Thrombin-Induced Secretion of Serotonin From Platelets Can Occur in Seconds

By Adrian R. L. Gear and David Burke

The platelet release reaction was studied by a new quenched-flow approach. Platelets labeled with $^{3}C$-serotonin were reacted for short times (up to 5 sec) with thrombin and then quenched with glutaraldehyde or paraformaldehyde. Serotonin secretion began within 1 sec and was nearly complete by 4 sec. Aggregation recorded by a resistive-particle counter was similarly fast. Therefore, the quenched-flow system reveals that serotonin secretion can occur more rapidly than estimated in earlier studies.

THERE ARE GOOD REASONS why efficient hemostasis requires very rapid platelet activation. Some evidence exists that the hydrolysis of metabolic adenosine triphosphate (ATP) begins within a second of a stimulus. Phospholipases apparently are activated by 10 sec, and significant morphological changes occur by 5 sec. The ability to follow readily the kinetics of such early reactions therefore becomes important for understanding the nature and control of the stimulus–response sequence. We have developed a general quenched-flow approach for this purpose and have demonstrated that platelet aggregation is very rapid. After a lag period of less than a second, about 30% of particles then aggregate per second. We now report that the thrombin-induced secretion of serotonin can be much faster than previously believed. It begins within 1 sec of stimulation and is nearly complete by 4 sec.

The method of quenched-flow aggregometry was used to study the kinetics and extent of serotonin release. Shear forces within a narrow tube (about 0.3 mm internal diameter) provide platelet activation, and particle morphology and volume are not altered by passage through the reaction tubing. The reaction is stopped by addition of a quenching agent and reaction times from 100 msec to minutes can be selected by varying syringe-pump speed or tubing diameter and length.

MATERIALS AND METHODS

Platelet Isolation and Labeling

Platelet-rich plasma (PRP) was prepared from normal human volunteers using acid-citrate dextrose (ACD) as anticoagulant. The PRP (5 ml) was then exposed to 1 μCi of serotonin (2$^{14}$C, Amersham) and incubated for 20 min at 37°C. This resulted in over 80% uptake of serotonin. After labeling, the PRP was stored at room temperature and “gassed” with a 95% air and 5% CO₂ mixture to help maintain plasma pH and platelet function.

Quenched-Flow Methods

Imipramine (4.2 μM) was added to 1.5-ml aliquots of PRP, which had been warmed for 5 min at 37°C, and the PRP placed in one syringe of the quenched-flow apparatus (Fig. 1), which was kept at 37°C in a hot air chamber. The imipramine prevents reuptake of serotonin during the experiment, although we found no significant influence on rapid-release kinetics. Bovine thrombin, adenosine diphosphate (ADP), or adrenalin, (Sigma, St. Louis, Mo.) were diluted in 0.15 M NaCl and placed in a second syringe. The quenching agent was either 2% glutaraldehyde or 1.9% paraformaldehyde and was introduced into the third syringe of the quenched-flow apparatus. Reaction loops normally provided initial reaction times of 1.5 sec; shorter or longer ones were occasionally employed for 0.5 or 3.0 sec.

RESULTS

The time course for secretion of serotonin in comparison with aggregation is shown in Fig. 2. Thrombin induced a rapid release with an “onset time” of approximately 0.8 sec and a half-maximal change at about 4 sec. The onset time was obtained by extrapolating backwards, a tangent to the slope of serotonin release. This is analogous to the procedure used for aggregation. Platelet aggregation was similar to that of

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previously noted for thrombin; namely, there was an onset time of less than 1 sec and about 20% of single platelets aggregated per second. Control experiments revealed no release of $^{14}$C-serotonin when platelets were pumped through the quenched-flow system in the absence of thrombin for up to 5 sec of shear at less than 50 dynes/sq cm. Some preparations did, however, exhibit an initial small loss of serotonin (less than 7%), which did not increase during longer pumping times in the quenched-flow loop.

The effect of thrombin concentration on the early release of serotonin is presented in Fig. 3. No $^{14}$C label was lost in this particular experiment at thrombin levels of 0.1 or 0.25 U/ml, even though 0.25 U caused 40% of single platelets to aggregate by 4 sec (data not shown). However, another preparation exposed to 0.25 U/ml exhibited a similar extent of aggregation but showed a serotonin release of 25% by 5 sec. Such variability between the platelets of different individuals is similar to that described by ADP as an inducing agent. In other experiments with thrombin at 5 U/ml and employing shorter reaction loops, about 6% serotonin was secreted at 0.5 sec, reaching 21% by 1.7 sec. These direct measurements support the onset times of less than 1 sec discussed above. Use of longer reaction loops also indicated that with some preparations over 90% secretion could occur by 8–10 sec, with a mean release of 70%.

When PRP is stirred in an aggregometer, secretion of serotonin occurs faster with thrombin than with ADP, adrenaline, or collagen. Consistent with this, we have found with the quenched-flow system that ADP (10 $\mu$M) or adrenaline (15 $\mu$M) did not induce more than 10% release of $^{14}$C-serotonin by 10 sec, even though massive aggregation (>80%) had occurred with ADP. Therefore, at high levels, thrombin differs markedly from ADP and epinephrine in being able to elicit major release within seconds of induction.

In view of the report that formaldehyde may be preferable to glutaraldehyde as a quenching agent, we compared the effectiveness of the two in the quenched-flow system. No significant difference was
found in the extent and rate of \(^{14}\)C-serotonin release (5 PRP preparations). Thus, at 2 sec after exposure to thrombin (1 U/ml), 18.7% \(\pm\) 10.9% of serotonin was released when glutaraldehyde was used for quenching; with formaldehyde it was 23.7% \(\pm\) 10.2%. The values at 5 sec were 57.0% \(\pm\) 24.8% and 62.6% \(\pm\) 25.5%, respectively (means \(\pm\) 1 standard deviation). It might be noted that only 0.25% glutaraldehyde was employed earlier,\(^{12}\) and the higher concentrations we used (1% in the reaction loop, Fig. 1) could be responsible for a minimal loss of serotonin from control platelets. Glutaraldehyde added to control PRP before isolation of PPP did not cause any significant release (\(p > 0.1, 12\) PRP preparations).

**DISCUSSION**

The quenched-flow approach used in this research provides the opportunity to investigate the kinetics and sequence of events occurring within seconds, or fractions of a second, of platelet stimulation. We have shown that aggregation begins before 1 sec and is half complete by about 3 sec.\(^8\) The new information presented here indicates that serotonin release may occur much more rapidly than has been estimated in earlier studies of secretion,\(^{14-17}\) although data obtained with a manual fixation technique involving formaldehyde\(^{12}\) did suggest a release half-time of about 15 sec at 25°C.

Thrombin can induce very rapid changes in platelet function and morphology. The speed of these changes is evident in recent measurements of alterations in intracellular viscosity with a fluorescent probe.\(^{18}\) Three mechanisms for thrombin's effects have been proposed,\(^{13,19}\) and further studies of these mechanisms employing quenched-flow aggregometry would be profitable. For example, use of chlortetracycline as a fluorescent probe suggests a lag time of 1.8 sec at 22°C before cytoplasmic calcium levels increase.\(^{17,20}\) At 37°C the lag would be shorter and consistent with activation of phospholipases and other calcium-dependent enzymes within 1 sec.

Horne et al.\(^{21}\) have reported that thrombin can cause changes in cytoplasmic pH and membrane potential within 5 sec. They indicated that analysis of the temporal relationship between these parameters was not possible because of experimental limitations of their fluorimeter. It would appear valuable to combine the fluorimetric and quenched-flow methods to evaluate such questions. Depolarization of the platelet membrane could well be an important element in the signals leading to the rapid release of serotonin, analogous to neurosecretion.

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AR Gear and D Burke