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OXYGEN CONSUMPTION OF HUMAN BLOOD PLATELETS

I. EFFECT OF THROMBIN*

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SUMMARY

The effect of thrombin on the oxygen consumption of washed human platelets was measured polarographically with the Clark oxygen electrode. The average basal respiratory rate was 18 ± 1.6 (mean $\pm$S.E.) natoms oxygen per min per 10^9 platelets. Thrombin (1.9 units/ml) caused a 4–13-fold increase in the rate of oxygen consumption (138 ± 14 (mean $\pm$S.E.) natoms oxygen per min per 10^9 platelets). The thrombin-stimulated increase of oxygen consumption was transient, lasting from 1 to 1.5 min before returning to the respiratory rate observed before the thrombin addition. Release of platelet constituents appeared to precede the stimulation of oxygen consumption. These results may provide a basis for explaining the discrepancy in the literature concerning the effects of thrombin on platelet respiration.

INTRODUCTION

The interaction between thrombin and platelets and the subsequent aggregation has been implicated as an important event in hemostasis [1, 2]. In vitro, thrombin is known to induce the release of various platelet constituents [3, 4], notably adenine nucleotides and calcium, both of which are believed to be involved in platelet aggregation [5, 6].

Thrombin also causes an increase of lactate production concomitantly with the release of adenine nucleotides and calcium [7]. In contrast, the thrombin effects on oxygen consumption of human platelets [8–13] and the role of energy metabolism associated with the thrombin-stimulated release reaction [14] are controversial subjects. Presumably, the controversy concerning the effect of thrombin on platelet

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respiration has resulted from the use of different techniques for determining the oxygen consumption as well as other variations in experimental conditions. The present study was undertaken to resolve this controversy and to investigate the relationship of aerobic energy metabolism to the thrombin-stimulated release reaction.

METHODS

Isolation of platelets

Platelets were isolated from venous blood obtained from normal adult males who had not taken any medication for the previous 10 days. The drawn venous blood, 240 ml, was mixed with 3.6 ml of 0.27 M EDTA (pH 7.2–7.4) and centrifuged at $1400 \times g$ for 4 min. The platelet-rich plasma was centrifuged at $2250 \times g$ for 12.5 min and the platelet pellet resuspended in about 200 ml of a buffer containing 140 mM sodium chloride, 25 mM Tris · HCl and 0.3 mM EDTA (pH 7.4). This suspension was centrifuged at $120 \times g$ for 10 min to sediment leukocytes and erythrocytes. The supernatant fluid was centrifuged at $2250 \times g$ for 12.5 min to obtain a platelet pellet which was resuspended in 8–10 ml of 140 mM sodium chloride, 25 mM Tris · HCl and 1.5 mM EDTA (pH 7.4) to yield a final count (phase microscopy) of $1.2 \cdot 10^9$–$1.3 \cdot 10^9$ platelets per ml. These suspensions contained less than 1 % contaminating leukocytes and erythrocytes. In some experiments the protein concentrations of the suspensions also were determined [15] and were proportional to the platelet counts. Preparative steps and storage of the platelet suspensions during polarographic determinations were done at 4 °C.

Oxygen consumption

Oxygen uptake was determined polarographically with the Clark oxygen electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, U.S.A.). 1 ml of the platelet suspension was preincubated at 37 °C for 2 min, the electrode inserted, forming an air-tight seal and recording begun. Additions to this suspension were done with microliter syringes through a capillary port in the cuvette. Details of the technique were essentially as described by Chappell [16] and Estabrook [17].

Release reaction

Samples of washed platelet suspensions were preincubated for two min at 37 °C prior to the addition of thrombin at a final concentration of 1.9 units/ml. After varying periods of incubation (5–300 s), all samples were cooled rapidly to 4 °C and centrifuged at $2250 \times g$ for 12.5 min. Perchloric acid (0.5 M) was added to the supernatant fluid and the precipitated protein was removed by centrifugation at $2250 \times g$ for 15 min. Adenine nucleotides and calcium were determined as described by Wolfe and Shulman [7].

MATERIALS

U.S. Standard Thrombin (human lot H-1) was provided by Dr D. Aronson, Bureau of Biologics, Food and Drug Administration. All other reagents were of highest quality available commercially. Glass-distilled or deionized water was used throughout.

RESULTS

A basal respiratory rate for washed human platelets of 18 ± 1.6 (mean $\pm$S.E.) natoms oxygen per min per 10^9 platelets was obtained from 75 assays in 13 experiments using 10 normal adult male blood donors. The variation of the basal rate between individual assays within a single experiment was less than 20 %. The addition of thrombin to human platelets caused a burst in oxygen consumption (Fig. 1). The maximum rate of oxygen consumption induced by thrombin will be designated "thrombin burst rate".

Thrombin, 1.9 units/ml, stimulated a 4–13-fold increase in the basal rate of oxygen consumption. In 13 experiments, using the first assay of each experiment, an average thrombin burst rate of 138 ± 14 (mean $\pm$S.E.) natoms oxygen per min per 10^9 platelets was obtained. The burst of oxygen consumption stimulated by thrombin was transient, lasting from 45 to 90 s before returning to the basal rate (Fig. 1). A delay of 8–10 s occurred after the addition of thrombin before the onset of the burst in oxygen uptake was detected. The response time of the electrode was less than 2 s; therefore, the delay of the oxygen burst after the addition of thrombin apparently was not an electrode artifact.

In 16 out of 20 experiments, the oxygen uptake after the thrombin-stimulated burst (post-thrombin rate, Fig. 1) did not differ significantly ($P < 0.7$) from the basal rate. In the other four experiments the post-thrombin oxygen uptake rate was 10–20 % greater than the basal rate.

In Fig. 1, the amount of oxygen consumption induced by thrombin is designated as "extent of burst". The extent of burst is a measure of the ability of thrombin to increase oxygen consumption above the basal level. In 13 experiments, the extent of burst was 36 ± 4 (mean $\pm$S.E.) natoms oxygen per 10^9 platelets. In view of the correlation ($r = 0.80$) between the thrombin-stimulated burst rate of oxygen consumption and the extent of the burst, all subsequent results are expressed only in terms of the thrombin burst rate.

The thrombin burst rate decreased with each subsequent assay during the

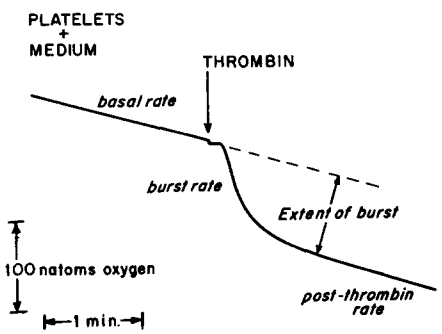

Fig. 1. A typical polarographic trace of basal platelet oxygen consumption and the burst of oxygen consumption induced by thrombin. The "burst rate" is the maximum rate of oxygen consumption induced by thrombin. The "extent of burst" is the amount of oxygen consumption induced by thrombin above basal respiration. The "post-thrombin rate" is the steady-state oxygen consumption after the thrombin-stimulated burst of oxygen consumption. Addition of thrombin (1.9 units/ml) is shown by the arrow.

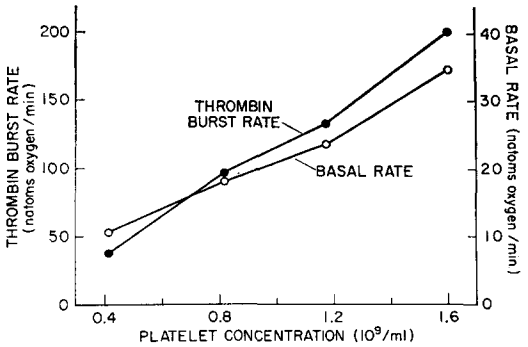

Fig. 2. Effect of platelet concentration on oxygen consumption. The thrombin concentration was 1.9 units/ml. Each point represents a single determination.

2.5–3 h of each experiment. Taking the first determination of the thrombin burst rate in each experiment as the maximum rate, the burst rate decreased from 20 to 60 % in 18 out of 20 experiments. Because the response of platelets to thrombin decreased during their storage, platelets were isolated within 70–90 min after blood was drawn. Individual oxygen determinations were initiated immediately and completed within 2.5 h. Control experiments disclosed that the diminished response was attributed to changes in the platelets during storage, and not to inactivation of the thrombin. Addition of heated thrombin (90 °C for six min) did not stimulate oxygen consumption; however, the subsequent addition of unheated thrombin to these same platelets elicited the typical burst of oxygen consumption. Addition of thrombin alone to the incubation medium also did not elicit oxygen uptake.

The magnitude of the thrombin-stimulated burst rate was proportional to the amount of thrombin used. It was possible to detect a minimal increase in oxygen consumption with a thrombin concentration of 0.2 unit/ml. However, in order to obtain a thrombin burst rate of sufficient magnitude for accurate quantitation, a thrombin concentration of 0.45 unit/ml and a platelet concentration of $1.5 \cdot 10^9$ platelets/ml were required.

As shown in Fig. 2, both the basal respiratory rate and the thrombin-stimulated burst rate were proportional to the platelet concentration. The parallel patterns of the thrombin burst rate and the basal rate versus platelet concentration are additional evidence that the burst of oxygen consumption induced by thrombin is not an artifact.

In these studies, an initial addition of thrombin caused the platelets to be refractory to subsequent additions of thrombin. This was shown by adding a submaximum amount of thrombin (0.5 unit/ml) that induced a small uptake of oxygen. Further additions of thrombin (1.9 units/ml) had no significant stimulatory effect on the oxygen consumption.

Release reaction. Release of adenine nucleotides and calcium induced by thrombin was about 60 % complete at 5 s and 90 % complete at 30 s after the addition of thrombin. The release of adenine nucleotides apparently preceded the onset of the thrombin-stimulated burst of oxygen consumption as shown by the data summarized in Fig. 3.

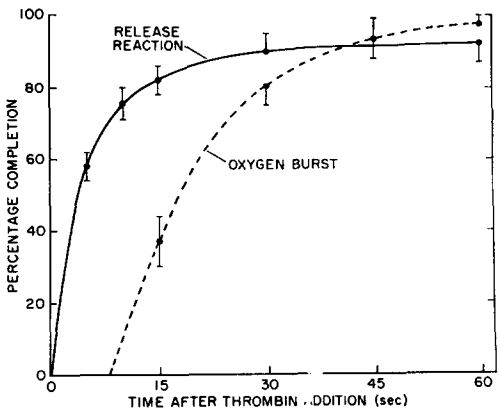

Fig. 3. Time course of the adenine nucleotide release compared to the thrombin-stimulated burst of oxygen consumption. The release reaction and the oxygen consumption studies were performed under similar experimental conditions with a final concentration of 1.9 units/ml of thrombin used throughout. The release reaction data is a summary of three experiments with platelet concentrations from $1.4 \cdot 10^9$ to $1.8 \cdot 10^9$ platelets/ml. The oxygen burst data is a summary of 17 determinations with platelet concentrations from $1.2 \cdot 10^9$ to $3.2 \cdot 10^9$ platelets/ml. The bars represent one standard deviation from the mean value.

DISCUSSION

Previous studies [8, 9, 11, 12] have shown that thrombin had no effect, or diminished the oxygen consumption of human platelets. In contrast, Mürer [13] reported that thrombin stimulated a burst in oxygen consumption. This observation was confirmed and extended in our studies. The investigators [8, 9] who employed a manometric technique would have failed to detect the thrombin-stimulated burst, since it is impossible to observe changes in oxygen consumption of short duration with this technique. The inability of Detwiler [11] and Kitchen and Newcomb [12] to observe an effect with thrombin on oxygen consumption may be attributed to the low platelet concentration used and a temperature-dependent effect on the burst [13].

In our laboratory, the use of platelet-rich plasma was unsatisfactory for determinations of oxygen consumption. Although the basal respiration of platelets could be measured in plasma, addition of thrombin led to clumping and highly erratic results. We recognize that the use of EDTA as a chelating agent to prevent platelet aggregation may introduce an undesirable parameter [14], although concentrations of EDTA were varied from 0.3 to 1.5 mM without any detectable difference in the thrombin-stimulated burst of oxygen consumption. However, the aggregation of platelets upon addition of thrombin when EDTA was omitted from the incubation medium precluded accurate determinations of oxygen consumption.

Our data indicate that the increase of the respiratory rate may be an effect secondary to the thrombin-induced release of platelet constituents, since the release reaction was shown to precede the onset of the thrombin-stimulated burst. The difference in time observed between the onset of the release reaction and the oxygen burst may represent a methodological artifact, particularly in regard to measurements of the release reaction. Our conclusion, however, is consistent with a recent kinetic

study by Detwiler and Feinman [18], which demonstrated that the onset of calcium release occurred immediately upon addition of thrombin. Further experiments are required to elucidate the factors involved in the control of energy metabolism in human platelets and the relationship of this control to the initiation and maintenance of the release reaction.

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