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Research Article

The mechanism of stimulus-response coupling in human platelets was investigated with a new instrument that simultaneously monitors aggregation and secretion in the same sample of platelet-rich plasma. When platelets were stimulated by high concentrations of ADP, secretion began only after aggregation was almost complete. With lower concentrations of ADP or with epinephrine, biphasic aggregation was observed, and secretion began simultaneously with, or slightly after, the second phase of aggregation. When platelets were stimulated with high concentrations of γ -thrombin or A23187, secretion and aggregation began essentially together. With very low concentrations of γ -thrombin or A23187, biphasic aggregation was observed with secretion paralleling the second phase. At every concentration of collagen, secretion and aggregation appeared to be parallel events. Under every condition where the beginning of secretion lagged behind aggregation, secretion was dependent upon aggregation and was inhibited by indomethacin; this is referred to as aggregation-mediated platelet activation. When secretion began at the same time as aggregation, it also occurred in the absence of aggregation and was not blocked by indomethacin; this is referred to as directly induced platelet activation. These observations are consistent with a simple model of platelet stimulus-response coupling that includes two mechanisms for activation; aggregation-mediated activation is inhibited by indomethacin, while direct activation does not depend upon aggregation and is not inhibited by indomethacin. Secretion and second wave [...]

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Interrelations of Platelet Aggregation and Secretion

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ABSTRACT The mechanism of stimulus-response coupling in human platelets was investigated with a new instrument that simultaneously monitors aggregation and secretion in the same sample of platelet-rich plasma. When platelets were stimulated by high concentrations of ADP, secretion began only after aggregation was almost complete. With lower concentrations of ADP or with epinephrine, biphasic aggregation was observed, and secretion began simultaneously with, or slightly after, the second phase of aggregation. When platelets were stimulated with high concentrations of γ -thrombin or A23187, secretion and aggregation began essentially together. With very low concentrations of γ -thrombin or A23187, biphasic aggregation was observed with secretion paralleling the second phase. At every concentration of collagen, secretion and aggregation appeared to be parallel events. Under every condition where the beginning of secretion lagged behind aggregation, secretion was dependent upon aggregation and was inhibited by indomethacin; this is referred to as aggregation-mediated platelet activation. When secretion began at the same time as aggregation, it also occurred in the absence of aggregation and was not blocked by indomethacin; this is referred to as directly induced platelet activation. These observations are consistent with a simple model of platelet stimulus-response coupling that includes two mechanisms for activation; aggregation-mediated activation is inhibited by indomethacin, while direct activation does not depend upon aggregation and is not inhibited by indomethacin. Secretion and second wave aggregation appear to be parallel events, with little evidence for second wave aggregation being a consequence of secretion as usually described.

INTRODUCTION

Understanding the interrelations between the *in vitro* platelet functions usually measured (shape change, aggregation, and secretion) has always seemed to be a

key to understanding stimulus-response coupling in these cells. While under certain conditions one or more of these functions may be independent of the others, it is generally assumed that some secreted substances cause aggregation (1, 2) and, conversely, that aggregation can lead to secretion (3). Interpretation of published results is complicated by two factors; (a) a variety of stimuli are used and each gives somewhat different results, and (b) the two major functions, aggregation and secretion, are usually measured under different conditions, precluding precise comparisons.

We have therefore studied the response of platelets to several types of stimuli with a new instrument to measure shape change, aggregation, and secretion simultaneously in the same sample (4, 5) of platelet-rich plasma. We used indomethacin, an inhibitor of prostaglandin synthesis and the "second wave" of aggregation, to distinguish different aspects of the responses. We propose a model of stimulus-response coupling that includes two mechanisms of platelet activation; one is mediated by aggregation and is inhibited by indomethacin, while the other is independent of aggregation and is not inhibited by indomethacin.

METHODS

Preparation of platelet-rich plasma. Blood was collected by venipuncture from healthy volunteers into 0.1 vol of 3.8% trisodium citrate. All donors denied taking any drugs for at least 1 wk before phlebotomy. Platelet-rich plasma was prepared by centrifugation of the whole blood at 300 g for 20 min at 25°C. Platelet-poor plasma was obtained by centrifugation of an aliquot of platelet-rich plasma for 5 min in an Eppendorf microfuge (model 320, Brinkmann Instruments, Inc., Westbury, N. Y.).

Simultaneous measurement of shape change, aggregation, and secretion. The continuous recording of platelet function was carried out with a new instrument that continuously and simultaneously monitors secreted ATP and light transmittance (shape change and aggregation) in the same sample. This instrument, described in detail elsewhere (4, 5), utilizes the luminescent firefly luciferase system for detection of secreted ATP (6, 7), while aggregation is measured by the usual turbidometric method (8). The geometry of the optics, as well as the use of near-infrared light for the

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transmittance measurement and a narrow response photomultiplier tube for the luminescence measurement, prevent interference of scattered light with the secretion measurement. Reactions were at 37°C in a commercial aggregometer cuvette (Chrono-Log Corp., Havertown, Pa.) with stirring by means of a Teflon-coated stirring bar driven at 1,100 rpm. Results were displayed on a 2-pen Houston Omniscrite Chart Recorder (Houston Instrument Div. of Bausch & Lomb, Inc., Austin, Tex.). The reaction mixture consisted of 0.6 ml platelet-rich plasma; 50 μ l of 80 mg/ml Dupont luminescence assay mixture (Biometer luminescence kit, Dupont Co., Wilmington, Del.) dissolved in 100 mM Tris-HCl, pH 7.4; and either 10 or 20 μ l of 154 mM MgSO₄ (the lower amount was preferred, but it results in less luminescence so that for some experiments the higher concentration was used to avoid excessive instrument noise accompanying high signal amplification). The responses to added ATP were instantaneous and linear with ATP concentration and there was no appreciable interference due to adenylate kinase (or to any other enzyme that could convert ADP to ATP) as manifested by the lack of increased luminescence on addition of ADP. The sensitivity of the measurement under the conditions of these experiments is such that a pen deflection of twice the noise amplitude is caused by less than 50 nM ATP, which corresponds to about 1% of the maximum releasable ATP. Further details and necessary precautions for this type of assay have been discussed by Charo et al. (7). The aggregation trace was calibrated with platelet-free plasma (theoretical maximal aggregation) at about half the full chart width (see Fig. 1). Secretion was calibrated by adjusting photomultiplier gain with two different standards. The preferred method is to relate the amount secreted to the maximal that can be secreted, usually taken as the maximal secreted in response to thrombin; this was used in Figs. 1, 4, and 5. Since this would cause only a slight deflection in response to weaker stimuli, some traces were calibrated with the maximal secretion in response to collagen (Figs. 2, 3, and 6). Collagen consistently caused from 50 to 60% as much secretion as did thrombin.

The results presented here are representative of many experiments with platelet-rich plasma obtained from 25 different donors. The data within a figure are from a single sample of platelet-rich plasma, and, in addition, comparisons between figures are valid because both aggregation and secretion curves are standardized to maximum responses (see preceding paragraph), thereby normalizing differences in platelet counts of different donors. Results have been highly consistent except for the well-known variation among donors for biphasic aggregation (9); from a single sample of platelet-rich plasma, biphasic aggregation is fully reproducible, but the concentration of stimulus required for biphasic aggregation is slightly different for each sample of platelet-rich plasma. Although biphasic aggregation may be an *in vitro* artifact (10), it is believed to reflect important physiological functions and is especially important as the only *in vitro* parameter of platelet function that is dependent upon prostaglandin synthesis. We have also observed some variation between donors in the rate of response of platelets to ADP; the traces selected for Fig. 1 (fifth trace) and for Fig. 2 (third trace) illustrate the range of responses.

Materials. ADP (Boehringer-Mannheim, Indianapolis, Ind.) and ATP (Sigma Chemical Co., St. Louis, Mo.) were dissolved in 100 mM Tris-HCl, pH 7.4. L-Epinephrine (Sigma Chemical Co.) was dissolved in 0.1 N HCl and indomethacin (Sigma Chemical Co.) in absolute ethanol. A23187 was a gift of Eli Lilly and Co., Indianapolis, Ind., and was dissolved in di-

methylsulfoxide. Collagen (Hommon Co., Munchen 45, W. Germany) was an insoluble, particulate preparation in dilute acid and was added as the stock solution. All additions of ethanol and dimethylsulfoxide were less than 0.5% of the total volume. Highly purified human α -thrombin and human γ -thrombin were the generous gifts of Dr. John W. Fenton, II, Division of Laboratories and Research, New York State Department of Health, Albany, N. Y. γ -Thrombin (11-13) is a product of limited tryptic hydrolysis of purified α -thrombin, which is presumed to be the predominant physiological form. γ -Thrombin has essentially the same esterase activity as α -thrombin, but less than 0.1% activity when compared to α -thrombin using fibrinogen as substrate. When γ -thrombin is used to induce secretion of Ca²⁺ from washed platelets, its activity is about 1% the activity of α -thrombin (i.e., 100 times as much γ -thrombin is required for full secretion).¹

RESULTS

The use of the presumed physiologically active form of thrombin, α -thrombin, with platelet-rich plasma is difficult because high concentrations catalyze the quick formation of a clot that interferes with the aggregation measurement, while at low concentrations blood coagulates at about the time of second wave aggregation. We avoided these problems by using γ -thrombin, a product of limited hydrolysis of α -thrombin; it has essentially no clotting activity, but about 1% of the platelet stimulating activity of α -thrombin (see Materials). Secretion and the primary wave of aggregation induced by α - or γ -thrombin appear identical (unpublished observations). We thus consider results with γ -thrombin to be qualitatively the same as with α -thrombin.

Fig. 1 shows typical secretion and aggregation progress-time curves obtained with saturating levels of either thrombin, A23187, collagen, or ADP. When either thrombin, A23187, or collagen was the stimulus, secretion and aggregation began essentially simultaneously. In contrast, when ADP was the stimulus, shape change and aggregation began immediately, but no detectable secretion was observed for at least 30 s (note the different time scale with the ADP trace). This suggests that ADP does not induce secretion directly, but instead induces aggregation, which in turn leads to secretion. Indeed, if the platelet-rich plasma was not stirred, ADP-induced aggregation was prevented and there was no secretion (data now shown), whereas thrombin induced secretion equally well when aggregation was prevented by not stirring (Fig. 1). (Similar experiments with collagen are not meaningful because the initial interaction of particulate collagen with platelets probably requires stirring).

The relationship between aggregation and secre-

¹ Wasiewski, W. W., and T. C. Detwiler. Unpublished observations.

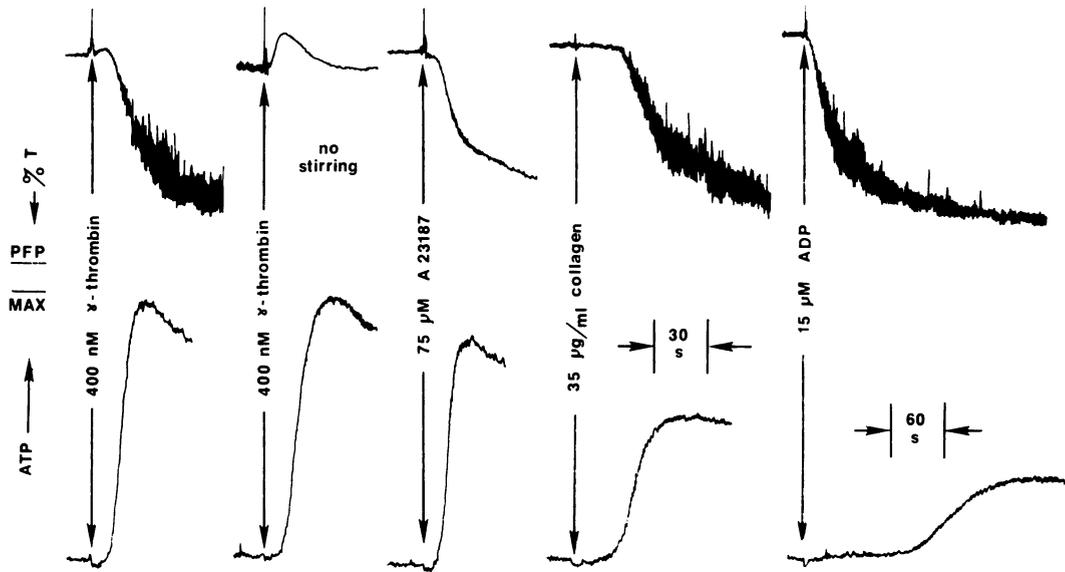


FIGURE 1 Simultaneous measurements of aggregation and secretion induced by high levels of stimuli added to platelet-rich plasma as described in Methods. Secretion of ATP (lower traces) was measured by the firefly luminescence assay, with the gain of the instrument set so that maximum release with thrombin gave half-chart deflection (indicated by MAX). The decrease in the signal seen at the end of some curves does not represent uptake or metabolism of ATP by platelets, but is due to the well-known property of the assay system to show a decay in luminescence (23, 24). Aggregation (upper traces) was measured simultaneously in the same cuvette, with the limit set with platelet-free plasma (PFP) as indicated. Reactions were initiated by addition of the stimulus. In the second set of traces, the stirring motor was turned off to prevent aggregation. The first four experiments were recorded at 30 s/inch (space between vertical bars) and the fifth was at 60 s/inch.

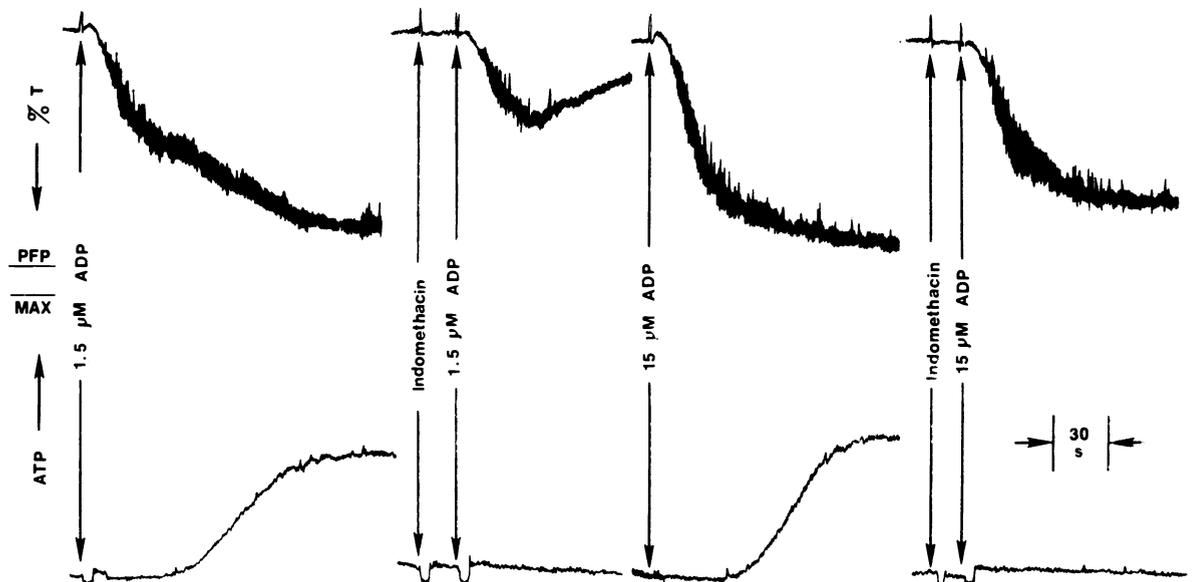


FIGURE 2 Effect of indomethacin on aggregation and secretion induced by ADP. In these experiments the gain of the luminescence detector was calibrated so that collagen curves gave half-chart deflection (MAX) to amplify the lower ADP curves. All traces were at 30 s/inch. Addition of up to 70 μM ADP (highest concentration tried) did not cause secretion until aggregation was almost complete. Indomethacin was added at a final concentration of 25 μM . This concentration was found to completely block production of detectable malondialdehyde, a measure of prostaglandin synthesis (22); the necessary concentration of indomethacin varied with the stimulus. PFP, platelet-free plasma.

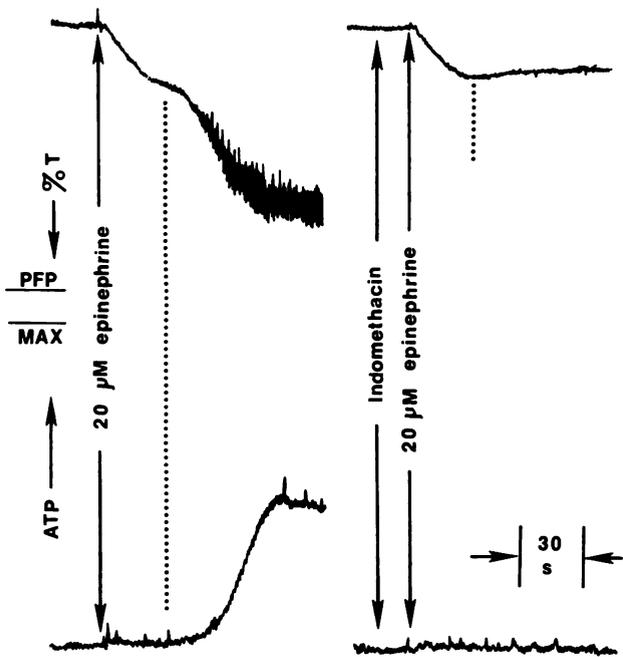


FIGURE 3 Effect of indomethacin on epinephrine-induced aggregation and secretion. The experiments were as described for Fig. 2. Indomethacin was added at a final concentration of $25 \mu\text{M}$. The dotted line indicates the beginning of the second wave of aggregation. We determined this point in two ways: (a) the inflection point in the biphasic curve, and (b) the point where the reversible aggregation curve (on the right) deviated from the biphasic curve. Both methods gave virtually identical results. PFP, platelet-free plasma.

tion was examined by comparison of curves with different concentrations of stimuli with and without indomethacin, an inhibitor of prostaglandin synthesis and "second wave" aggregation. Fig. 2 shows the effects of indomethacin on aggregation and secretion induced by levels of ADP low enough to cause two waves of aggregation or by saturating levels. Indomethacin clearly blocked both second wave aggregation and secretion. Similar results were obtained with epinephrine-stimulated platelets, where biphasic aggregation was more distinct (Fig. 3). Note that the second wave of aggregation precedes the onset of detectable secretion, and again indomethacin blocks both secretion and second wave aggregation.

With thrombin as the stimulus, two different relationships between aggregation and secretion were observed (Fig. 4). At low levels of thrombin, aggregation was biphasic, with secretion detectable only with the beginning of the second wave of aggregation; both secretion and second wave aggregation were blocked by indomethacin. At saturating levels of thrombin, a single wave of aggregation was observed, with secretion and aggregation beginning simultaneously; indomethacin had essentially no effect. Similar results were seen with stimulation by the divalent cation ionophore A23187 (Fig. 5); at low levels there were two waves of aggregation, with secretion and the second wave of aggregation blocked by indomethacin, while at high levels indomethacin had little effect.

A somewhat different pattern of responses was observed with collagen stimulation (Fig. 6). Secretion

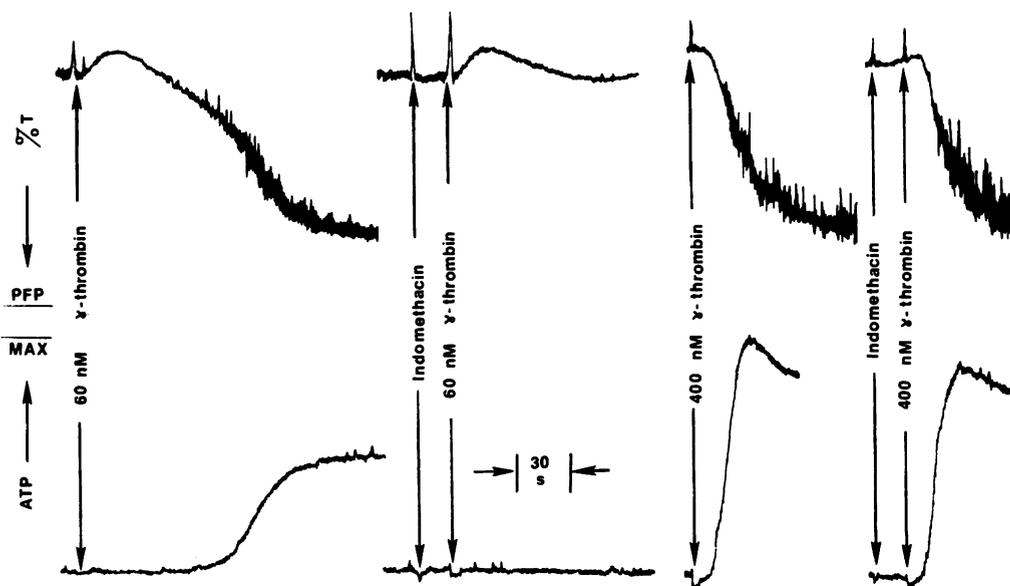


FIGURE 4 Effect of indomethacin on thrombin-induced secretion and aggregation. The experiments were as described in Fig. 2 but with γ -thrombin as stimulus. Indomethacin was added at a final concentration of $25 \mu\text{M}$. Instrument gain was adjusted for high concentrations of thrombin to give half-scale deflection (MAX). PFP, platelet-free plasma.

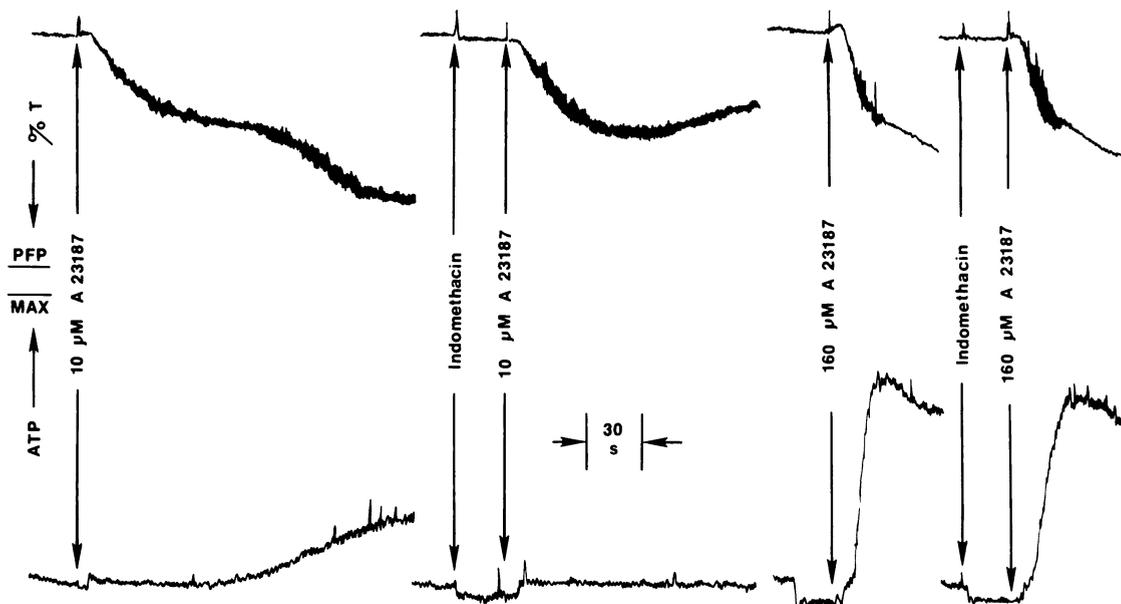


FIGURE 5 Effect of indomethacin on A23187-induced secretion and aggregation. The experiments were as described for Fig. 2 but with A23187 as stimulus and $50 \mu\text{M}$ indomethacin. Instrument gain was adjusted for thrombin to give half-scale deflection (MAX). PFP, platelet-free plasma.

began with, or slightly before, shape change and aggregation, and we have never been able to observe biphasic aggregation or aggregation in the absence of secretion. Indomethacin had essentially no effect at high levels of collagen and only partially

inhibited the responses to low levels. Interpretation of collagen results is complicated by the fact that secretion was significantly inhibited by hirudin (data not shown), but not by heparin plus antithrombin III, suggesting that part of the response may have

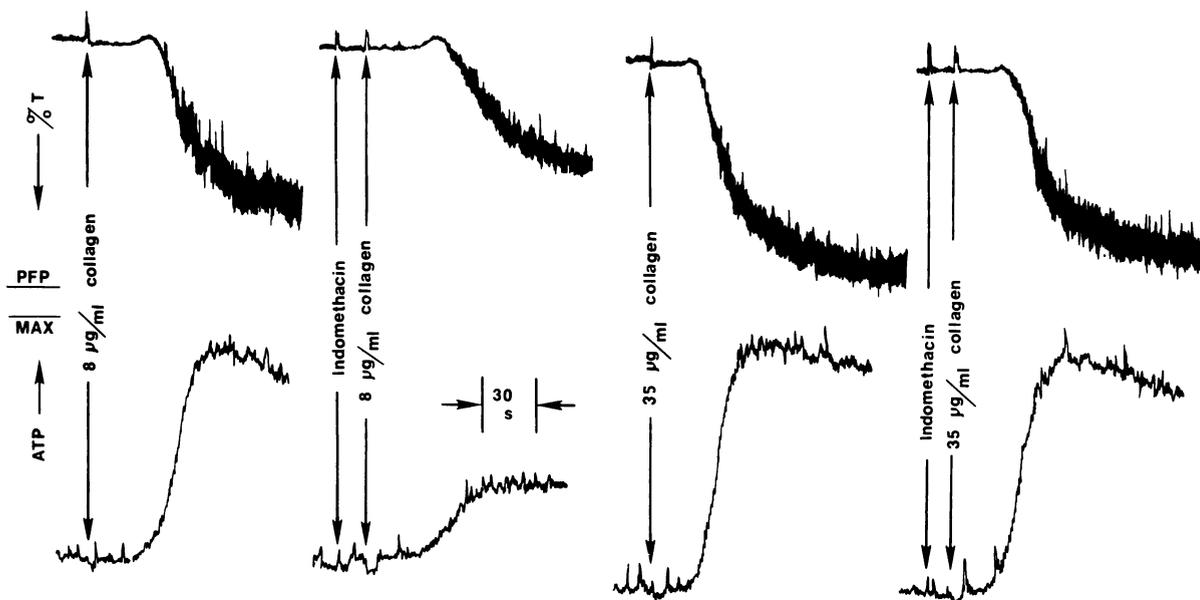


FIGURE 6 Effect of indomethacin on aggregation and secretion induced by collagen. The experiments were the same as for Fig. 2. Indomethacin was added at a final concentration of $25 \mu\text{M}$. Instrument gain was adjusted to give half-scale deflection (MAX) with a saturating level of collagen. PFP, platelet-free plasma.

been due to generation of thrombin. The failure of the antithrombin-heparin complex to give similar inhibition suggests either that hirudin inhibits something other than thrombin or, if thrombin is involved, that it may be generated on the platelet surface where it is not accessible to the larger inhibitor.

DISCUSSION

The progress-time curves of secretion and aggregation presented depict the precise temporal relationship between secretion, shape change, and aggregation. Our data suggest that there are two fundamentally different ways of inducing secretion. One way is dependent upon aggregation, is blocked by indomethacin, and has a substantial lag between induction of aggregation and secretion; the other mechanism is independent of aggregation, is not affected by indomethacin, and shows simultaneous aggregation and secretion. Some stimuli (e.g., ADP and epinephrine) are capable of inducing only aggregation-dependent secretion. Secretion induced by these agents is inhibited by indomethacin and occurs well after aggregation under all conditions and regardless of concentration of stimulus (ADP curve in Fig. 1). Other stimuli (e.g., thrombin and A23187) induce aggregation-dependent secretion at very low levels of stimulus but are also capable of inducing aggregation-independent secretion at higher levels of stimulus.

An important feature of these curves is the relationship between second wave aggregation and secretion, which begin together, with second wave aggregation actually appearing to slightly precede secretion in some traces (Fig. 3). It thus appears that second wave aggregation and secretion are parallel events and may not be causally related. This result suggests that the widely held concept that second wave aggregation is the consequence of secreted substances (i.e., an inter-platelet positive feedback) may be incorrect (14–16). Although it is possible that some active substance (e.g., a prostaglandin intermediate) is released before ATP,² or that at the time of second wave aggregation the platelets have become exquisitely sensitive to traces of secreted substances, we suggest an alternative interpretation. A model that is consistent with our data, that does not presume inter-platelet “feedback” reactions, and that represents a somewhat different way of considering stimulus-response coupling in platelets is shown in Fig. 7.

We postulate two distinct mechanisms leading to

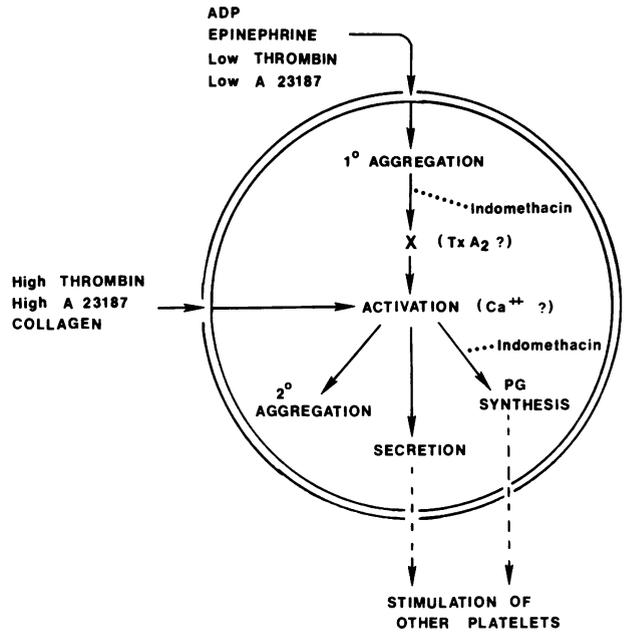


FIGURE 7 Model depicting the proposed relationships between aggregation and secretion induced by various stimuli. Platelets are activated by aggregation by two processes; the first (from the top) is mediated by aggregation via a mechanism that is inhibited by indomethacin, while the second (from the left) bypasses this process and is thus insensitive to indomethacin. Activation leads to secretion, secondary aggregation, and, tentatively, prostaglandin (endoperoxide and thromboxane) synthesis as parallel events; secretion is the most easily measured parameter of activation. Since indomethacin inhibits prostaglandin synthesis in platelets, the indomethacin-sensitive step is tentatively shown to involve some prostaglandin derivative (e.g., thromboxane A₂, TxA₂). Activation is believed to involve an intracellular calcium flux; shape change and primary aggregation may also involve a separate (or weaker) flux of a divalent cation. Released products are presumed to be capable of stimulating other platelets, but this is not essential to account for biphasic aggregation in our model and is suggested by our data only with collagen, where we interpret the partial inhibition by indomethacin (in the absence of any evidence of primary aggregation) to be due to partial activation with a positive feedback through prostaglandin synthesis. The diagram is not meant to imply separate membrane reactions for high and low thrombin and A23187, although this is not excluded.

platelet activation; (a) an aggregation-mediated, indomethacin-sensitive mechanism, and (b) a direct activation mechanism that bypasses the indomethacin-sensitive, aggregation step. Our data suggest that secretion and secondary aggregation follow platelet activation³ as parallel events. We speculate that intra-

² It is believed, but has not been conclusively proved, that secreted substances are stored in the dense bodies and secreted as a packet, so that measurement of any one represents all others. Prostaglandin intermediates are not stored in dense bodies and may be released with a different time course.

³ Aggregation is considered in terms of “primary” and “secondary” aggregation, terms that have not been precisely defined and appear to be used somewhat differently by different authors. We consider primary aggregation to be aggregation by platelets that have not been fully activated

platelet phenomena lead to second wave aggregation and that inter-platelet feedback phenomena are not required as generally believed. While inter-platelet reactions are certainly possible (perhaps even likely under some conditions), such second order effects need not be considered to explain the results presented here. In addition, with aggregation-mediated activation, the circumstance where inter-platelet phenomena have been considered significant, the amount of ATP secreted is much less than with direct activation. This seems inconsistent with a positive feedback mechanism, but is consistent with our model if it is assumed that aggregation-mediated activation is not as complete as direct activation.

Although the model in Fig. 7 is independent of actual intermediates or second messengers, we have considered how the overall scheme might relate to intermediates that have been implicated in stimulus-response coupling in platelets. There is some evidence for a central role for Ca^{2+} in regulation of platelet function (17–20), so that activation may involve an intracellular Ca^{2+} flux. One line of evidence for a Ca^{2+} flux is the ability of the divalent cation ionophore to activate platelets (17–19), and the ability of A23187 to cause shape change and primary aggregation suggests that these processes may also involve Ca^{2+} . Since shape change and aggregation can be seen in the absence of secretion, this would involve Ca^{2+} in a different compartment or simply as a weaker flux, consistent with Holmsen's suggestion that graded Ca^{2+} fluxes lead to graded responses (21). It should be noted, however, that this key role of an intracellular Ca^{2+} flux is still largely hypothetical.

In the model, we suggest that prostaglandin synthesis may be involved in two ways. Inhibition of aggregation-mediated activation by indomethacin, a known inhibitor of cyclooxygenase, implies that prostaglandin synthesis is a necessary intermediate in this mechanism of activation. However, thrombin and A23187 stimulate very rapid prostaglandin synthesis even though platelet activation by these agents is not inhibited by indomethacin, suggesting that prostaglandin synthesis may also be a consequence of platelet activation. Stimuli that induce the greatest yield of secretion (e.g., thrombin) also induce the greatest prostaglandin synthesis (22), while stimuli that induce less secretion (e.g., ADP) also induce

(under most circumstances this will be aggregation without secretion), whereas secondary aggregation is aggregation by fully activated platelets (under most circumstances this will be accompanied by secretion). We define an "activated" platelet phenomenologically as one that secretes, rapidly synthesizes prostaglandins, and undergoes secondary aggregation. Since primary and secondary aggregation are frequently not distinguishable by an aggregometer trace, secretion is usually the best indicator of platelet activation.

less prostaglandin synthesis (22). A major part of the evidence that prostaglandin synthesis is a necessary step in aggregation-mediated activation is that inhibitors of cyclooxygenase block it; however, it is possible that these inhibitors may have other, unknown effects.

Collagen is an especially complicated platelet stimulus and requires detailed discussion. Since native or particulate collagen fibrils are larger than the platelets themselves, the platelet-collagen interaction is best considered a platelet-surface reaction. Since two platelets stuck to a surface are unlikely to have sufficient freedom to aggregate, the concept of primary aggregation may not be meaningful for collagen stimulation. Aggregation never preceded secretion with collagen, and we have been unable to detect aggregation in the absence of secretion. Thus, it appears that collagen induces only direct, or aggregation-independent activation. These considerations suggest to us that with collagen, most aggregation is due to substances released from collagen-activated platelets. Since we only see direct activation by collagen, the partial inhibition of secretion by indomethacin might seem inconsistent with our model. However, the inhibition is seen only at lower levels of collagen where not all platelets react directly with collagen; under these conditions a substantial part of the observed secretion must be due to the second order effects of substances released from collagen-stimulated platelets. The contribution of these second order effects to total secretion would be inhibited by indomethacin if they were due to either released prostaglandin intermediates or secreted ADP (or any agent that activated by an aggregation-mediated mechanism).

In conclusion, the stimuli we have studied can be categorized according to the mechanism by which they activate platelets. ADP and epinephrine activate only by an aggregation-mediated mechanism. Thrombin and A23187 activate by either an aggregation-mediated or a direct activation mechanism, depending upon the concentration. Collagen appears to activate only by the direct activation mechanism. Secondary effects due to released substances appear to be important only with collagen. It should be emphasized that our proposal that secreted substances do not play a major role with most stimuli is based not on the absence of detectable nucleotides before second wave aggregation, but rather on the consistency of a model that considers second wave aggregation and secretion as parallel events and that does not require the previously postulated involvement of secreted substances.

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