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Disaggregation of aggregated platelets by apyrase from the tick, *Ornithodoros savignyi* (Acari: Argasidae)

BEN J. MANS[1], JAN COETZEE[2], ABRAHAM I. LOUW[1], ANABELLA R. M. GASPAR[1] and ALBERT W. H. NEITZ[1,*]
[1]*Department of Biochemistry, University of Pretoria, Pretoria 0002, South Africa*
[2]*Laboratory for Microscopy and Microanalysis, University of Pretoria, Pretoria 0002, South Africa*

Abstract. Apyrase, secreted by ticks during feeding, is a platelet aggregation inhibitor that functions as a regulator of the host's hemostatic system. This present study concerns the disaggregation effect of salivary gland apyrase from the tick *Ornithodoros savignyi*. Secondarily aggregated platelets, disaggregated by apyrase, exhibited a reversal of shape from a spherical (aggregated) form to a discoid form, reminiscent of reversible aggregation at low ADP concentrations in citrated platelet-rich plasma. However, they showed a dilatory open canaliculary system and an absence of granules indicating disaggregation after degranulation had taken place. In contrast, disaggregation by the fibrin(ogen)olytic enzyme, plasmin, showed that platelets degranulated, but retained a spherical form with numerous extended pseudopods. While thrombin had no effect on aggregation or clotting of platelets disaggregated with plasmin, it did activate those platelets disaggregated with apyrase and clotted the plasma. This is the first study to describe the disaggregating effects of tick derived apyrase on aggregated platelets. It also shows that apyrase can disaggregate platelets even after secondary aggregation and degranulation of platelets has taken place. Platelet aggregation is one of the main barriers encountered by ticks during feeding and counteraction of this process by ticks is an important factor for successful feeding.

Key words: apyrase, disaggregation, secondary platelet aggregation, tick

Introduction

Ticks are the most economically important ectoparasites of man and domestic animals. Annually several billion dollars are lost due to tick infestation, while the search for suitable tick control methods continues as ticks become resistant to acaricides (de Castro and Newson, 1993). The focus in recent years has been the identification of potential targets for vaccine development, although the understanding of naturally acquired immunity is still rudimentary

* Author for correspondence: Department of Biochemistry, Faculty of Biological and Agricultural Sciences, University of Pretoria, Pretoria 0002, South Africa (*Tel.:* +27-12-420-2906; *Fax:* +27-12-346-5302; *E-mail:* albert.neitz@bioagric.up.ac.za)

(Willadsen and Jongejan, 1999). An understanding of the mechanisms by which ticks control their environment can assist in the development of rational tick control methods.

Ticks are obligate parasites that depend on a blood meal for their survival. The defenses of the host, such as the immune and the hemostatic system must be suppressed or regulated during the feeding process to obtain a blood meal. Ticks control the hemostatic system of their hosts by secreting numerous bioactive compounds (Sauer *et al.*, 1995). Blood coagulation is prevented by inhibitors specific for key serine proteases involved in the blood coagulation cascade, like factor Xa (Gaspar *et al.*, 1996; Joubert *et al.*, 1998) and thrombin (Nienaber *et al.*, 1999). Vasoconstriction is prevented by vasodilatory agents such as prostaglandins and nitric oxide secreted by the tick (Ribeiro *et al.*, 1985; Schoombie *et al.*, 1998). Antagonists that prevent the action of different platelet agonists have been described. Collagen (Waxman and Connolly, 1993), thrombin (Nienaber *et al.*, 1999) and ADP (Mans *et al.*, 1998a) specific inhibitors of platelet aggregation have been identified.

Apyrase is an ATP-diphosphohydrolase (EC 3.6.1.5) that hydrolyzes ATP and ADP to AMP and phosphate (Leibecq *et al.*, 1962). Several functions have been ascribed to it depending on its localization. Apyrase associated with the endoplasmic reticulum and Golgi apparatus is implicated in the glycosylation of proteins (Gao *et al.*, 1999; Zhong and Guidotti, 1999) while it functions in nervous tissues as a neuromodulator (Wang and Guidotti, 1998). It is present in the epithelial linings of the circulation system of mammals where it is involved in the regulation of hemostasis by inhibition of platelet aggregation (ADP hydrolysis) and control of blood pressure (ATP hydrolysis) (Komozsyñski and Wojtczak, 1996).

Apyrase was also identified in all blood feeding organisms where it functions as an inhibitor of platelet aggregation during the feeding process. It has been identified in the hard tick *Ixodes dammini* (now referred to as *Ixodes scapularis*) (Ribeiro *et al.*, 1985) and the soft ticks *Ornithodoros moubata* (Ribeiro *et al.*, 1991) and *Ornithodoros savignyi* (Mans *et al.*, 1998a). Apyrase has also been purified from the mosquito *Aedes aegypti* (Champagne *et al.*, 1995), the assassin bug *Rhodnuis prolixus* (Sarkis *et al.*, 1986) and *O. savignyi* (Mans *et al.*, 1998b).

It was shown that apyrase inhibits ADP and collagen induced aggregation in citrated plasma, but not that induced by thrombin. Addition of apyrase to aggregated platelets also suggested that it could cause disaggregation (Mans *et al.*, 1998b). This disaggregating effect was investigated in more detail in the present study.

Materials and Methods

Purification of tick apyrase

Apyrase was purified as described previously (Mans *et al.*, 1998b). The apyrase preparation (15 ml) was concentrated by ultrafiltration (1,800 × *g*, 30–60 min) using an Ultrafree-15 centrifugal filter device with 10,000 Da cutoff (Millipore Corporation, USA) to approximately 1 ml giving an activity of 2 U/ml with ADP-Ca as substrate. The buffer composition was also changed during this step to 20 mM Tris–HCI, 0.15 M NaCl pH 7.2. Activity was determined as described previously (Mans *et al.*, 1998a). One unit of enzyme activity is defined as the amount of enzyme that causes the release of 1 μmole inorganic phosphate per minute at 37°C.

Platelet aggregation and disaggregation studies

Platelet aggregation was monitored with an aggregometer (Chronolog Corporation, Havertown, PA) using the photometric method (Born and Cross, 1963). Fresh blood was collected in Vacutainer tubes, containing citrated buffer solution (0.109 M), from human donors that had used no substances known to inhibit platelet aggregation, such as alcohol, garlic and aspirin for at least two weeks prior to donation. Platelet-rich plasma (PRP) was prepared by centrifugation (120 × *g*, 10 min) and collected in plastic tubes. Platelet-poor plasma (PPP) was prepared by further centrifugation (180 × *g*, 30 min). PRP was diluted with PPP to a final count of 300×10^9 platelets per liter using a Coulter counter. For aggregation, 400 μl of platelet preparation was added to siliconized glass cuvettes and incubated for 5 min prior to aggregation to monitor spontaneous aggregation. Platelet-poor plasma was set at 100% transmission and platelet-rich plasma at 0% transmission. Aggregation was induced by the addition of 20 μl ADP (Diagnostica Stago, USA) at a final concentration as indicated in the results. For disaggregation studies platelets were allowed to aggregate for 4 min until a plateau was reached, before either 0.1 U apyrase or 0.4 U plasmin (Boehringer Mannheim GmbH, Germany) were added to the aggregated platelets. The effect of thrombin (Ortho Diagnostic Systems, USA) on disaggregated platelets was determined by allowing platelet aggregation to take place for 4 min after which disaggregation was induced and allowed to proceed for 4 min. Thrombin (0.4 U) was then added to these disaggregated platelets and incubated for at least 2 min.

Preparation of platelet aggregates for electron microscope analysis

The disaggregated platelets and their respective controls were analysed by scanning (SEM) and transmission (TEM) electron microscopy. The platelet preparations were diluted with 0.1% glutaraldehyde solution (0.075 M phosphate buffer, pH 7.2) and centrifuged at 180 × *g* for 10 min. The supernatant was discarded and the pellet resuspended in 2.5% glutaraldehyde (0.075 M phosphate buffer, pH 7.2) and fixed for 1 h before pelleting at 180 × *g* for 10 min. The pellet was washed twice with 0.075 M phosphate buffer (15 min) after which it was fixed in 1% osmium tetroxide (water, 20 min) and washed twice in 0.075 M phosphate buffer (15 min). The platelets were then dehydrated sequentially with 30%, 50%, 70% and 100% (x3) ethanol. For SEM, a fraction of the prepared platelets was then critical-point-dried in CO_2 and sputter-coated with gold prior to SEM analysis. Platelets were analyzed on a JEOL 840 SEM using 5 kV and a magnification of 5,000 ×. For TEM analysis, the remaining platelets were sequentially infiltrated with 30%, 60%, 90% and 100% (x3) Quetol resin (23) diluted with 100% ethanol. Platelets were then embedded in 100% Quetol resin and polymerized at 60°C for 48 h. Sections (90 nm) were prepared with an ultramicrotome and mounted on copper grids, contrasted with uranyl acetate (5 min) and lead citrate (2 min). Samples were studied using a Philips 301 TEM.

Results

Disaggregation of aggregated platelets with plasmin or apyrase

To investigate the disaggregating effect of apyrase, three control experiments were done for comparative purposes. The first control consisted of platelets activated with low ADP concentrations (0.5 μM) to induce shape change and reversible aggregation (Figure 1a). Platelets not stimulated with any agonist were also analyzed and gave the same SEM and TEM patterns as that observed for platelets activated by 0.5 μM ADP (results not shown). The second control was to induce secondary aggregation of platelets with 5 μM ADP (Figure 1b). The third control was used to compare the effect of fibrinogenolysis with that of apyrase on aggregated platelets. Plasmin which is known to be a fibrin(ogen)olytic enzyme (Humphries *et al.*, 1993) was used to induce disaggregation by fibrinogenolysis (Figure 1c). Disaggregation of aggregated platelets with apyrase (0.1 U) is indicated in Figure 1d.

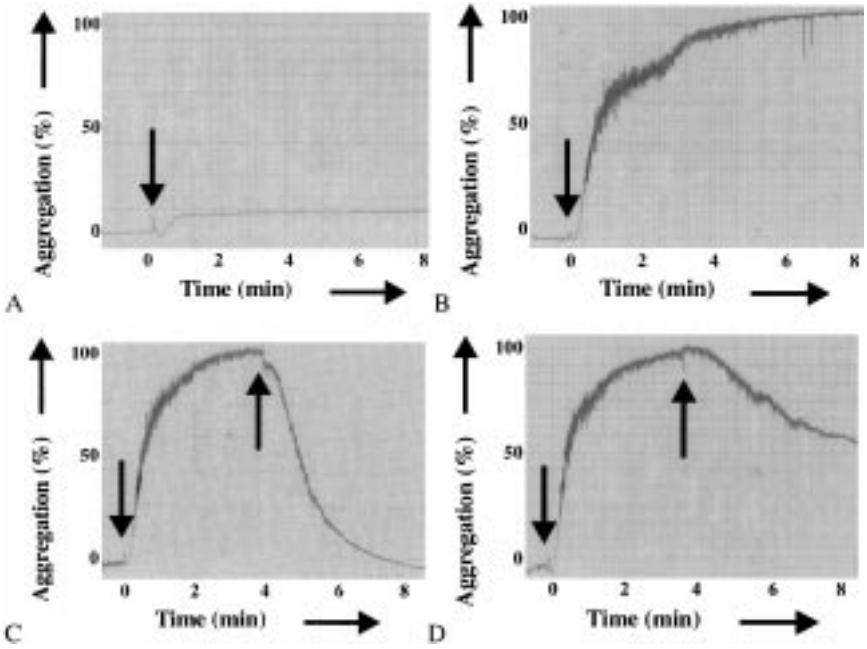

Figure 1. Aggregation tracings of platelets activated with ADP and disaggregated with either plasmin or apyrase. (A) Platelets activated with 0.5 μM ADP, (B) platelets aggregated with 5 μM ADP, (C) platelet aggregation induced with 5 μM ADP and disaggregated with 0.5 U plasmin after 4 min of aggregation. (D) Platelet aggregation induced with 5 μM ADP and disaggregated with 0.1 U apyrase after 4 min of aggregation. Arrows pointing down indicate initiation of aggregation with ADP and arrows pointing upward the addition of disaggregating agents.

The effect of thrombin on aggregated platelets disaggregated with plasmin or apyrase

To determine whether disaggregation caused by apyrase, might be due to a fibrinogenolytic activity, the effect of thrombin on platelets disaggregated with either plasmin or apyrase was tested. Previous studies have shown that apyrase did not inhibit thrombin induced aggregation (Mans *et al.*, 1998b), while fibrinogenolysis would inhibit aggregation induced by all agonists as it removes fibrinogen which serves as molecular anchor between platelets. Thrombin is known to activate and aggregate platelets in platelet-rich plasma after which clotting takes place rapidly, marked by oscillation above the maximum aggregation recorded with other agonists like ADP (Zucker, 1989). Platelets disaggregated with apyrase still showed a shape change and aggregated when thrombin was added followed by clotting (Figure 2a). Platelets disaggregated with plasmin showed no aggregation or clotting upon addition of thrombin, indicating complete fibrinogenolysis (Figure 2b).

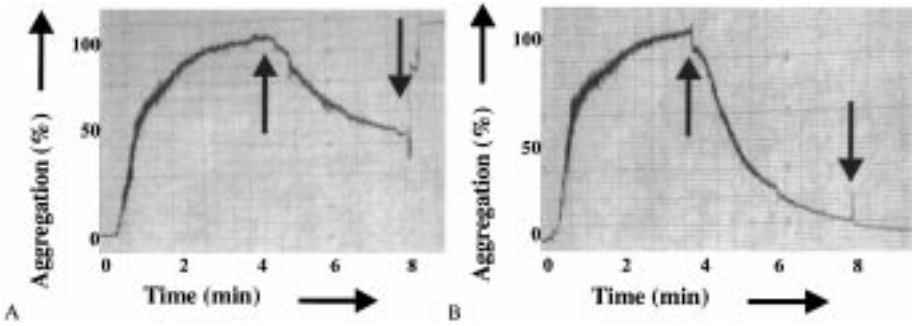

Figure 2. The effect of thrombin on platelets disaggregated with (a) apyrase or (b) plasmin. Platelets were aggregated and allowed to aggregate for 4 min before (a) apyrase (0.1 U) or (b) plasmin (0.5 U) were added (arrows pointing upwards). Disaggregation were allowed for 4 min after which 0.4 U of thrombin were added (arrows pointing downwards).

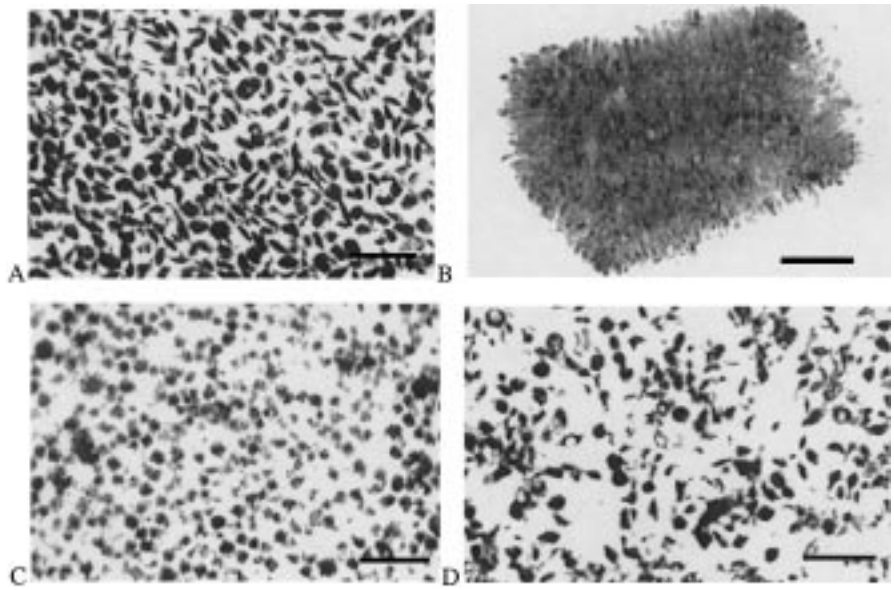

Figure 3. Light microscope analysis of aggregated and disaggregated platelets. (A) Platelets activated with 0.5 μM ADP, (B) platelets aggregated with 5 μM ADP, (C) platelets aggregated with 5 μM ADP and disaggregated with 0.5 U plasmin, (D) platelets aggregated with 5 μM ADP and disaggregated with apyrase (0.1 U). Scale bars indicate 10 μm.

External changes observed for aggregated and disaggregated platelets

The external change in platelet morphology during disaggregation was investigated using SEM and light microscopy. Platelets activated with 0.5 μM ADP showed that activation took place as indicated by the presence of pseudopods extending from the platelet surface (Figures 3a and 4a). The platelets did,

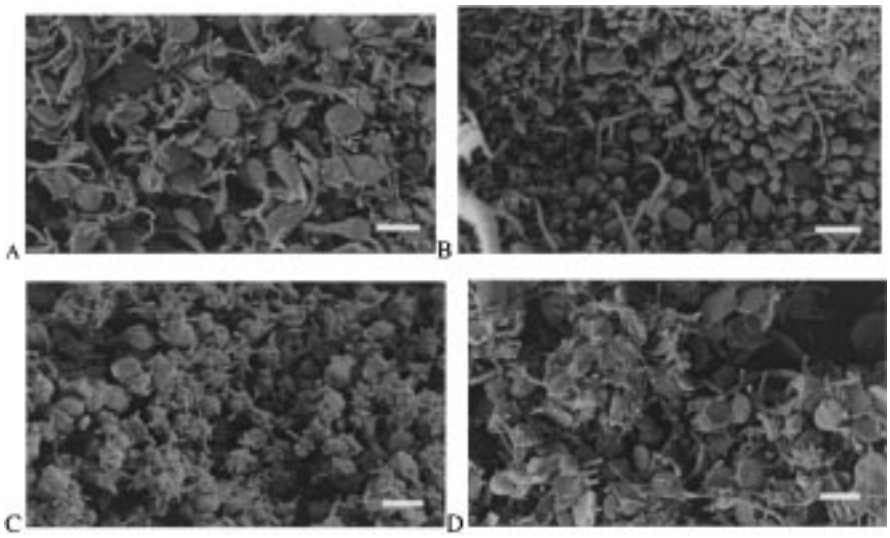

Figure 4. Scanning electron microscope (SEM) analysis of aggregated and disaggregated platelets. (A) Platelets activated with 0.5 μM ADP, (B) platelets aggregated with 5 μM ADP, (C) platelets aggregated with 5 μM ADP and disaggregated with 0.5 U plasmin, (D) platelets aggregated with 5 μM ADP and disaggregated with 0.1 U apyrase. Scale bars indicate 5 μm.

however, retain a discoid form characteristic of resting platelets. Platelets aggregated with 5 μM ADP showed extensive shape change and formation of large aggregates. Those observed on the periphery of the aggregate still retained a spherical form normally associated with activated platelets (Figures 3b and 4b). Platelets disaggregated with plasmin had a spherical activated shape with a large number of pseudopods protruding from the platelet surface giving it a fuzzy appearance (Figures 3c and 4c). Platelets disaggregated with apyrase showed the platelets to have a similar appearance to those activated with 0.5 μM ADP with a discoidal form and extended pseudopods (Figures 3d and 4d).

Internal changes associated with aggregation and disaggregation of platelets

The internal changes in platelet morphology were investigated using TEM. The discoid form associated with the resting state can clearly be seen for platelets activated with 0.5 μM ADP. They retained their electron dense granules as is characteristic for platelets undergoing reversible aggregation (Figure 5a). Platelets aggregated with 5 μM ADP show aggregates in which individual platelets cannot be distinguished clearly, and are generally devoid of granules, indicating irreversible aggregation (Figure 5b). Platelets disaggregated with plasmin were spherical with extended pseudopods and showed

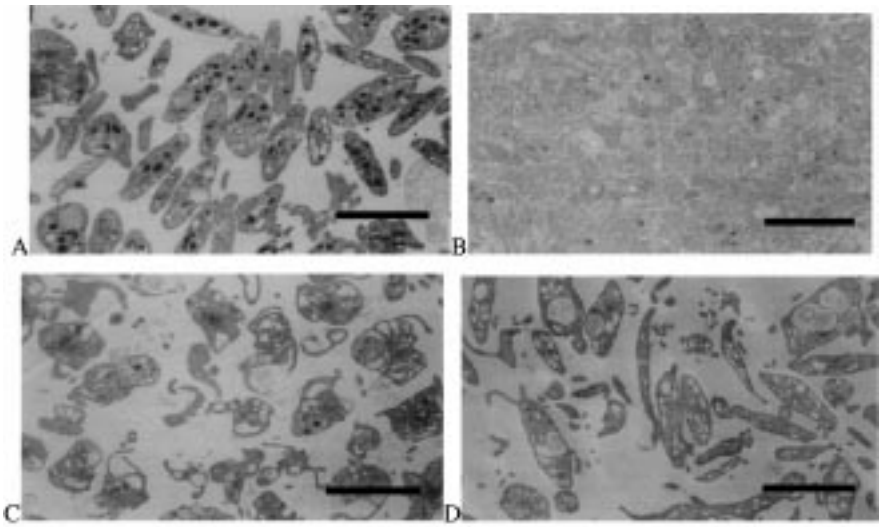

Figure 5. Transmission electron microscope (TEM) analysis of aggregated and disaggregated platelets. (A) Platelets activated with 0.5 μM ADP, (B) platelets aggregated with 5 μM ADP, (C) platelets aggregated with 5 μM ADP and disaggregated with 0.5 U plasmin, (D) platelets aggregated with 5 μM ADP and disaggregated with 0.1 U apyrase. Scale bars indicate 5 μm.

definitive signs of degranulation and a dilatory open canaliculary system (Figure 5c). Platelets disaggregated by apyrase were degranulated as exemplified by the dilated open canaliculary system and absence of electron dense granules. Exept for platelets in an elongated form, most showed a similar shape to those that had undergone reversible aggregation (Figure 5d).

Discussion

The results of this study are the first to describe the previously suggested disaggregation effect of a tick-derived apyrase (Mans *et al.*, 1998b) and emphasizes the important role that this enzyme plays during tick feeding. Feeding and the accompanying probing for blood by a tick, damages and lacerates mainly capillary blood vessels and epithelial cells (Binnington and Kemp, 1980). It is known that such damage is one of the main causes of ADP release and subsequent activation and aggregation of platelets induced by ADP (Gachet and Cazenave, 1991). Because platelet aggregation is the main event in the maintenance of hemostasis, it is also probably the most important hemostatic barrier that soft ticks face during feeding (Law *et al.*, 1992). Apyrase has been previously shown to inhibit aggregation induced by ADP

(Mans *et al.*, 1998b). A further crucial function for apyrase during tick feeding might be the dissolution of platelet aggregates in the blood meal.

Platelets activated by ADP undergo a shape change and a concomitant conformational change in the fibrinogen receptor which enable binding of fibrinogen and subsequent aggregation. When platelets are prepared in citrated plasma (low Ca^{2+} concentrations), primary aggregation or secondary aggregation is observed (Gachet and Cazenave, 1991). Low ADP concentrations (0.5–1 μM) leads to a primary wave response with subsequent disaggregation. The platelets revert from a spherical to a discoid form, normally in less than 1 min, with no release of granule contents (Zucker, 1989) as observed in Figures 1a and 5a. With critical ADP concentrations (1–2 μM), both primary as well as secondary wave responses can be distinguished. The secondary response commences after approximately 1 min due to secretion of granule contents and further activation by ADP. At this stage, platelets are loose aggregates and still retain their granules and ability to disaggregate (Mattson, 1985). With high ADP concentrations (2–5 μM), the primary and secondary wave responses cannot be distinguished and irreversible aggregation occurs. After approximately 3 min platelets become tightly organized aggregates. The secondary wave associated with secretion of granule contents is believed to be due to liberation of arachidonic acid from platelet membranes and subsequent conversion to thromboxane A_2, a potent platelet-aggregation agonist (Clemetson, 1995). It is generally accepted that this is not a true reflection of ADP activation at physiological Ca^{2+} concentrations (Gachet *et al.*, 1997). Although not a physiological response, several things could be concluded from the disaggregation of secondarily aggregated platelets by apyrase as observed during this study. Apyrase is able to inhibit aggregation of platelets mediated by metabolites of the cyclo-oxygenase pathway. It can also disaggregate platelets aggregated in this manner. This result also explains in part the inhibition previously observed for collagen-induced platelet aggregation (Mans *et al.*, 1998b). Collagen-induced platelet aggregation is due to secretion of granule contents with subsequent activation of platelets by ADP and the cyclo-oxygenase pathway (Clemetson, 1995). Disaggregation of secondary aggregated platelets implies that the effects of the cyclo-oxygenase pathway can be reversed which would also abolish the effects of collagen-induced platelet aggregation.

Previous studies showed that the rate of reversible platelet aggregation was increased by addition of potato apyrase (Rozenberg and Holmson, 1968), and it was postulated that dispersal is mediated by removal of ADP through hydrolysis. It has also been shown that bovine aorta apyrase could disaggregate platelets aggregated with critical concentrations of ADP (2 μM) (Coté *et al.*, 1992). The proposed function of the enzyme is the regulation of plate-

let aggregation by dispersion of platelets at the site of injury. While these apyrases have been shown to be able to disaggregate aggregated platelets, the current study is the first to describe disaggregation under conditions normally associated with irreversible aggregation such as degranulation.

This present study has shown that the disaggregation curve for plasmin was much steeper than that for apyrase (slope of -3 vs. -1, respectively) and the transmission level decreased to pre-aggregation levels (Figure 1c). This suggests that the platelets disaggregated and retained a spherical form as confirmed by SEM (Figure 4c). The spherical form and the presence of the pseudopods indicate that no signalling processes have occurred that are involved in the regulation of platelet shape change. These results are concordant with the idea that plasmin disaggregates platelets by fibrinogenolysis alone.

A possible reason why the curve associated with apyrase disaggregation (Figure 1d) does not return to a lower transmission, not even if left for more than 20 min (results not shown), may lie in the fact that these platelets have degranulated and that some retain an elongated shape (Figure 4d). Subsets of platelets may also still be in smaller aggregates although Figure 3c shows that most of the platelets have disaggregated. It was also found that platelets regain the discoid form in greater measure the longer disaggregation is allowed (results not shown). The longer time needed for disaggregation by apyrase in contrast to plasmin could reflect an extensive cytoskeletal rearrangement needed for shape change.

Thrombin had no effect on platelet aggregation or clotting after disaggregation by plasmin (Figure 2b). This can be expected if fibrinogen is degraded and thus removed from the system. It should be noted that plasmin can inhibit or activate platelets (Penny and Ware, 1992) although the conditions used in this study and the results obtained would suggest fibrinogenolysis. Platelets disaggregated by apyrase were, however, still activated by thrombin (shape change exemplified by the decrease in light transmittance) and aggregation took place before clotting of the fibrinogen to form a fibrin clot. This suggests that apyrase (known to hydrolyse ADP to AMP and pyrophosphate) disaggregated the platelets by hydrolysis of ADP and that pathways specific for ADP regulation of platelet aggregation were implemented in the disaggregation process. Thrombin, however, uses a different activation pathway than ADP (Clemetson, 1992) and disaggregation by removal of ADP would thus have no effect on its activation pathways.

Apyrase appears to be an important part of the regulation of host hemostasis by the feeding tick. The dual role of apyrase as an inhibitor as well as a disaggregation agent emphasizes the extraordinary co-evolution of these parasites to adapt to their host's environment. Elucidation of the molecular

mechanisms of action of different anti-hemostatic components might not only shed new light on the biology of hemostasis, but might also assist in the development of tick control strategies.

Acknowledgements

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