

# Synergism of Platelet-aggregating Agents

## Role of Elevation of Cytoplasmic Calcium

J. Anthony Ware, Marianne Smith, and Edwin W. Salzman

Charles A. Dana Research Institute and Harvard-Thorndike Laboratory, Departments of Medicine (Cardiovascular Division) and Surgery, Harvard Medical School, Beth Israel Hospital, Boston, Massachusetts 02215

### Abstract

When a pair of platelet agonists, each in subthreshold concentration, is added together or in sequence to a platelet suspension, the platelet response is enhanced. Addition of two agonists to platelets loaded with aequorin also enhanced the observed rise in cytoplasmic ionized calcium ( $[Ca^{2+}]_i$ ) in response to the second agonist if the agonists were added within 20 s of each other. Enhancement of aggregation and secretion required that an increase in  $[Ca^{2+}]_i$  (as indicated by aequorin but not necessarily indo-1) followed the first agonist, but not that the  $[Ca^{2+}]_i$  remain elevated until addition of the second agonist. Enhancement was not prevented by aspirin, ADP scavengers, or chelators of extracellular  $Ca^{2+}$ . We conclude that a rise in  $[Ca^{2+}]_i$  induced by a first agonist "primes" platelets for an augmented functional response to a second agonist, which is not, however, determined by the  $[Ca^{2+}]_i$  at the time of addition of the second agonist.

### Introduction

That one platelet agonist added to platelet-rich plasma in a concentration too low to cause aggregation enhances the response and leads to induction of aggregation upon addition of another platelet agonist in similar subthreshold concentration has been demonstrated for several pairs of agonists (1–5). This phenomenon is of great interest, since experiments using low concentrations of several agonists may mimic the conditions under which thrombosis occurs in vivo (6, 7); however, the biochemical basis for this synergistic response remains unclear. Many investigators have suggested that synergism between agonists results from an increase in the concentration of cytoplasmic second messengers, including ionized calcium (1, 4), although some attribute the synergistic relation between ADP and epinephrine to an alteration by epinephrine of the ability of ADP to interact with its platelet receptor (8).

Our laboratory has previously described the relationship be-

tween platelet functional changes induced by several agonists and a rise in cytoplasmic ionized calcium concentration ( $[Ca^{2+}]_i$ )<sup>1</sup> as measured by the bioluminescent protein aequorin and the fluorescent indicator 2-methyl-6-methoxy 8-nitroquinoline (quin2) (9–12). These studies have shown that platelet aggregation and secretion by common agonists are invariably preceded or accompanied by an increase in  $[Ca^{2+}]_i$  as indicated by aequorin, but this rise in  $[Ca^{2+}]_i$  is not always shown by quin2 (10–12). Furthermore, inhibition of ADP-induced platelet aggregation by the calcium antagonists verapamil and diltiazem is accompanied by a reduction in the ADP-induced  $[Ca^{2+}]_i$  rise indicated by aequorin, but the ADP-induced  $[Ca^{2+}]_i$  increase reported by quin2 is unchanged (12). Therefore, we have suggested that these indicators reflect different aspects of  $Ca^{2+}$  homeostasis in stimulated platelets, with aequorin being linked more closely to platelet activation.

The purpose of this study was to compare  $[Ca^{2+}]_i$  changes associated with platelet aggregation and secretion in response to several agonists alone and in combination, with particular emphasis on the relationship of enhanced  $[Ca^{2+}]_i$  responses to functional synergism between agonists.

### Methods

Platelet-rich plasma was obtained and the platelets were loaded with aequorin as described previously (9). Calcium measurements were performed using a modified whole-blood Lumiaggregometer (Chronolog Corp., Havertown, PA), which simultaneously provided tracings of platelet aggregation in the same sample (10, 12). In some experiments, EGTA 2 mM was added 30 s before the (first) agonist; in experiments designed to test the influence of contaminating or released ADP, creatine phosphate and creatine kinase (CP/CK) (3) were added in sufficient quantities to prevent aggregation by ADP 2  $\mu$ M, as determined for each experiment in a sample of platelets from the same donor. For experiments involving aspirin-treated platelets, acetylsalicylic acid 1 mM was incubated with the platelet-rich plasma for 30 min before further processing; an aliquot of washed platelets was then stimulated with sodium arachidonate 10  $\mu$ M, and absence of aggregation was regarded as confirmation of the aspirin effect.

Some aliquots were incubated with  $^{14}C$ -labeled 5-hydroxy tryptamine 0.1  $\mu$ Ci/ml before gel filtration. In these samples, incubations with agonists were terminated 2 min after addition of the final agonist; the sample underwent immediate centrifugation through silicone oil and the supernatant was collected for  $^{14}C$ -liquid scintillation counting.

1. *Abbreviations used in this paper:* ASA, acetylsalicylic acid;  $[Ca^{2+}]_i$ , cytoplasmic ionized calcium concentration; CP/CK, creatine phosphate/creatin kinase; quin2, 2-methyl-6-methoxy 8-nitroquinoline; U46619, 9,11-dideoxy-11a, 9a-epoxymethano-prostaglandin F2a.

Address reprint requests to Dr. Ware, Cardiovascular Division, Department of Medicine, Beth Israel Hospital, SL-4, 330 Brookline Avenue, Boston, MA 02215.

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To load platelets with indo-1, a procedure modified from that employed for loading with quin2 was followed (9). Because of the increased brightness of the newer  $\text{Ca}^{2+}$ -sensitive fluorophores such as fura-2 and indo-1 (13), final loading concentrations of 1–2  $\mu\text{M}$  of the acetoxymethyl ester form of the indo-1 were adequate to produce a useful signal-to-noise ratio, as compared with the 5–20- $\mu\text{M}$  concentrations required for quin2 (9).

In preliminary experiments, indo-1 was found to leak from the cytoplasm of washed platelets more rapidly than does quin2, as assessed by quenching extracellular fluorescence with  $\text{MnCl}_2$  (9). The presence of increased amounts of extracellular dye and the 30-fold greater brightness of these newer compounds (13) resulted in an apparent rise in "resting  $[\text{Ca}^{2+}]_i$ " in experiments done in  $\text{Ca}^{2+}$ -containing media (from 60 to 130 nM in 60 min). Therefore, experiments with platelets loaded with indo-1 were carried out within 20 min after gel filtration. Indo-1 signals were calibrated as described by Grynkiewicz et al. (13); experiments at each wavelength of the emission pair were performed on separate aliquots of platelets in parallel.

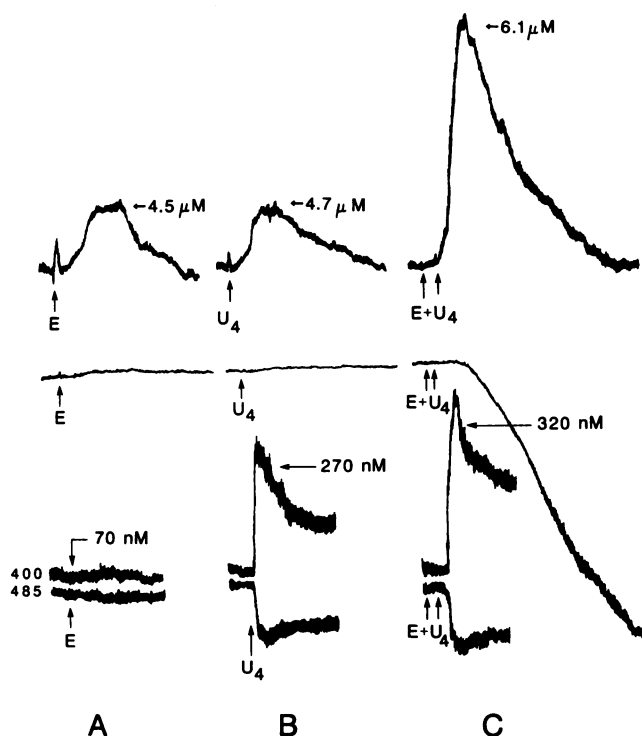
Particular attention was given to finding a concentration of each agonist that was just below the threshold for aggregation or shape change. Concentrations of agonists that did not cause aggregation are referred to as "subthreshold"; in some instances, such concentrations did result in a rise in  $[\text{Ca}^{2+}]_i$ . The agonist pairs studied included epinephrine, ADP, or the  $\text{Ca}^{2+}$ -ionophore A23187 followed by thrombin; thrombin followed by ADP; and epinephrine followed by the thromboxane  $\text{A}_2$  analogue U46619 (Upjohn Co., Kalamazoo, MI). Tracings displayed in the figures are representative of three to five similar experiments. Indo-1 was purchased from Calbiochem-Behring Corp., La Jolla, CA and aequorin was purchased from Dr. John R. Blinks (Mayo Clinic, Rochester, MN). Purified human fibrinogen was kindly provided by Dr. Jack Lindon (Beth Israel Hospital, Boston, MA).

## Results and Discussion

As noted previously (11), each agonist, in concentration-dependent fashion, caused platelet shape change, aggregation, secretion, and a  $[\text{Ca}^{2+}]_i$  rise indicated by aequorin (except for epinephrine, which did not cause shape change). In no instance was shape change or aggregation seen without a concomitant or preceding rise in aequorin-indicated  $[\text{Ca}^{2+}]_i$ ; however, epinephrine did not consistently produce a  $[\text{Ca}^{2+}]_i$  elevation as shown by indo-1 (Fig. 1), even when the platelets aggregated; similar observations were previously made with quin2 (11). With low concentrations of agonists, an elevation in aequorin-indicated  $[\text{Ca}^{2+}]_i$  occurred without aggregation or secretion (Fig. 1), as had been noted with several agonists previously (11).

When a second agonist was added within 20 s of the first, addition of two agonists at subthreshold concentrations resulted in a  $[\text{Ca}^{2+}]_i$  rise greater than that seen with either agonist alone. Full aggregation and enhanced secretion accompanied the enhanced  $[\text{Ca}^{2+}]_i$  rise (Fig. 2 A). If the time interval between addition of the two agonists was longer (Fig. 2 B), there was still enhancement of aggregation and secretion in response to the second agonist, even though enhancement of the  $[\text{Ca}^{2+}]_i$  rise induced by the second agonist was proportionately less obvious, and did not occur after  $\sim 120$  s (Fig. 2 C). These general characteristics were true of all agonist pairs studied.

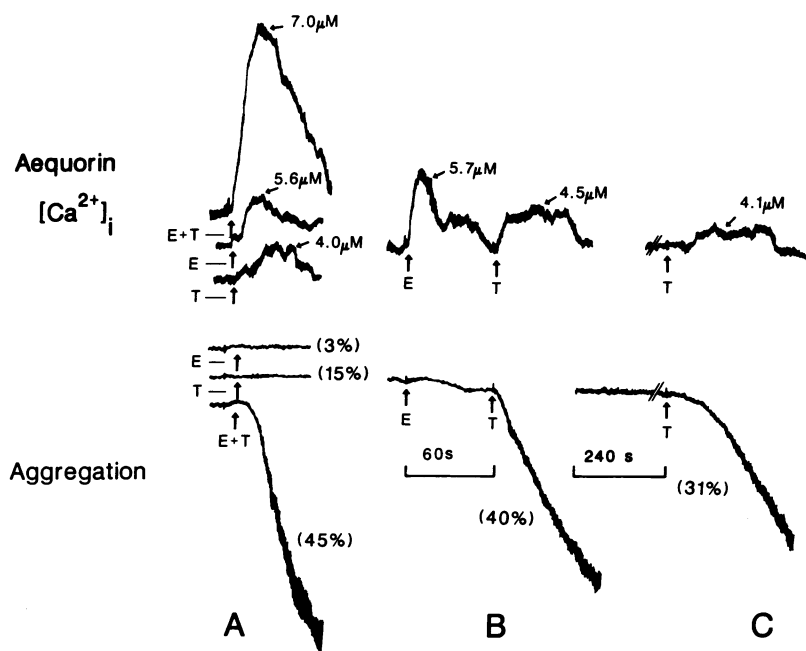
Thus, enhancement of the  $[\text{Ca}^{2+}]_i$  rise and aggregation in response to the second agonist could be uncoupled. Furthermore, persistence of elevation of  $[\text{Ca}^{2+}]_i$  after addition of the first agonist to the time of addition of the second agonist was not required for an enhanced aggregation response to the second agonist.



**Figure 1.** Elevation of  $[\text{Ca}^{2+}]_i$  and aggregation induced by epinephrine and the thromboxane  $\text{A}_2$  analogue U46619. In (A), top and middle tracings represent the response of aequorin-loaded platelets to epinephrine (E), 0.5  $\mu\text{M}$ ; a parallel sample of platelets loaded with indo-1 is seen on the bottom. Excitation wavelength for the indo-1 platelets was 355 nm, and the emission wavelengths corresponding with increased or decreased  $[\text{Ca}^{2+}]_i$  were 400 nm and 485 nm, respectively (13). In (B) the top and middle tracings are from aequorin-loaded platelets treated with subthreshold concentrations of U46619 (U4-50 nM); these agonists aggregate platelets when added together (C). The bottom tracings from indo-1 loaded platelets demonstrate that this concentration of epinephrine enhances U46619-induced aggregation, even though it does not produce an elevation in  $[\text{Ca}^{2+}]_i$  as detected by indo-1.

We studied the influence of the concentration of the first agonist on the enhanced response to the second agonist (Fig. 3). The  $[\text{Ca}^{2+}]_i$  rise, aggregation and secretion produced by thrombin, e.g., were enhanced by ADP in a concentration-dependent manner; however, enhancement of these effects occurred only at concentrations of ADP sufficient to cause an elevation of aequorin-indicated  $[\text{Ca}^{2+}]_i$  if ADP was present by itself (0.5  $\mu\text{M}$  in Fig. 3). No aggregation resulted from ADP alone at this concentration. In experiments using indo-1-loaded platelets, however, enhancement of the  $[\text{Ca}^{2+}]_i$  rise and aggregation produced by a second agonist was possible even if the first agonist did not elevate indo-1-indicated  $[\text{Ca}^{2+}]_i$  (as with epinephrine, Fig. 1). Thus, a first agonist-induced rise in the aspect of platelet  $[\text{Ca}^{2+}]_i$  detected by aequorin, but not necessarily of that reported by the fluorophores, appeared to be essential for enhancement of aggregation or secretion after a second stimulus.

The importance of the rise in  $[\text{Ca}^{2+}]_i$  produced by the first agonist to enhanced aggregation after the second agonist is illustrated by experiments using the  $\text{Ca}^{2+}$ -ionophore A23187 (Fig. 4). As with epinephrine and ADP, only concentrations of A23187 that produced a rise in  $[\text{Ca}^{2+}]_i$  enhanced thrombin-induced ag-



**Figure 2.** Enhancement of thrombin-induced aggregation and secretion by epinephrine. Secretion of  $^{14}\text{C}$ -labeled-5 hydroxytryptamine is expressed as a percentage of total platelet content and is in parentheses. Top tracings are of aequorin luminescence, and the bottom tracings are parallel aggregation tracings. In *A*, epinephrine ( $E$ - $0.5 \mu\text{M}$ ) and thrombin ( $T$ - $0.01 \text{ U/ml}$ ) are added simultaneously to produce aggregation. In *B*) thrombin is added 60 s after epinephrine; aggregation and secretion are still enhanced, but the  $[\text{Ca}^{2+}]_i$  rise is only slightly greater than that seen with thrombin alone. This effect persists even at prolonged time intervals after addition of epinephrine (*C*).

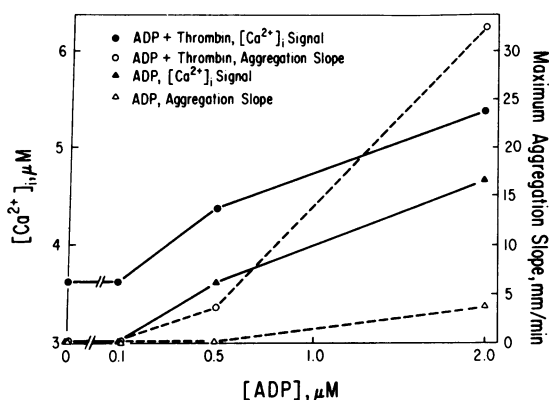
gregation. This enhancement occurred even though the elevated  $[\text{Ca}^{2+}]_i$  following the ionophore had returned to baseline. Also, enhanced aggregation was not dependent on a simultaneously enhanced  $[\text{Ca}^{2+}]_i$  rise.

Addition of EGTA 2 mM to the external media to chelate the extracellular  $\text{Ca}^{2+}$  reduced but did not eliminate the rise in  $[\text{Ca}^{2+}]_i$  and aggregation in response to any agonists, except epinephrine. Epinephrine did not elevate aequorin-indicated  $[\text{Ca}^{2+}]_i$  when EGTA was present in the media (11), nor did it enhance the thrombin-induced  $[\text{Ca}^{2+}]_i$  rise or aggregation under these conditions (Fig. 5). Epinephrine's effect appeared to be totally dependent on the availability of external  $\text{Ca}^{2+}$ . However, enhancement of thrombin-induced aggregation by ADP did not

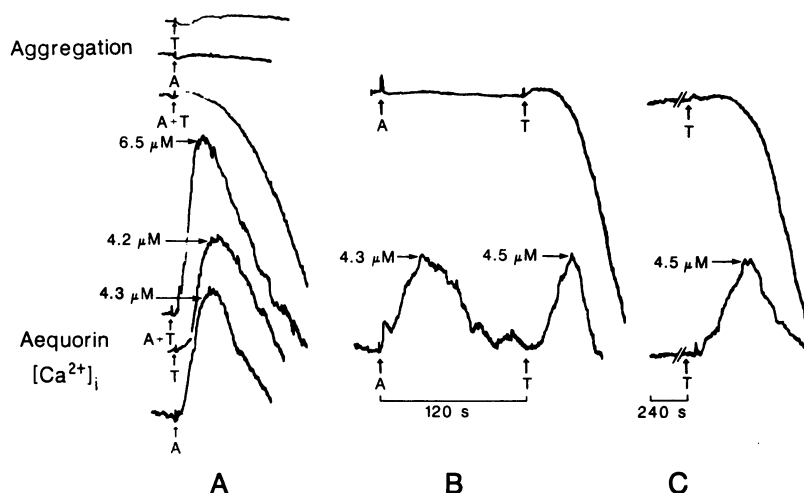
show an absolute dependence on extracellular  $\text{Ca}^{2+}$ , although aggregation in the presence of EGTA was much reduced (data not shown). Thus, the synergistic functional response of two agonists required only that the first agonist increase  $[\text{Ca}^{2+}]_i$ ; the source of the  $\text{Ca}^{2+}$  could be either intra- or extracellular, although both were required for a maximal response.

Experiments were also performed to determine the contributions of cyclooxygenase products and of ADP released from storage granules (Fig. 5). Addition of aspirin or the ADP scavenger CP/CK did not limit the rise in  $[\text{Ca}^{2+}]_i$  or aggregation induced by thrombin alone. Aspirin treatment also did not affect epinephrine's enhancement of aggregation in response to other agonists, confirming other reports (14, 15), or of its enhancement of the  $[\text{Ca}^{2+}]_i$  rise after thrombin administration. However, both the rise in  $[\text{Ca}^{2+}]_i$  and the enhanced aggregation induced by thrombin were reduced, although not eliminated, by phosphorylating released ADP. The combination of aspirin and CP/CK did not lower the enhanced  $[\text{Ca}^{2+}]_i$  rise or aggregation more than did CP/CK alone. Thus, although others have found that epinephrine-induced platelet-binding of fibrinogen (16), aggregation, and secretion are totally inhibited by aspirin (17) or by CP/CK (2, 16), it appears that epinephrine's enhancement of thrombin-induced  $[\text{Ca}^{2+}]_i$  and aggregation is not dependent on cyclooxygenase products and is only partially attributable to released ADP.

Holmsen (18) has postulated that the progression of platelet functional changes from shape change to aggregation and thence to secretion of dense granular contents occurs as a result of increasing concentration of cytoplasmic second messengers. The generality of positive interaction between physiologic agonists (1-6), and the similarities in time dependency (4), might suggest further that the synergistic effect of pairs of agonists occurs as a result of their cumulative effect on the cytoplasmic concentration of ionized  $\text{Ca}^{2+}$  (1, 4, 5). The findings of the present study indicate that the mechanism of both these phenomena is more complex. We found that the  $[\text{Ca}^{2+}]_i$  after addition of a second agonist does not determine enhanced platelet aggregation and secretion. In-



**Figure 3.** Effect of increasing concentrations of ADP on thrombin-induced rises in  $[\text{Ca}^{2+}]_i$  and aggregation. Thrombin,  $0.01 \text{ U/ml}$ , was added 10 s after ADP; platelets were pretreated with aspirin and suspended in  $\text{Ca}^{2+}$ -containing media. Parallel determinations of  $[\text{Ca}^{2+}]_i$  rise and aggregation slope in response to ADP alone are shown for comparison. Only concentrations of ADP sufficient to raise  $[\text{Ca}^{2+}]_i$  were capable of producing aggregation when combined with thrombin; it was not necessary for ADP to produce aggregation for enhancement of the thrombin response to occur.



**Figure 4.** Enhancement of thrombin-induced aggregation by the  $\text{Ca}^{2+}$ -ionophore A23187. Bottom tracings are of aequorin luminescence and the top tracings are parallel aggregation tracings. In *A* A23187 (A-20 nM) and thrombin (T-0.01 U/ml) are added simultaneously to produce aggregation and enhancement of the rise in  $[\text{Ca}^{2+}]_i$ . *B* and *C* show that the enhancement of thrombin-induced aggregation by A23187 persists, even though the  $[\text{Ca}^{2+}]_i$  level elevated by the ionophore has returned to baseline.

stead, a rise in aequorin-indicated  $[\text{Ca}^{2+}]_i$  in response to an initial agonist "primes" the platelet for an augmented response to a second agonist. The degree of augmented response is a function of the peak  $[\text{Ca}^{2+}]_i$  after the first agonist, which mediates a cellular event that has no obvious simultaneous functional correlate, perhaps activation of an enzyme, phosphorylation of some other protein, or modification or expression of receptors on the cell

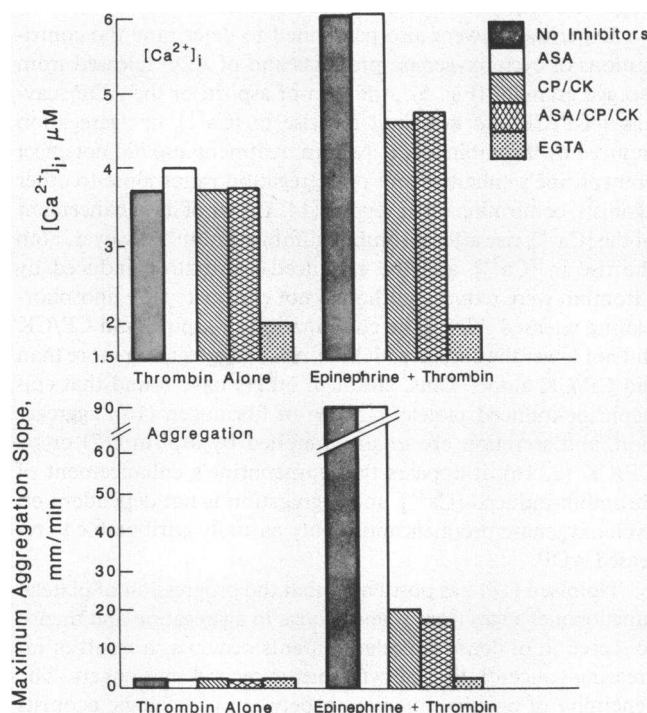
surface, and that persists even after the initiating rise in  $[\text{Ca}^{2+}]_i$  has returned to baseline. This  $\text{Ca}^{2+}$ -mediated event, at present unidentified, effectively lowers the activation threshold for subsequent agonists.

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**Figure 5.** Effect of inhibitors on enhancement of thrombin-induced  $[\text{Ca}^{2+}]_i$  rise (top panel) and aggregation (bottom panel). Media contained  $\text{Ca}^{2+}$  1 mM, except for EGTA samples. No fibrinogen was added. Epinephrine 1  $\mu\text{M}$  was added 10 s before thrombin 0.005 U/ml (0.01 U/ml for the EGTA-containing samples). The bars represent the values obtained in a single experiment, which is representative of four determinations on samples from different donors.

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