h)*	*ΔP_i*	*Stimulation (%)*
Na^+, 115 mM	1.28	—	—
Na^+, 115 mM + K^+, 10 mM	1.66	0.38	30

in addition to Mg^{2+}, a further increase in ATPase activity was observed, varying from 15 to 35 % with different platelet preparations (Table I). Maximal activity, achieved due to the combined effects of Na^+ and K^+, was observed when the concentrations of Na^+ and K^+ were 115 and 10 mM, respectively. The pH optimum for the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity was 7.30–7.40 and the K_m for ATP was approx. 0.4 mM. The platelet enzyme was half-maximally inhibited by ouabain at a concentration of 0.15 μM.

Subcellular Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity was more pronounced in the membrane layer, although activity was present in the membrane–granule–mitochondria fraction as well (Table II).

Time-course of the ATPase reaction

When the incubation time was prolonged or the platelet protein concentration increased, we detected no further significant increase in ΔP_i after approx. 10 % of the substrate ATP was hydrolyzed (Table IIIA and IIIB). Table IIIC shows that this phenomenon is not due to inactivation of the enzyme during incubation, since constant levels of both Mg^{2+}-dependent and Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activities were obtained when incubation time was prolonged to 60 min and platelet protein concentration correspondingly decreased to maintain ATP hydrolysis below 10 %. The reason for this phenomenon is not entirely clear, but could reflect product inhibition of enzyme activity *in situ*. In view of these findings, the concentration of added lysate protein in all experiments reported was adjusted so that the extent of ATP hydrolysis was less than 10 %.

Effects of ADP and P_i on platelet ATPase activities

We investigated the effects of added ADP over a wide range of concentrations on platelet lysate ATPase activities. ADP (varying from 0.1 μM to 1 mM) inhibited both the Mg^{2+}-dependent ATPase activity and the ATPase activity in the presence of $Mg^{2+} + Na^+ + K^+$ by approximately the same percentage (Table IV). Moreover, when ΔP_i figures were calculated according to the definition given in MATERIALS AND METHODS, a consistent inhibition of ΔP_i (Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated

TABLE II

LOCALIZATION OF ENZYME ACTIVITIES IN SUBCELLULAR PLATELET FRACTIONS

The specific activity of cytochrome *c* oxidase is defined as log unit cytochrome *c* oxidized per mg protein per min. The specific activity figures for acid phosphatase, glucose-6-phosphatase, and Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase refer to μmoles P_i split per mg protein per h. Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase is ΔP_i, as defined in MATERIALS AND METHODS.

Enzyme	*Location*	*Specific activity*	
		Membrane fraction	*Membrane–granule–mitochondria fraction*
Cytochrome *c* oxidase	Mitochondria	0	0.30
Acid phosphatase	Lysosomes	13.70	16.30
Glucose-6-phosphatase	Microsomes	0.67	1.81
Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase	Membranes	1.18	0.73

TABLE III

EFFECT OF INCUBATION TIME AND PROTEIN CONCENTRATION ON ATPase ACTIVITIES

In Expt. A the incubation time was varied while the platelet protein concentration was kept constant. In Expt. B the protein concentration was varied while the incubation time remained constant. In Expt. C both incubation time and protein concentration were varied so that the product of the two remained constant. Expts. A, B and C employed different platelet preparations. All other details were as described in METHODS AND MATERIALS.

Expt.	*Incubation time (min)*	*Protein (μg)*	*μmoles P_i split in presence of*		*ΔP_i (μmoles)*
			$Mg^{2+} + Na^+$	*$Mg^{2+} + Na^+ + K^+$*	
A	10	675	0.14	0.20	0.06
	15	675	0.23	0.33	0.10
	20	675	0.26	0.37	0.11
	30	675	0.40	0.57	0.17
	40	675	0.53	0.67	0.14
	45	675	0.60	0.78	0.18
B	20	405	0.22	0.35	0.13
	20	675	0.35	0.56	0.21
	20	945	0.51	0.72	0.21
	20	1215	0.65	0.85	0.20
C	15	816	0.38	0.50	0.12
	30	408	0.39	0.53	0.14
	45	272	0.39	0.52	0.13
	60	204	0.39	0.51	0.12

TABLE IV

INHIBITION OF ATPase ACTIVITIES BY ADP AND P_i

Figures in parentheses refer to percent inhibition of control specific activity or ΔP_i figures. ΔP_i is Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity as defined in MATERIALS AND METHODS. Specific activity figures refer to μmoles P_i split per mg protein per h. In Expt. B ATPase activities were estimated by measuring $^{32}P_i$ released from [γ-^{32}P]ATP as described in MATERIALS AND METHODS. Expts. A and B employed different platelet preparations.

Expt.	*Substance added to reaction mixture*	*Specific activity*		*ΔP_i*
		$Mg^{2+} + Na^+$	*$Mg^{2+} + Na^+ + K^+$*	
A	0 (Control)	3.04	3.47	0.43
	ADP, 0.1 μM	2.89 (5)	3.26 (6)	0.37 (14)
	ADP, 1 μM	2.53 (17)	2.87 (17)	0.34 (21)
	ADP, 10 μM	2.49 (18)	2.83 (18)	0.34 (21)
	ADP, 0.1 mM	2.13 (30)	2.43 (30)	0.30 (30)
	ADP, 1 mM	1.67 (45)	1.88 (46)	0.21 (51)
B	0 (Control)	3.24	3.72	0.48
	P_i, 0.1 mM	2.88 (11)	3.00 (19)	0.12 (75)
	ADP, 0.1 mM	2.28 (30)	2.52 (32)	0.24 (50)
	P_i, 0.1 mM + ADP, 0.1 mM	1.92 (41)	2.04 (45)	0.12 (75)

ATPase) was observed. If ADP had inhibited only the "basal" Mg^{2+}-dependent ATPase activity, no effect on ΔP_i would have been demonstrated. Thus, increasing ADP concentrations produced increasing inhibition of the Mg^{2+}-dependent,

$(Na^+ + K^+)$-stimulated ATPase activity, as well as inhibition of the "basal" Mg^{2+}-dependent ATPase activity. In thirty experiments employing fifteen different platelet donors the mean ΔP_i was 0.336 ± 0.022 μmole P_i split per mg protein per h in the absence of ADP and 0.228 ± 0.020 μmole P_i split per mg protein per h in the presence of 1 mM ADP ($P < 0.001$). No change in the inhibitory effect of 1 mM ADP was found when the Mg^{2+} concentration in the incubation medium was increased from 3 to 4 mM, ruling out the possibility that added ADP might exert its effect by binding Mg^{2+} in the medium. The inhibitory effect of 1 mM ADP on the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity (ΔP_i) was not altered when several different ATP concentrations were used (0.6, 1.0, 1.5, 2.0, and 3.0 mM), implying that the inhibition produced by ADP on ΔP_i was not competitive with respect to ATP.

GDP, which does not aggregate platelets[14], did not inhibit ATPase activities in the presence of either $Mg^{2+} + Na^+$ or $Mg^{2+} + Na^+ + K^+$ (and thus did not affect ΔP_i) even when the concentration of GDP was 1 mM.

It is also demonstrated in Table IV that P_i, at a concentration of 0.1 mM, produced an inhibitory effect on ATPase activities in the presence of $Mg^{2+} + Na^+$ and $Mg^{2+} + Na^+ + K^+$. The inhibition of ΔP_i (Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase) by P_i was somewhat greater than the inhibition produced by ADP at the same concentration. It should be pointed out that when the method of analysis was based on the measurement of ^{32}P hydrolyzed from [γ-^{32}P]ATP we observed a greater percentage inhibition of ΔP_i by 0.1 mM ADP, and found that ΔP_i was more sensitive to inhibition by ADP and P_i than was Mg^{2+}-dependent ATPase activity. Under these conditions ADP and P_i, at concentrations of 0.1 mM, produced a 75 % inhibition of ΔP_i. It is, therefore, conceivable that complete inhibition of ΔP_i (observed in Table III after 10 % ATP hydrolysis) was the result of a similar effect, since 10 % hydrolysis of 3 mM ATP would presumably yield 0.3 mM ADP and P_i.

Effect of ADP on p-nitrophenyl phosphatase activity

As in the case of ATPase, there was a considerable basic Mg^{2+}-activated *p*-nitrophenyl phosphatase activity which was further stimulated by K^+ (10–20 % in different platelet preparations). The Mg^{2+}-dependent, K^+-stimulated *p*-nitrophenyl phosphatase activity (ΔP_i) was inhibited by ouabain. There was an inhibitory effect of ADP on basal Mg^{2+}-dependent, *p*-nitrophenyl phosphatase activity, and in addition, an inhibition of Mg^{2+}-dependent, K^+-stimulated activity (ΔP_i, Table V). In seven experi-

TABLE V

INHIBITION OF *p*-NITROPHENYL PHOSPHATASE BY ADP

The concentration of Mg^{2+} was 4 mM and K^+, when present, was 10 mM. All other details were as described in MATERIALS AND METHODS.

Cations	*ADP concn. (mM)*	*P_i split (μmole P_i per mg protein per h)*	*ADP inhibition (%)*	*ΔP_i*	*Inhibition (%)*
Mg^{2+}	0	0.500			
$Mg^{2+} + K^+$	0	0.584		0.084	
Mg^{2+}	1	0.400	20		
$Mg^{2+} + K^+$	1	0.444	24	0.044	48

ments employing three different platelet donors the mean ΔP_i was 0.101 ± 0.024 μmole P_i split per mg protein per h in the absence of ADP and 0.040 ± 0.025 μmole P_i split per mg protein per h in the presence of 1 mM ADP ($P < 0.01$). Thus, both the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity and the Mg^{2+}-dependent, K^+-stimulated *p*-nitrophenyl phosphatase activities were inhibited by ADP.

DISCUSSION

Platelets are capable of actively transporting ions (Na^+ and K^+)[15] and organic compounds (5-hydroxytryptamine[16] and amino acids[17]) in a manner similar to other mammalian cells. By analogy with other cell systems, the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase system of the human platelet membrane is most likely involved in active cation transport. The fact that the ouabain sensitivity of the ATPase system parallels the ouabain sensitivity of Na^+-K^+ transport in platelets is a strong indication that this is so.

The general properties of the platelet Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase system are quite similar to those of the enzyme preparations from other tissues. These include a requirement for Mg^{2+} and the presence of Na^+ and K^+ in definite proportions, and a requirement for ATP as substrate. The specific activity and ouabain sensitivity of the platelet enzyme is similar to that reported for erythrocyte preparations[18].

Our investigation of the effects of low concentrations of ADP and P_i on the ATPase activities has shown that the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase (ΔP_i) is consistently and significantly reduced in the presence of these agents. The inhibition of ΔP_i by ADP is apparently not competitive. The ability of ADP to induce platelet aggregation in the presence of Ca^{2+} and plasma protein(s) has led BORN[19] to suggest that it may exert its effects by transiently complexing with platelet membrane components. It is known that platelets increase in volume when ADP-induced aggregation occurs[20]. Inhibition of platelet Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity by ADP, perhaps by an allosteric mechanism, could provide a basis for these observations. It is noteworthy that GDP, which does not cause platelet aggregation, has no effect on platelet Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase.

Adenine nucleotides are not transported into platelets[21], and ADP-induced platelet aggregation may result from ADP interaction with the external membrane surface. That ADP can inhibit the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase by affecting the external membrane surface is suggested by its inhibition of the Mg^{2+}-dependent, K^+-stimulated *p*-nitrophenyl phosphatase activity. This phosphatase activity is believed to be a model for the externally oriented, K^+-stimulated, ouabain-sensitive phosphatase component of the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase system[11].

Presumably, inhibition of Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase activity results in diminished Na^+ and K^+ transport and consequently, diminished platelet membrane potential. The altered surface charge distribution might produce changes in the stereochemical configuration of charged groups in the platelet membrane, and this "exposure of adhesive sites" could then result in platelet aggregation "through intermediate secondary bridges containing Ca^{2+}, fibrinogen, or other protein"[22].

A Ca^{2+}-Mg^{2+}-activated, actomyosin-like ATPase (thrombosthenin)[23] has been shown to be inhibited by ADP in intact platelet preparations[24]. The relative role of this "ecto-ATPase" and the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase in ADP-induced platelet aggregation merits further investigation.

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