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Research Article

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Effects of Thrombin on the Potassium and ATP Content of Platelets *

PHILIP D. ZIEVE,† JAMES L. GAMBLE, JR., AND DUDLEY P. JACKSON

(From the Departments of Medicine and Physiology, Johns Hopkins University and Hospital, Baltimore, Md.)

The blood platelets have many of the characteristics of living cells including an active metabolism. As in other cells, the concentration of potassium within platelets is high in comparison to that of extracellular fluid and is similar to that of red blood cells (1). ATP also is present in high concentration within platelets (2) and is considered to provide the energy necessary for clot retraction, a process that requires metabolically active platelets (3). Unlike other cells, platelets are uniquely susceptible to the effects of thrombin, a proteolytic enzyme formed during clotting of blood. During coagulation, the platelets undergo a complex series of morphologic and biochemical changes (4), and there is a decrease in the concentration of some of the constituents of platelets, including potassium (1) and ATP (5). The present studies were designed to investigate some of the factors that influence changes in potassium and ATP within platelets.

Methods

All glassware with which platelets had contact was coated with silicone.[1] Blood was drawn from normal human donors through 15-gauge, uncoated, disposable metal needles attached to plastic tubing and was collected in tubes containing an aqueous solution of the disodium salt of EDTA as an anticoagulant (1 part EDTA: 9 parts blood). The final concentration of EDTA in blood was 0.005 M. Platelet-rich plasma was obtained by differential centrifugation (6). Unless specified otherwise, platelets from 3-ml portions of platelet-rich plasma were sedimented (6) and washed twice with and finally resuspended in 1.8 ml of Tris-buffered saline, pH 7.5 (6), containing potassium chloride (0.004 M), glucose (0.0055 M), and EDTA (0.0013 M). The washing procedures were performed at 4° C and required approximately 30 minutes. Platelet counts were performed on these suspensions by the method of Brecher and Cronkite (7). The final concentration of platelets was approximately 250,000 per mm^3. The washed platelets then were incubated without agitation at 37° C under various experimental conditions. After incubation, the platelets to be analyzed for potassium and ATP were washed once with Tris-buffered saline containing EDTA but no potassium or glucose. Those to be analyzed for sodium were washed once with Tris-buffered potassium chloride (0.155 M) containing EDTA. The final washing procedure was performed at 4° C and required approximately 10 minutes. The platelets then were sedimented by centrifugation at 22,000 *g* for 5 minutes at 4° C and lysed in 3 ml of a 3% solution of trichloroacetic acid. Potassium and sodium in the lysates were measured by use of a flame photometer employing an internal lithium standard. ATP, in most experiments, was measured after fractionation of the lysate on a Dowex-1 anion exchange column by use of a Beckman model DU spectrophotometer reading at 260 mμ (8). In other experiments ATP was measured either by the firefly luminescence method [2] or enzymatically in neutralized perchloric acid lysates of platelets by use of hexokinase and glucose-6-phosphate dehydrogenase (G-6-PD) (9). Radioactive potassium (K^{42}) [3] was measured by use of a well-scintillation detector. Thrombin [4] and trypsin [5] solutions were

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† Postdoctoral fellow, U. S. Public Health Service.

[1] General Electric silicone SC-87, Dri-Film.

[2] Assays kindly performed by Dr. William D. McElroy, McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Md.

[3] Obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

[4] Thrombin, topical. Parke, Davis and Co., Detroit, Mich.

[5] Trypsin, twice crystallized, salt-free, lyophilized, minimum activity 10,000 U per mg. Worthington Biochemical Corp., Freehold, N. J.

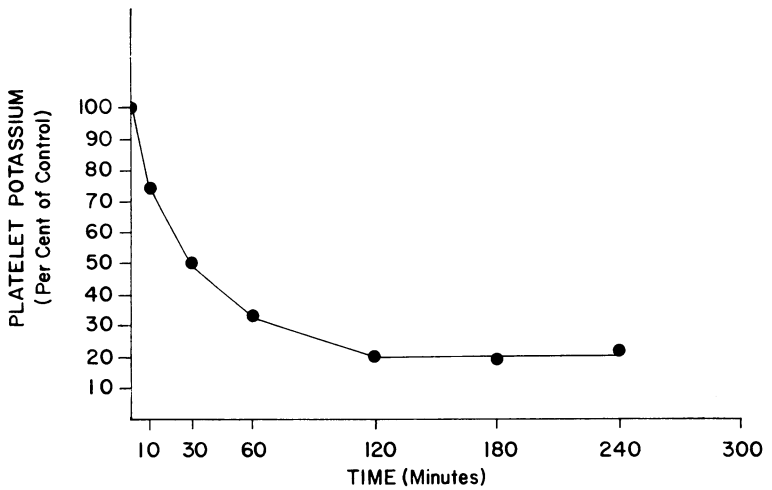

FIG. 1. NET LOSS OF POTASSIUM FROM PLATELETS INCUBATED IN POTASSIUM-FREE BUFFER. Results of a representative experiment are shown.

prepared from commercial products. Plasminogen was prepared by the method of Kline (10) and was activated with an equal amount, by weight, of streptokinase [6] (11). The plasmin preparation had an activity of 60 of Norman's caseinolytic U per mg of plasminogen as measured [7] by a modification (12) of a previously described method (13).

Results

Release of potassium from platelets. The potassium content of control platelets washed twice in Tris-buffered saline containing EDTA, glucose, and potassium and once in Tris-buffered saline containing EDTA was 89 (SD 23) μmoles per 10^{11} platelets in 12 experiments. Platelets washed three times at 4° C in potassium-free buffer had a similar content of potassium in two experiments. In 20 experiments, there was no significant change in the potassium content of platelets washed with and then incubated at 37° C for periods up to 4 hours in buffer containing EDTA, glucose, and potassium. In contrast, in four experiments, platelets washed twice with and then incubated at 37° C in buffer containing EDTA but no potassium were depleted of 25 to 50% of their potassium within 10 minutes and of 80 to 90% of their potassium within 2 hours when compared to control platelets incubated in buffer containing potassium. The presence or absence of glucose in the buffer did not influence the results. A representative experiment is summarized in Figure 1. Platelets that had lost potassium during incubation in potassium-free buffer for 2 hours did not lose additional potassium after addition of thrombin (100 U per ml). Studies of the release of potassium by various agents, therefore, were performed using platelets that were washed with and incubated in Tris-buffered saline containing EDTA, glucose, and potassium.

Washed platelets incubated with thrombin contained 35% less potassium than did controls. The decrease was maximal after 10 minutes and was the same between concentrations of platelets from 100,000 to 500,000 per mm^3 and concentrations of thrombin from 1.0 to 100 U per ml. Thrombin in the presence of calcium ions produced a greater net loss (64%) of potassium from platelets than did thrombin alone, and the loss again was maximal after 10 minutes. Platelets incubated with calcium chloride in the absence of thrombin were not depleted of potassium. Results of these studies are summarized in Table I. In six of the experiments, platelets incubated with thrombin (100 U per ml) contained, on an average, 27% more sodium than did controls, but the sodium content of platelets incubated with thrombin and calcium chloride (0.005 M) was the same as that of controls. The sodium content

[6] Streptokinase-streptodornase (Varidase), Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[7] Assays kindly performed by Dr. Philip S. Norman, Johns Hopkins Hospital, Baltimore, Md.

TABLE I

*Net loss of potassium from platelets by various agents**

Agent with which platelets were incubated (final concentration)		Time of incubation	Experiments	Mean decrease of potassium ± 1 SD
		minutes	*no.*	%
Thrombin	100 U/ml	10	20	35 ± 8
Thrombin + $CaCl_2$	100 U/ml + 0.005 M	10	20	60 ± 11
Trypsin	25 μg/ml	10	9	40 ± 13
Trypsin + $CaCl_2$	25 μg/ml + 0.005 M	10	8	63 ± 14
Plasmin	1.0 mg/ml	10	3	0
NaF	0.024 M	60	8	50 ± 8
NaF + Thrombin	0.024 M + 100 U/ml	60	6	67 ± 3
NaF + Thrombin + $CaCl_2$	0.024 M + 100 U/ml + 0.005 M	60	5	68 ± 13

* Platelets from 3-ml portions of platelet-rich plasma were washed twice with and then resuspended in 1.8 ml of Tris-buffered saline (pH 7.5) containing potassium (0.004 M), glucose (0.0055 M), and EDTA (0.0013 M). After addition of 0.2 ml of the agents indicated, the suspensions were incubated for varying periods. The platelets then were washed in potassium-free Tris-buffered saline, and the potassium content of the platelets was measured. Results are expressed as percentage of change from control platelets incubated in the absence of the various agents.

of control platelets was approximately 35 μmoles per 10^{11} platelets.

In five experiments, twice washed human red blood cells (50,000 to 600,000 per mm^3) incubated with thrombin (100 U per ml) or thrombin and calcium chloride (0.005 M) under the conditions of these experiments showed no change in content of potassium.

Platelets incubated with thrombin and calcium chloride tend to aggregate and cannot be counted accurately. To determine whether the decrease in concentration of potassium was due to a loss of platelets, the procedure was modified in two experiments. The platelets were lysed in 0.02 N HCl rather than in trichloroacetic acid after the period of incubation and washing. The concentrations of potassium and protein of the lysates were measured, and the concentration of potassium was related to that of protein. Platelets incubated with thrombin contained 45% less potassium per mg of platelet protein than did controls, and those incubated with thrombin and calcium chloride contained 66% less potassium.

Trypsin or trypsin and calcium chloride produced a net loss of potassium from platelets that was comparable to that produced by thrombin or thrombin and calcium chloride. Plasmin, in contrast, did not alter the potassium content of platelets even after incubation for periods up to 60 minutes. Sodium fluoride (NaF) produced a net loss of 50% of potassium from platelets; and sodium fluoride in the presence of thrombin, a net loss of 67% of the potassium. The addition of calcium chloride did not enhance the change of potassium in platelets produced by sodium fluoride and thrombin. Results of these experiments are summarized in Table I.

Uptake of radioactive K^{42} by platelets and the effect of thrombin. In six experiments, twice washed platelets from 3-ml portions of platelet-rich plasma were incubated at 37° C in Tris-buffered saline containing radioactive potassium (K^{42}) in addition to stable potassium, glucose, and EDTA. The concentration of stable potassium in the buffer differed with each experiment in order to maintain the amount of radioactivity (counts per minute) sufficiently high for accurate counting. This variation did not influence the results. After varying periods of time the platelets were separated from the buffer, washed once, and the radioactivity as well as the total potassium of the buffer and of lysates of the platelets was measured. The K^{42} in the platelets reached an apparent maximum within 60 minutes (Figure 2), and the average specific activity [K (counts per minute)/total K (mmoles)] of the potassium in the platelets was 64% of the specific activity in the buffer (Table II).

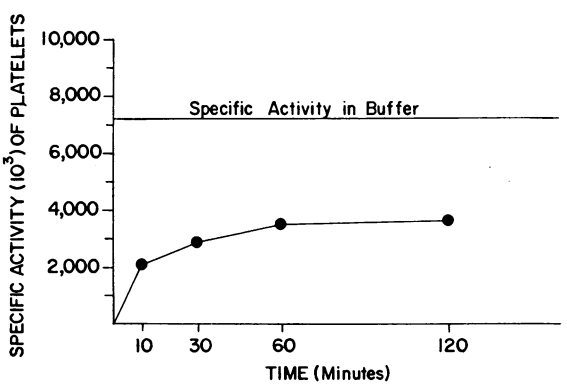

FIG. 2. UPTAKE OF RADIOACTIVE POTASSIUM (K^{42}) BY PLATELETS. Results of a representative experiment are shown. SA = K^{42} (counts per minute)/total K (millimoles).

TABLE II

*Uptake of radioactive potassium by platelets**

Additive	Specific activity† $\times 10^3$			Measured total K in platelets
	Buffer	Platelets	Platelets/Buffer	
				mmoles $\times 10^{-3}$
Buffer	5,525	3,550	0.64 (±0.17)	0.45
Thrombin 100 U/ml	5,350	6,113	1.14 (±0.10)	0.30
Thrombin 100 U/ml + $CaCl_2$ 5 mmoles/L	5,100	5,120	1.00 (±0.10)	0.16

* Twice washed platelets from 3.0-ml portions of platelet-rich plasma were resuspended in 1.8 ml of Tris-buffered saline containing radioactive potassium (K^{42}), stable potassium (0.004 to 0.009 M), glucose (.0055 M), and EDTA (0.0013 M). The suspensions were incubated at 37° C for 60 minutes after addition of 0.2 ml of the agents indicated (final concentrations are shown). The platelets then were washed once with potassium-free Tris-buffered saline and lysed, and the radioactivity (counts per minute) and total potassium of the platelet lysates and of the buffer were measured. Results are mean values of six experiments. The numbers in parentheses represent ± 1 SD.

† SA = K^{42} (counts per minute)/total K (millimoles).

In contrast, the reduced amount of potassium present in platelets exposed to thrombin or thrombin and calcium chloride was completely equilibrated with the potassium in the media in 30 minutes (i.e., the specific activity of K^{42} in platelets incubated with thrombin or thrombin and calcium chloride was approximately the same as that in the buffer). The accumulation of radioactive potassium (counts per minute) in platelets exposed to thrombin was the same as that of controls, but the total content of potassium was lower and the specific activity correspondingly higher in thrombin-treated platelets. These experiments are summarized in Table II.

Effect of thrombin on ATP content and clot retraction activity of platelets. The ATP content of control platelets washed twice in Tris-buffered saline containing EDTA, glucose, and potassium and once in Tris-buffered saline containing EDTA was 6.38 (SD 2.13) μmoles per 10^{11} platelets in 12 experiments in which ATP was isolated by anion exchange chromatography and assayed by measurement of absorption at 260 mμ, and was 6 μmoles in each of three experiments in which ATP was measured by the method of firefly luminescence.

In 11 experiments, platelets from 3-ml portions of platelet-rich plasma were washed twice with and resuspended in 1.8 ml of Tris-buffered saline containing EDTA, glucose, and potassium. The suspensions were incubated at 37° C for 30 minutes after addition of 0.2 ml of buffer, thrombin (final concentration, 100 U per ml), or thrombin and calcium chloride (final concentration, 0.005 M). The platelets then were washed once in Tris-buffered saline containing EDTA. The ATP content was measured by various techniques in lysates of a portion of the platelets, and the clot retraction activity of the remaining platelets was measured as described below. Results are expressed as percentage of change from control platelets incubated in buffer alone. Platelets incubated with thrombin or thrombin and calcium chloride contained, respectively, 61% (SD 14) and 75% (SD 8) less ATP than controls in seven experiments in which ATP was isolated by anion exchange chromatography and assayed by measurements of absorption at 260 mμ. In these studies other peaks coinciding with the elution characteristics of AMP and ADP showed a parallel decrease in 260 mμ absorbing material. The percentage of decrease in ATP of platelets exposed to thrombin or thrombin and calcium chloride was, in each instance, greater than the percentage of decrease of potassium. In three experiments ATP was measured by the method of firefly luminescence, and platelets incubated with thrombin contained 76, 77, and 82% less ATP and those incubated with thrombin and calcium chloride contained 90, 94, and 95% less ATP than controls. In one experiment ATP was assayed with use of hexokinase and G-6-PD, and platelets incubated with thrombin contained 73% less ATP and those incubated with thrombin and calcium chloride contained 88% less ATP than controls.

The clot retraction activity of platelets that had been depleted of ATP by incubation with thrombin was measured in seven of these experi-

ments. Portions (0.5-ml) of the suspensions of platelets that had been incubated with thrombin or thrombin and calcium chloride as described in the preceding paragraph were added to 0.5 ml of EDTA platelet-free plasma. The plasma was clotted by addition of calcium chloride (0.2 ml, 0.05 M) and thrombin (0.1 ml, 100 U) and incubated for 2 hours at 37° C in silicone-coated tubes. In each instance, clots containing platelets (final concentration, approximately 125,000 per mm^3) that had been depleted of 60 to 90% of their ATP by incubation with thrombin retracted maximally, and the degree of retraction was comparable to that of clots containing control platelets.

The ability of thrombin-treated platelets to support clot retraction also was measured semiquantitatively. In two experiments, twice washed platelets were incubated for 30 minutes with thrombin (100 U per ml) as described above. After incubation, the platelets were sedimented and resuspended in EDTA platelet-free plasma to give concentrations of platelets ranging from 30,000 to 700,000 per mm^3. The plasma samples were clotted by addition of calcium chloride and thrombin and the clots incubated in silicone-coated tubes at 37° C for 2 hours. Retraction of the clots containing platelets previously incubated with thrombin was comparable to that of clots containing platelets previously incubated in buffer alone. Thrombin-treated and control platelets produced maximal retraction at concentrations of platelets > 90,000 per mm^3 and partial retraction at lower concentrations of platelets. In a similar experiment, platelets incubated with thrombin were resuspended at various concentrations (45,000 to 350,000 per mm^3) in platelet-free plasma prepared from blood collected with sodium citrate as an anticoagulant (1 part 3.8% sodium citrate: 9 parts blood). The plasma then clotted within 2 minutes without the subsequent addition of thrombin or calcium chloride, and the clots retracted. Retraction again was maximal at concentrations of platelets of > 90,000 per mm^3 and partial at lower concentrations of platelets.

Discussion

The present studies provide further evidence of the unique susceptibility of platelets to the action of thrombin. The loss of potassium from platelets produced by thrombin apparently was not due to a general effect on all cells, since thrombin did not induce a loss of potassium from red blood cells. The mechanism of action of thrombin on platelets is not completely understood but involves, at least in part, proteolysis of a clottable protein (platelet fibrinogen) that appears to be associated with the platelet membrane (6). Platelets treated with thrombin no longer contain clottable protein (14). It has been suggested that the release of 5-hydroxytryptamine from platelets by thrombin is due to an effect of thrombin on the platelet membrane leading either to increased permeability (15) or to expulsion (release) of intraplatelet material (15, 16).

Our studies have shown that thrombin in the presence of divalent cation (calcium) induced a greater net loss of potassium from platelets than did thrombin alone. Divalent cations are known to influence other effects of thrombin on platelets (17) and also to affect the reaction of thrombin and plasma fibrinogen (18). The enhanced release of potassium from platelets by thrombin in the presence of divalent cation may be due to an alteration of the reaction of thrombin with platelet fibrinogen, but other possibilities exist.

Trypsin, another proteolytic enzyme, which affects plasma fibrinogen in a manner similar to thrombin (19) and which removes clottable protein (6, 14) and releases 5-hydroxytryptamine from platelets (15, 16), caused a loss of potassium from platelets comparable to that produced by thrombin. In contrast, plasmin, which splits different bonds in plasma fibrinogen than does thrombin (20) and does not release 5-hydroxytryptamine from platelets (14), did not affect the concentration of potassium in platelets. Recent studies have shown that the concentration of plasmin employed in our studies does not remove clottable protein from platelets (21).

Sodium fluoride, a potent inhibitor of glycolytic metabolism, was demonstrated in the present studies to produce a net loss of 50% of potassium from human platelets and to augment the effect of thrombin on the loss of potassium from platelets to approximately the same degree as divalent cation. Platelets treated with fluoride, in contrast to those treated with thrombin, are not capable of supporting clot retraction (22). Sodium fluoride, therefore, apparently disrupts metabolism of plate-

lets to a greater extent than does thrombin and presumably acts by a different mechanism.

The specific activity of K^{42} within platelets after incubation for 60 minutes was only one-half to two-thirds that of the surrounding medium, but the potassium remaining in platelets after treatment with thrombin was essentially equilibrated completely with the labeled potassium in the media. The results suggest that potassium may be distributed within two compartments in the human platelet. The data are compatible with the view that the potassium lost from platelets by exposure to thrombin is more inert or less freely exchangeable. Alternatively, however, factors influencing permeability may have produced an increase in the rate of exchange after exposure to thrombin.

The ATP content of platelets is diminished by thrombin in the presence of divalent cations (3, 16), but reports of the effect of thrombin alone on platelet ATP have been conflicting (3, 16, 23). We have consistently noted a profound fall of ATP in washed platelets exposed to thrombin alone or thrombin and calcium chloride. The ATP in platelets has been reported to supply the energy necessary for clot retraction (3). In the present studies, platelets depleted of 60 to 90% of their ATP by exposure to thrombin were capable, under appropriate conditions, of supporting clot retraction. It is possible that the small fraction of ATP remaining in platelets after exposure to thrombin was sufficient to supply the energy required for retraction. The ATP content of platelets exposed to thrombin in the presence of glucose increases transiently (3), and it is also possible that ATP was regenerated by thrombin-treated platelets that were resuspended in fresh plasma.

Summary

Washed human platelets incubated in potassium-free buffer lost approximately 80% of their potassium, but this loss did not occur from platelets incubated in buffer containing potassium. Thrombin induced a rapid net loss of approximately 35% of the potassium from platelets incubated in buffer containing potassium. Calcium augmented the effect of thrombin on potassium loss from platelets to approximately 60%. These agents did not cause a net loss of potassium from human red blood cells. Trypsin was similar to thrombin in its effect on the loss of potassium from platelets, but plasmin had no effect.

Studies employing radioactive potassium (K^{42}) revealed equilibration of one-half to two-thirds of the potassium in the platelets with that in the surrounding media in control studies but with all of the potassium remaining after treatment with thrombin. The results suggest that potassium may be distributed within two compartments in the human platelet.

Washed platelets exposed to thrombin or thrombin and calcium were depleted of 60 to 90% of their ATP. Platelets depleted of ATP by exposure to thrombin were capable of supporting clot retraction.

Addendum

Since this manuscript was prepared, Buckingham and Maynert (J. Pharmacol. exp. Ther. 1964, **143**, 332) have described the release of potassium from platelets by various agents including thrombin, trypsin, and sodium fluoride. These authors provide evidence that approximately 70% of the potassium in platelets is free within the cytoplasm.

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