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RELEASE REACTION AND ENERGY METABOLISM IN BLOOD PLATELETS WITH SPECIAL REFERENCE TO THE BURST IN OXYGEN UPTAKE*

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SUMMARY

1. Release of adenine nucleotides into the suspending medium was studied in suspensions of washed blood platelets in buffered saline in the presence of EDTA. Release-inducers were thrombin, latex particles and NaF.

2. 2-Deoxy-D-glucose and antimycin in combination caused pronounced inhibition of release with all agents. The metabolic blockers had less effect when used separately.

3. When thrombin or latex particles were added, most of the nucleotide release took place within 30 sec.

4. A burst in oxygen uptake occurred concomitantly with the release of adenine nucleotides. Blockers of mitochondrial respiration did not significantly inhibit the burst except in combination with inhibitors of glycolysis.

5. The release induced by NaF was slow and had a 6-min lag phase. NaF did not induce an oxygen burst.

6. It was concluded that both release and oxygen burst were dependent upon ATP supplied either from glycolysis or oxidative phosphorylation. The burst in oxygen uptake seemed not to be linked to ordinary platelet respiration. The possibility for a similarity to the increased oxygen uptake in phagocytizing leucocytes has been discussed.

INTRODUCTION

Platelets exposed to thrombin[1], collagen[2] or latex particles[3] release adenine nucleotides which cannot be labeled when the platelets are incubated with $^{32}P_i$ or [8-^{14}C]adenosine prior to release[4,5]. GRETTE[1] found that thrombin released adenine nucleotides in the presence of EDTA and citrate at 37°, whereas these chelators inhibited the reaction at 15°. Latex particles adhered to platelets in the presence of EDTA[6], while phagocytosis of the particles seemed to take place only in the absence of this chelator[3].

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The action of thrombin upon platelets induced changes in metabolism, as was illustrated by a transient increased production of lactic acid[7]. HUSSAIN AND NEWCOMB[8] reported a burst in oxygen uptake effected by thrombin and explained their findings as the result of opening of platelet membranes with consequent exposure of mitochondria to the oxygen in the surrounding medium. These findings have lately been challenged by DETWILER[9], who claimed that the effect was an artifact which was not demonstrable when a Clark electrode was applied, but was dependent upon the use of an uncoated platinum electrode. Although cyanide completely inhibited the respiration measured with the Clark electrode, the burst measured with an uncoated electrode was not affected by this inhibitor[9].

The present study is designed to test these claims. The role of platelet energy metabolism in release reactions induced with latex particles, thrombin and fluoride and a possible connection between burst and release reaction are also investigated. All assays are performed in the presence of EDTA and absence of extracellular divalent cations.

MATERIALS AND METHODS

Chemicals

Antimycin A, Type 3, 2-deoxy-D-glucose and *N*-ethylmaleimide were acquired from Sigma Chemical Co., St. Louis, Mo., U.S.A. Polystyrene latex particles (of diameter 1.099 and 0.234 μ) from Dow Chemical Co., Midland, Mich., U.S.A., were dialyzed extensively against distilled water to remove detergents. The thrombin used was Topostasin from Hoffman-LaRoche, Basle, Switzerland, made up as 50 National Institutes of Health units per ml 0.12 M NaCl with 25 mM Tris–HCl (pH 7.4).

Biological materials

Human blood was collected in 1/14 vol. of 0.077 M EDTA buffered to pH 7.4 with Tris. Erythrocytes and leucocytes were removed by 15 min centrifugation at 300 $\times$ *g* (maximum) and platelets separated from the plasma by centrifugation at 900 $\times$ *g* (maximum). The platelets were washed (once for the oxygraph experiments, twice for the release experiments) with 0.154 M NaCl (12.5 ml wash-solution was used for platelets from 40 ml blood). The sediment was easily resuspended in 0.154 M NaCl. All centrifugations took place in the cold. As a precaution 0.3 mM EDTA buffered to pH 7.4 with Tris was added during washing in most preparations as stated in the legends for tables and figures.

Aggregation was observed visually.

Protein content was determined by a biuret method[10].

Oxygen uptake was measured as the decrease in oxygen tension recorded with a Clark electrode, Model No. 4004, Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A. At 37° a 0.13 M NaCl solution was assumed to contain 0.4 μatom O_2 per ml at equilibrium with the atmosphere, and the setting of the instrument was adjusted accordingly. For the purpose of the study this was judged sufficiently accurate.

Adenine nucleotides were determined from the ultraviolet spectrum recorded against a waterblank with a Beckman DK 2A Ratio spectrophotometer. The basic non-specific absorption was subtracted to give the net adenine nucleotide absorption at 260 mμ. The amount was calculated from the absorption curve of a known sample of ATP in 0.5 M $HClO_4$.

RESULTS AND DISCUSSION

Washed platelets suspended in buffered NaCl released adenine nucleotides in the presence of thrombin, latex particles or NaF when incubated at 37°. The release did not require extracellular divalent cations and took place even in the presence of EDTA at concentrations where aggregation was not noticeable, thus supporting GRETTE's findings. However, inhibitors of mitochondrial respiration and of glycolysis, added in combination, suppressed the release reaction 70–80 % (Table I). Determination of total ADP + ATP released gave good indication that the major part of the 260 mμ-absorbing material consisted of these two compounds (Table I), in agreement with earlier reports[11,12]. The discrepancy found in the presence of inhibitors between the amount of ADP + ATP and the amount of 260 mμ-absorbing material may be caused by AMP, which will not register in the firefly method.

The major part of the release took place during the first 30 sec after addition of thrombin (Fig. 1). Rate of release depended on the amount of release-inducer (Fig. 2).

TABLE I

RELEASE OF ADENINE NUCLEOTIDES FROM PLATELETS INITIATED BY THROMBIN, POLYSTYRENE LATEX OR NaF

The reaction mixtures contained washed platelets (4–6 mg protein), Tris (25 mM buffered to pH 7.4 with HCl), EDTA (0.2–0.3 mM, buffered to pH 7.4 with Tris), and NaCl (0.1 M) in a total volume of 4 ml. In Expts. 2–4 the mixture was made isotonic by addition of NaCl (separate studies revealed no difference between results obtained under isotonic conditions and under conditions reported in Table I, Expt. 1). 2.5 N.I.H. units thrombin were added where indicated. 50 ng antimycin (anti) was added in 5 μl 95 % ethanol, 40 μl 0.5 M 2-deoxy-D-glucose (dGlc) in 0.154 M NaCl and 0.1 M NaF and 0.1 ml 10 % polystyrene latex (PLx) in water. Incubation for 35 min took place in 25-ml erlenmeyer flasks in a Dubnoff metabolic incubator at 37°, and the reaction was stopped by cooling in ice water. The samples were centrifuged in the cold for 15 min at 900 × *g* (maximal), and the supernatants were made 0.5 M with respect to $HClO_4$. Precipitate was removed by centrifugation (20 min at 4500 × *g* (maximal)) and the ultraviolet spectrum of the supernatant recorded. Numbers in parentheses are nmoles ADP + ATP per mg platelet protein as determined by the firefly method worked out by HOLMSEN, HOLMSEN AND BERNHARDSEN[13] (determinations kindly done by Dr. H. HOLMSEN and Dr. J. DAY). Platelets for Expts. 2–4 were prepared in the presence of EDTA.

Condition No.	*Addition at 0 min*	*Addition at 30 min*	*nmoles adenine nucleotides in the supernatant per mg platelet protein in the incubation medium*			
			Expt. 1	*Expt. 2*	*Expt. 3*	*Expt. 4*
1	No	No	5.1*	5.1 (4.4)	5.7 (3.6)	8.7
2	No	Thrombin	25.2	21.9 (20.0)	25.1 (15.3)	33.2
3	dGlc	Thrombin	13.7*		21.4 (11.8)	28.0
4	Antimycin	Thrombin			13.4 (7.1)	19.1
5	Antimycin, dGlc	Thrombin	7.6*	10.1 (4.3)	11.0 (4.3)	13.8
6	No	PLx (1.1 μ)	14.1		21.6	
7	No	PLx (0.234 μ)	20.8	22.0 (21.4)	26.0	29.2
8	Antimycin, dGlc	PLx (0.234 μ)	9.7			13.0
9	NaF (1.7 mM)	No	8.5			
10	NaF (10 mM)	No	28.2			
		Addition at 15 min				
11	No	NaF (10 mM)			26.2 (28.9)	31.8
12	Antimycin, dGlc	NaF (10 mM)			10.9 (4.1)	11.6

* Experiment performed in the absence of EDTA.

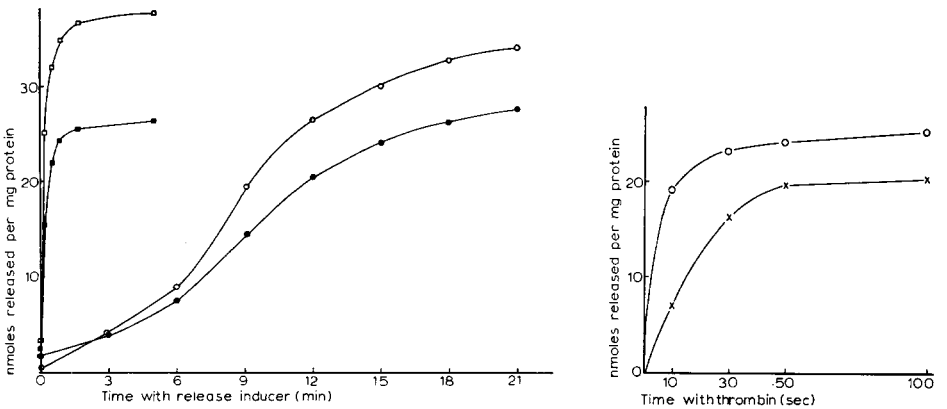

Fig. 1. Release of adenine nucleotides as a function of time after addition of release inducer. A mixture of washed platelets (1–1.5 mg protein per ml) in 25 mM Tris (pH 7.5), 0.3 mM EDTA and either 0.8 National Institutes of Health unit thrombin per ml or 10 mM NaF was made isotonic with NaCl and incubated by shaking at 37° in a Dubnoff metabolic incubator. 3-ml samples were taken out at intervals and added directly to vessels immersed in ice water, centrifuged and measured as described in Table I. The zero-point was defined when a sample equilibrated at 37° without release inducer was added to a vessel in ice bath containing thrombin. When NaF was used as release-inducer, the zero-point was defined by a sample taken out of the incubation mixture immediately after the addition of fluoride. The samples were read against the sample without release inducer (corrected for dilution by addition of 0.154 M NaCl). □—□ or ■—■, release started with thrombin; ○—○ or ●—●, release started with NaF. The black figures represent numbers from the same preparation. Platelets were prepared in the presence of EDTA.

Fig. 2. Release of adenine nucleotides as a function of time after addition of different concentrations of thrombin. Experiments performed as described in Fig. 1. ○—○, release induced by 0.8 National Institutes of Health unit thrombin per ml; ×—×, release induced by 0.08 N.I.H. unit thrombin per ml. No samples were taken at zero time. Platelets were prepared in the presence of EDTA; same preparation was used for both experiments.

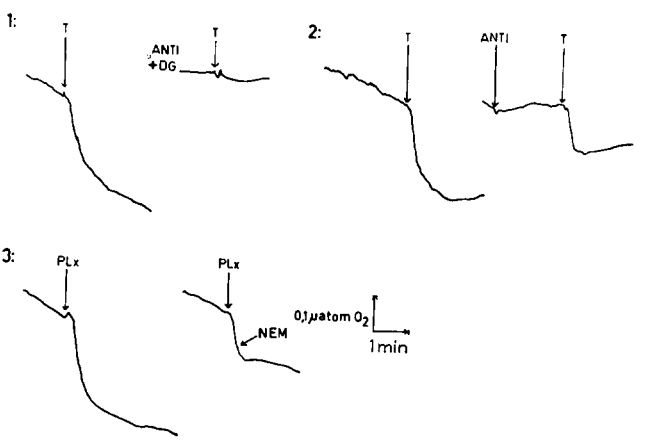

Fig. 3. Burst in oxygen uptake induced by releasing agents. Experiments performed as described in Table II. Abbreviations: ANTI, antimycin; PLx, polystyrene latex particles; DG, 2-deoxy-D-glucose, T, thrombin; NEM was 50 µl 83 mM *N*-ethylmaleimide. Three experiments are presented, demonstrating the effect of (1) a combination of antimycin and deoxyglucose on the burst, (2) the insensitivity of the burst to antimycin (400 ng added), and (3) the quick response to the addition of NEM during burst. In the antimycin experiment platelets were prepared in the presence of EDTA. In Expts. 1 and 3 were used 9–10 mg platelet protein; in Expt. 2, 13 mg platelet protein.

NaF caused release far more slowly, and a 6-min lag period could be observed (Fig. 1). Latex particles caused the same rapid release as thrombin.

Concomitant with the release of nucleotides was a burst in oxygen uptake visualized on the graph over oxygen tension in the suspension by a steep fall for 10–20 sec followed by a smoother curve of varying shape and length (Fig. 3). The burst took place in the absence of extracellular divalent cations and in the presence of EDTA, a 5-fold increase in EDTA concentration not reducing the size of the burst. However, it was blocked by the combined addition of antimycin and 2-deoxy-D-glucose (Fig. 3, Table II). It should be noted that deoxy-D-glucose alone only partially inhibited the burst. Antimycin alone had little or no effect. The observation by DETWILER[9] that KCN alone did not inhibit the burst was confirmed. When latex particles induced the burst, the effect of inhibitors was less striking than in the thrombin experiments, but followed the same pattern (Table II).

The effect of inhibitors upon the release of adenine nucleotides and upon the burst in oxygen uptake provides strong evidence that both phenomena are dependent upon continuous synthesis of ATP either by glycolysis or mitochondria-linked oxidative phosphorylation. The variation in sensitivity to either antimycin or deoxy-

TABLE II

BURST IN OXYGEN UPTAKE INDUCED BY THROMBIN OR POLYSTYRENE LATEX

Oxygen uptake was measured during magnetic stirring at 37° of a mixture of washed platelets (8–10 mg protein) suspended in 2 ml 0.154 M NaCl, 0.5 ml 0.1 M Tris–HCl (pH 7.4) and 20 μl 0.077 M EDTA adjusted to pH 7.4 with Tris. 250 ng antimycin was added in 5 μl 95% ethanol, polystyrene latex (PLx) in a 10% suspension in distilled water, and 25 μmoles 2-deoxy-D-glucose (dGlc) as a 0.5 M solution in 0.154 M NaCl. 5 N.I.H. units thrombin were added where indicated. The increased amount of O_2 in solution at 25° and 30° was corrected for in the calculations. Numbers in the table are for representative and reproducible experiments. Platelets for Expts. 2, 4 and 5 were prepared in the presence of EDTA.

Expt. No.		*Maximal increased rate of oxygen uptake (natoms/min)*	*Size of burst (natoms)*
1	Thrombin	51.0	19.0
	Thrombin + antimycin	42.0	22.0
	Thrombin + dGlc	33.0	15.0
	Thrombin + dGlc + antimycin	0.0	0.0
	Thrombin	51.0	19.0
2	PLx (0.234 μ), 0.4%	45.0	21.6
	PLx (0.234 μ), 0.4% + antimycin	34.0	16.0
	PLx (0.234 μ), 0.4% + dGlc	32.0	14.0
	PLx (0.234 μ), 0.4% + antimycin + dGlc	18.7	8.0
	PLx (0.234 μ), 0.4%	51.0	21.0
3	Thrombin	66.0	18.0
	PLx (1.1 μ), 0.4%	13.0	10.0
	PLx (1.1 μ), 1.1%	39.0	19.0
4	Thrombin 25°	4.7	4.8
	Thrombin 30°	8.8	9.2
	Thrombin 37°	23.2	16.0
5	PLx (0.234 μ), 0.4% 25°	2.5	1.2
	PLx (0.234 μ), 0.4% 30°	11.2	11.0
	PLx (0.234 μ), 0.4% 37°	32.0	18.3

glucose added alone is probably caused by different levels of substrates for glycolysis in the platelets.

The burst was completely blocked 15 sec after the addition of *N*-ethylmaleimide (Fig. 3), which might indicate that easily accessible –SH groups are required for the oxygen uptake.

This study supports the findings of HUSSAIN AND NEWCOMB[8] with the modification that the effect was found in the presence of EDTA. However, it fails to support their explanation, since the burst in oxygen uptake was shown not to result from an increased antimycin A-sensitive respiration. The fact that the oxygen burst was obtained even when a Clark electrode was employed does not accord with the findings of DETWILER[9]. However, his experiments were performed at 30°, and the burst is shown to be highly temperature-dependent. An increase in incubation temperature from 30° to 37° increased the rate of burst by a factor of 2.5 (Table II)*.

The oxygen burst induced by latex particles seemed to depend upon particle size, or possibly on number of particles, as did the release reaction (Tables I and II). The difference could be overcome by an increase in the concentration of large particles (Table II).

10 mM NaF did not induce an oxygen burst. This may be related to the slow releasing effect of this agent. FANTL[14] has studied other effects of F^- on platelets and has suggested that these were caused by an interaction with thiol groups. It seems more likely that a slow building up of deposits of magnesium fluorophosphate triggered the release, indicating in fact an intracellularly induced release reaction.

Antimycin inhibits platelet respiration by 90 % (ref. 15; see also Fig. 3). One may therefore conclude that the oxygen burst was not an expression of an immediate increase in the rate of mitochondria-linked respiration. The action of the releasing agent may have exposed sites or compounds which were subsequently oxidized. On the other hand there are some striking similarities with the phenomena described in experiments with phagocytizing leucocytes[16–18]. SBARRA AND KARNOVSKY[16] find no decrease in phagocytosis-induced oxygen uptake in the presence of antimycin, while the respiration of resting cells is strongly inhibited. IYER, ISLAM AND QUASTEL[17] describe some experiments which may indicate that the NADPH oxidase activity, which is induced by phagocytosis, is of a different nature from the one found in resting cells. ZATTI AND ROSSI[18] find that the granules of phagocytizing polymorphonuclear leucocytes are the sites of such a NADPH oxidase. The activity is KCN-insensitive and is the main cause of the increased oxygen uptake during phagocytosis. By testing the granules of phagocytizing and resting cells (including treatment with saponin), they provide evidence that the NADPH oxidase activity is indeed a result of phagocytosis. They also point out that the induced oxidase appears in the very early phase of phagocytosis[19].

The oxygen burst caused by inducers of release from platelets is more dramatic and immediate than the one described for phagocytizing leucocytes. This would be in line with the rapid course of the release reaction, as contrasted to the slower phagocytotic process.

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* Attempts by the author to obtain a burst with platelets in Hank's solution in the presence of small amounts of platelet-free plasma as prescribed by HUSSAIN AND NEWCOMB[8] were mostly unsuccessful. The formation of aggregates and fibrin clots may introduce particularly erratic results when magnetic stirring is employed rather than a moving uncovered electrode.

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