Stochastic response of human blood platelets to stimulation of shape changes and secretion

(thrombin/stopped-flow/turbidimetry/scattering/Poisson kinetics)

DAVID A. DERANLEAU, ROLAND LÜTHY, AND ERNST F. LÜSCHER
Theodor Kocher Institute, University of Berne, CH-3000 Bern 9, Switzerland

Communicated by Earl W. Davie, November 21, 1985

ABSTRACT

Stopped-flow turbidimetric data indicate that platelets stimulated with low levels of thrombin undergo a shape transformation from disc to "sphere" to smaller spiny sphere that is indistinguishable from the shape change induced by ADP through different membrane receptor sites and a dissimilar receptor trigger mechanism. Under conditions where neither secretion nor aggregation occur, the extinction coefficients for total scattering by each of the three platelet forms are independent of the stimulus applied, and both reaction mechanisms can be described as stochastic (Poisson) processes in which the rate constant for the formation of the transient species is equal to the rate constant for its disappearance. This observation is independent of the shape assignment, and as the concentration of thrombin is increased and various storage organelles secrete increasing amounts of their contents into the external medium, the stochastic pattern persists. Progressively larger decreases in the extinction coefficients of the intermediate and final platelet forms, over and above those that reflect shape alterations alone, accompany or parallel the reaction induced by the higher thrombin concentrations. The excess turbidity decrease observed when full secretion occurs can be wholly accounted for by a decrease in platelet volume equal in magnitude to the fraction of the total platelet volume occupied by $\alpha$ granules. Platelet activation, as reported by the whole body light scattering of either shape changes alone or shape changes plus parallel (but not necessarily also stochastic) $\alpha$ granule secretion, thus manifests itself as a random series of transient events conceivably with its origins in the superposition of a set of more elementary stochastic processes that could include microtubule depolymerization, actin polymerization, and possibly diffusion. Although the real nature of the control mechanism remains obscure, certain properties of pooled stochastic processes suggest that a reciprocal connection between microtubule fragmentation and the assembly of actin-containing pseudopodal structures and contractile elements—processes that may exhibit reciprocal requirements for calcium—might provide a hypothetical basis for a rate-limiting step.

We have described (1) the transient kinetics of human blood platelet shape changes and inferred the presence of an unstable intermediate in the continuous transformation of discs to spiny "spheres" induced by the action of ADP. This result was obtained by fitting turbidimetric progress curves to an $A \rightarrow B \rightarrow C$ reaction scheme by means of the Beer-Lambert law and appears to be compatible with microscopic studies also demonstrating, particularly in patients with a giant platelet syndrome, the stimulus-induced appearance of a transient platelet form or forms (2). The turbidimetric study compared the extinction coefficients of the individual reaction species with extinction coefficients predicted from light scattering theory on the basis of idealized shapes, and as a working hypothesis, we have written the reaction as disc $\rightarrow$ "sphere" $\rightarrow$ smaller spiny sphere.

The description of the intermediate as a sphere is intended loosely. Simple scattering theory predicts a more or less spheroidal form that has an effective scattering volume much more closely resembling the initial disc than the final sphere with its long filiform pseudopodia, and it is probably significant that the extinction coefficient of rather uniformly "spherical" platelets, produced by chlorpromazine treatment of either discs or ADP-induced spiny spheres (1), is virtually the same as that of the real time intermediate. However, both our own calculations and those of Latimer (3) show that a randomly oriented collection of oblate spheroids with axial ratios above 0.7 or so could hardly be distinguished from true spheres by current turbidimetric methods and that it would be difficult to detect the presence of surface irregularities or short pseudopods in the process of formation. In this sense our results are seen to agree rather closely with the microscope study, which used axial ratios between 0.5 and 0.9 as one criterion for classifying a given cell as a transient form (2).

A remarkable result of the kinetic analysis of the ADP-induced shape change reaction is that the rate constants for the two reaction steps were found to be identical (1). This finding does not depend on the shape assignment, so that within the limitations imposed by the model itself, the overall reaction can be described as a stochastic (Poisson) process in which the rates of the individual physical transformations appear to be regulated by a common factor. In the present communication we present evidence that thrombin, a powerful physiological stimulator of platelet activity utilizing different primary membrane receptors than ADP and interacting with them in a different manner, behaves similarly. Not only are the extinction coefficients for stimulation by low, shape changing levels of thrombin the same as those found for ADP stimulation, but the overall pattern of reactivity is the same as well. Moreover, the stochastic response pattern is observed at thrombin concentrations that induce secretion as well as shape changes, and release of the contents of one or more types of storage granules appears to be reflected by parallel decreases in the extinction coefficients of both the intermediate and final platelet forms.

Blood platelets are the key cellular elements of the complex physiological process that prevents further loss of blood from small wounds. These small (2-3 $\mu$m diameter) cells build large aggregates that plug the wound within minutes, simultaneously losing their discoid shape, extending long filiform pseudopods that markedly increase their contact radii, and secreting or otherwise making available substances that promote aggregation and lead to the formation of a stable hemostatic plug. An analogous process occurs in arterial thrombosis, in which platelet aggregates large enough to occlude blood vessels can lead to heart infarction.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.


EXPERIMENTAL PROCEDURES

Experiments were carried out essentially as described for ADP stimulation (1) using stopped-flow mixing to initiate the reaction. In the present study, however, platelets were isolated from up to 20-hr-old samples of citrated blood obtained from the Blood Transfusion Service of the Swiss Red Cross, and they were gel filtered in acid-citrate buffer at 37°C as described by Wüthrich et al. (4). These platelets retained their flat discoid shape on the gel column and, after filtration, were diluted to a concentration of $4 \times 10^7$ platelets/ml with 30.8 mM N-tris(Hydroxymethyl)methyl-2-aminoethanesulphonate buffer (TES; pH 7.4 at 37°C) containing 118 mM NaCl, 3 mM KCl, and 4.8 mM glucose. The suspension was kept at 37°C for the duration of the experiments. EDTA (2 mM) was added just prior to mixing to prevent stimulus-induced aggregation, and aliquots of the platelet suspension were mixed 1:1 with an identically-buffered thrombin solution in a “zero-degree” stopped-flow laser turbidimeter (5) equipped with a beam stop to prevent light scattered outside the dimensions of the incident beam from reaching the detector and giving rise to spurious transmission values. The final platelet concentration ($2 \times 10^7$ cells/ml) was an order of magnitude less than the concentration at which deviations from Beer’s law begin to be observed. Light transmission data were digitized on line with a Data Translation LAB DATA SYSTEM and stored on magnetic media for later analysis. Transmission values were converted to extinctions, and the extinction vs. time progress curves were fitted to a pseudo first-order reaction scheme, $A \rightarrow B \rightarrow C$ with rate constants $k_1 (A \rightarrow B)$ and $k_2 (B \rightarrow C)$, through the aid of the light scattering equivalent of the Beer–Lambert law

$$E(t) = aA(t) + bB(t) + cC(t),$$

where $E(t)$ is the extinction for unit path length, $a$, $b$, and $c$ are total scattering extinction coefficients (cross sections), and $A(t)$, $B(t)$, and $C(t)$ are the respective time-dependent concentrations of the reaction species disc, “sphere,” and spiny sphere (1). Each individual experiment involved fitting not less than 800 digitized points by nonlinear least-squares techniques.

Comparative studies with ADP indicated that none of the differences in preparation had a significant effect on the results. Saturating amounts of ADP do not induce aggregation or secretion of cell granule contents under the experimental conditions used (1), but high levels of thrombin induce rapid secretion, and the optical change associated with this process is superimposed upon the optical changes associated with the shape change proper. For shape change measurements alone, this complication was avoided by using thrombin levels that were below the secretion threshold, as judged by the absence of release of acriflavine trapped within secretory granules (4).

RESULTS AND DISCUSSION

The fitted extinction coefficients and rate constants derived from progress curves generated by thrombin levels that induce shape changes but not secretion (0.01 unit/ml) are compared with the ADP data in Table 1. As expected, considerably different rate constants are observed for stimulation with saturating levels of ADP and with very low doses of thrombin, but apart from this, there are no statistically significant differences between the responses observed with either stimulator (Student’s t-test). The extinction coefficients reflect the size, shape, and refractive index of the individual reaction species, and the results demonstrate that the stimulus-induced changes in these parameters are virtually independent of the stimulator used, when the data are averaged over a very large number of cells as is the case in the present experimental arrangement. The rate constants for the formation and disappearance of the intermediate are equal to each other regardless of which stimulus is applied, so that the net reaction mechanisms are the same as well (both are characteristic of a Poisson-type stochastic process, implying, as discussed above and in more detail in ref. 1, the existence of a common rate-limiting step). The mechanistic identity and invariance of the size and shape parameters obtained on treating platelets with totally different stimulators is further, in this case quantitative, evidence that stimulation by agonists of diverse specificity and mode of action can result in a common pathway of cellular activity (6).

Equally important in the present context is the fact that the (stochastic) kinetic pattern obtained with low concentrations of thrombin persists as the thrombin concentration is increased and, in addition to shape changes, secretion occurs. At the same time, progressive decreases in the turbidity of the platelet suspensions are observed that appear to be correlated with secretory processes. Typical progress curves demonstrating the effect are shown in Fig. 1. Except for the initial extinction, the entire progress curves are modulated, and as the rate of the reaction increases, the curves are compressed toward shorter times. This and other evidence to be discussed below suggests that the effect is superimposed upon (occurs in parallel with) the optical changes that reflect shape factors alone (Fig. 1, curve a). In all cases the best fits were obtained with the simple $A \rightarrow B \rightarrow C$ model discussed above, and the rate constants for the formation of the intermediate were practically always found to be identical with those for its disappearance (one exception in 23 experiments). Significantly, the use of an $A \rightarrow B \rightarrow C \rightarrow D$ reaction scheme gave markedly poorer or even unacceptable fits to the experimental data, so that—assuming for the moment that it is secretion that is responsible for the additional decreases in extinction—a model in which secretion follows shape changes does not appear tenable.

The dependence of the fitted rate constants and extinction coefficients on thrombin concentration is shown in Fig. 2. The rate constants are given for completeness only and continue to increase with increasing receptor occupancy as commonly observed (the negative logarithm of the rate

---

Table 1. Light scattering extinction coefficients and kinetic rate constants for the $A \rightarrow B \rightarrow C$ shape change reaction of human blood platelets stimulated with thrombin and ADP at 37°C (stopped-flow mixing)

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Extinction coefficient at 632 nm, μm²/platelet</th>
<th>Rate constant, sec⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disk, A</td>
<td>Intermediate, B²</td>
</tr>
<tr>
<td>ADP, 10 μM*</td>
<td>1.42 ± 0.14</td>
<td>1.78 ± 0.20</td>
</tr>
<tr>
<td>Thrombin, 0.01 unit/ml†</td>
<td>1.38 ± 0.06</td>
<td>1.96 ± 0.07</td>
</tr>
</tbody>
</table>

*ADP, 8 experiments, >400 fitted data points per experiment (1).
†Present results, 5 experiments, >800 fitted data points per experiment.
‡For comparison, the extinction coefficient of “spherical” platelets produced by 100 μM chlorpromazine treatment is 1.9 in the same units.
constant shows an apparently secretion-related break, indicating that the rates do not increase without limit. More important for the present purposes are the extinction coefficients of the intermediate and the final spiny sphere, which decrease with increasing concentrations of thrombin over the range corresponding to from zero to essentially complete secretion (ca. 0.015–0.5 unit/ml; refs. 4 and 7). The extinction coefficient of the final spiny sphere levels off at thrombin concentrations large enough to induce full secretion, providing strong indirect evidence for assigning the additional turbidity changes to secretory events. The simultaneous decrease in the extinction coefficient of the intermediate (which does not level off because secretion by the transient species is never complete) and the identity of the rate constants in the presence of secretion both suggest that the secretory process occurs more or less in parallel with the shape changes. Note that it is the combination of shape change and secretion as reported by changes in whole body light scattering that appears as a random series of transient events; this does not necessarily imply that the secretory process is itself stochastic even if this were to be the case. The situation is shown somewhat differently in Fig. 3, where we have compared the decrease in the final steady state extinction, calculated as a fraction of the total change, with our own real time data for the release of acriflavine from dense granules (4). Although dense granule secretion is not likely to be observed by the turbidimetric method (see below), the similarity in the two dose–response curves is a further indication that shape changes and secretion induced by physiological stimuli are closely coupled events.

If the excess optical changes observed at high thrombin levels indeed reflect granule secretion, it should be possible to account theoretically for the effects. It is established that small spheres scatter less light than large spheres having the same refractive index, and since long thin pseudopods scatter

**Fig. 2.** Fitted extinction coefficients a, b, and c for total scattering by discs (solid ovals), transient spheres (open circles), and final spiny spheres (asterisks) (Top), and kinetic rate constants, \( k_1 = k_2 = k \) (Bottom) for the shape change/secretion reaction resulting from treatment of platelets (2 \( \times 10^5 \) cells/ml) with increasing concentrations of thrombin. Each point represents the average of three to six independently evaluated data sets.

**Fig. 3.** Fraction of the total decrease in the extinction coefficient of the final spiny sphere as a function of increasing thrombin concentration (asterisks) compared to the thrombin-induced secretion of acriflavine from preloaded platelets (solid circles; real time data are from ref. 4).
Table 2. Comparison of the experimental extinction coefficients for a series A → B → C reaction with those predicted by Rayleigh–Debye scattering theory

<table>
<thead>
<tr>
<th>Assumed parameters</th>
<th>Extinction coefficient, μm²/platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calc.</td>
</tr>
<tr>
<td>Disc</td>
<td>0.2</td>
</tr>
<tr>
<td>Sphere</td>
<td>1</td>
</tr>
<tr>
<td>Spiny sphere*</td>
<td>1</td>
</tr>
<tr>
<td>&quot;Empty&quot; spiny sphere†</td>
<td>1</td>
</tr>
</tbody>
</table>

*Shape changes only (10 μM ADP or 0.01 unit of thrombin/ml).
†Shape changes plus full secretion (0.5 unit of thrombin/ml).
‡Volume shown is the body volume, excluding pseudopods. The refractive index was taken as 1.044 in all cases (relative to water at 37°C).
§Measured 0.26 by light microscopy (20).

Little light in comparison to the platelet body (8, 9), a decrease in turbidity implies a reduction in either body size (effective scattering volume; total volume less the volume occupied by pseudopods), refractive index, or axial ratio (3). The latter is contraindicated since spiny "spheres" are observed in the microscope either with or without secretion, leaving a reduction in body size and/or refractive index as the most reasonable explanation. Because they contribute less than about 1% to the total platelet volume, a reduction in body size due to dense granule release probably could not be detected by the turbidimetric methods employed here. Scattering calculations indicate that these relatively small granules would have to have an impossibly high refractive index (approaching that of diamond or rutile) to account for the observed changes in the extinction coefficients. On the other hand, α-granules make up an estimated 11% of the total platelet volume (10), and it seems reasonable that extrusion of their contents under the influence of internal contraction would result in further shrinking of the platelet body, over that due to the reduction in body size that accompanies pseudopod extension. Assuming that the refractive index remains constant, the difference between the extinction coefficients of spiny spheres with intact secretory organelles and those with "empty" secretory organelles translates into a theoretical 10–11% reduction in body size with respect to the intact spiny sphere (9–10% reduction in total size with respect to the initial disc), or approximately the volume occupied by α-granules before secretion takes place. Details are presented in Table 2, where the extinction coefficients predicted by simple Rayleigh–Debye scattering theory are compared with the experimental extinction coefficients. The correctness of this reasoning is unequivocally demonstrated by the finding (11) that the additional, secretion-related changes in turbidity are not observed using platelets from a patient with an α-granule deficiency (gray platelet syndrome).

Scattering theory also provides an estimate of the overall decrease in body volume associated with the conversion of resting discoid platelets to fully-secreted spiny spheres. Painstaking morphometric measurements using phase contrast photomicroscopy demonstrate that such a volume change indeed takes place (12), and although the morphometric data were obtained using ADP rather than thrombin as a stimulus, secretion is promoted by the conditions employed (high platelet concentrations and stirring). The microscopically measured decrease in body volume amounts to 18–19% (cf. figure 6 of reference 12; average of the last six statistically indistinguishable values), which is just the figure predicted by scattering theory on the basis of simple models (18.4%, Table 2). While more in-depth studies may reveal the influence of other factors, the current morphometric and turbidimetric data both seem to indicate that (i) pseudopods are formed at the expense of the size of the platelet body in a process that takes place without a significant change in total platelet volume (see also ref. 1) and (ii) secretion of α-granule contents decreases the total platelet volume by an amount closely corresponding to the volume occupied by the loaded granules in the unstimulated platelet.

A known property of stochastic (Poisson) processes is that a set of n component processes, each having the same fundamental rate constant k, can form a pooled output that is in turn a Poisson process with rate constant K = nk (13). In the platelet, there are two such elementary processes that appear to be of crucial importance in effecting the shape changes. The first is (transient) depolymerization of at least portions of the microtubule ring that maintains the discoid form, thus allowing the cell to assume a spheroidal shape (the first reaction in our hypothetical shape change scheme). The second is linear actin polymerization, resulting in the formation of increasingly long actin filaments that may be (14) not only largely responsible for the extension of pseudopods (at the expense of a corresponding decrease in body size, the second reaction in our shape change scheme), but which are essential for assembly of the internal contractile system. It has been postulated that a local reciprocal connection exists between actin and tubulin polymers that can exercise control over cellular motility. In essence, this consists of a sort of push–pull arrangement, discussed in more detail for platelets by Gitler et al. (15) and for other forms of cellular motility by Yahara and Edelman (16) and by Fulton (17, 18), in which the extent of actin polymerization depends upon the extent of microtubule breakdown. While highly speculative, it is at least conceivable that such a system could provide the basis for a mechanism by which the rates of the component processes (including contraction when present, since it depends upon prior assembly of actin filaments) could be maintained equal to one another. Or, the rates of the component processes could be controlled by their reciprocal requirements for calcium. Such a mechanism, or something like it, could constitute a plausible if not overly simplistic or entirely realistic explanation of the observed results. Two facts suggest that further examination of these ideas is warranted. One is the apparent stochastic nature of the shape change/secretion process itself as detailed here and in ref. 1, and the other is the observation that microtubule depolymerization is itself a transient process (19), thus providing a means, if the reciprocity argument holds true, of stopping the activation cycle at an appropriate point.

We are grateful to the Sandoz Foundation (Basel, Switzerland) for the generous gift of the LAB DATAX data acquisition and handling system, without which this work would not have been possible. This work was supported by the Swiss National Science Foundation Grants 3.378.0.82 to E.F.L. and 3.353.0.82 to M. Baggiolini.