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Research Article

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Effect of Common Agonists on Cytoplasmic Ionized Calcium Concentration in Platelets

Measurement with 2-Methyl-6-methoxy 8-nitroquinoline (Quin2) and Aequorin

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Abstract

Because of controversy regarding the relationship of cytoplasmic ionized calcium concentration ($[Ca_i^{2+}]$) to platelet activation, we studied the correlation of platelet aggregation and ATP secretion with $[Ca_i^{2+}]$ as determined by 2-methyl-6-methoxy 8-nitroquinoline (quin2) and aequorin in response to ADP, epinephrine, collagen, the Ca^{2+} ionophore A23187, and thrombin. Both indicators showed a concentration-dependent increase in $[Ca_i^{2+}]$ in response to all agonists except epinephrine when gel-filtered platelets were suspended in media containing 1 mM Ca^{2+}. With epinephrine, a rise in $[Ca_i^{2+}]$ was indicated by aequorin, but not by quin2; $[Ca_i^{2+}]$ signals, aggregation, and secretion were suppressed by EGTA. ADP [0.5 μM] produced a rise in $[Ca_i^{2+}]$ that was registered by both aequorin and quin2 in platelets in Ca^{2+}-containing media; addition of EGTA to the medium raised the threshold concentration of ADP to 5.0 μM for both indicators. Collagen produced progressive concentration-related increases in $[Ca_i^{2+}]$ and aggregation in aspirin-treated aequorin-loaded platelets. Quin2 failed to indicate a rise in $[Ca_i^{2+}]$ at lower collagen concentrations with EGTA or aspirin. $[Ca_i^{2+}]$ response to A23187 and thrombin was reduced by addition of EGTA to platelets loaded with either aequorin or quin2. With all five agonists in all conditions tested, aequorin $[Ca_i^{2+}]$ signals occurred at the same agonist concentration as that or lower than that which produced platelet shape change, aggregation, or secretion. Platelet activation was better correlated with changes in $[Ca_i^{2+}]$ indicated by aequorin than with the response of quin2, possibly because aequorin is more sensitive to local zones of $[Ca_i^{2+}]$ elevation.

Introduction

It has long been recognized that both extracellular and cytoplasmic ionized calcium are important for platelet activation (1–3). Most conclusions regarding the role of cytoplasmic ionized calcium concentration ($[Ca_i^{2+}]$) in platelet shape change, aggregation, and secretion have been based on indirect evidence, including the effect of platelet stimulation on chlortetracycline fluorescence (4), platelet response to Ca^{2+} ionophores (5, 6), estimates of $^{45}Ca^{2+}$ flux (7), and comparison of Ca^{2+}-dependent and -independent phosphorylation of proteins (8). Other studies

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have employed methods providing access to the cell of external buffers by the use of digitonin or high-voltage electrical current (9, 10). Although these methods have yielded much valuable information, the lack of a direct nondestructive indicator of $[Ca_i^{2+}]$ has complicated attempts to correlate platelet activation with $[Ca_i^{2+}]$. Furthermore, the recent description of agonist-induced phospholipid breakdown (11–14), leading to the activation of protein kinase C at basal $[Ca^{2+}]$ levels (12), has led some investigators to postulate the existence of "Ca^{2+}-independent" pathways to platelet activation (15).

The first direct indicator of $[Ca_i^{2+}]$ in cells too small for microinjection was 2-methyl-6-methoxy 8-nitroquinoline (quin2),[1] synthesized by Tsien (16) and used to study agonist-induced changes in $[Ca_i^{2+}]$ homeostasis in platelets by Rink and colleagues (15, 17). The capacity of quin2 to buffer both resting Ca^{2+} and transient rises in $[Ca_i^{2+}]$ (18) renders it less than ideal for measuring agonist-induced changes, especially if these were confined to local areas of cytoplasm (19). Recently, our laboratory has successfully loaded the Ca^{2+}-sensitive photoprotein aequorin (M_r 20,000) into platelets by a nondestructive method and has compared $[Ca_i^{2+}]$ responses to thrombin and the calcium ionophore A23187 with those in quin2-loaded platelets (19). We have suggested that quin2 and aequorin might examine different aspects of $[Ca_i^{2+}]$ flux, aequorin having the capability of more sensitively detecting localized $[Ca_i^{2+}]$ elevations, which might not be revealed by the representation of quin2 of average or diffuse $[Ca_i^{2+}]$. Furthermore, the aequorin-loading procedure does not alter subsequent platelet aggregation or secretion in response to stimuli (19). In the present study, we used these methods to study levels of intraplatelet $[Ca_i^{2+}]$ achieved with varying concentrations of ADP, epinephrine, collagen, ionophore A23187, and thrombin. The relationship between these measurements and platelet aggregation and ATP secretion was examined, and the effects of chelating extracellular Ca^{2+} with EGTA and of blocking thromboxane formation with aspirin were assessed.

Methods

Blood collection. Blood was collected from apparently healthy volunteers taking no medications with known platelet-inhibiting properties. 45 ml of free-flowing blood from venipuncture was collected in 50 ml polypropylene tubes containing 5 ml of trisodium citrate (0.130 M). The sample was centrifuged at 2,000 g for 2 min at 20°C in a model PR 2 centrifuge (International Equipment Co., Boston, MA). Platelet-rich plasma (PRP) was then aspirated into a separate polypropylene tube for loading with either quin2 or aequorin. For studies of aspirin-treated platelets, 1 mM acetyl salicylic acid was added to PRP and incubated

1. *Abbreviations used in this paper:* F, fluorescence; L, luminescence; PGE_2, prostaglandin E_2; PRP, platelet-rich plasma; quin2, 2-methyl-6-methoxy 8-nitroquinoline.

for 15 min. For studies in media without extracellular calcium (i.e., $[Ca_i^{2+}] < 10^{-8}$ M), EGTA was added 30 s before the agonist.

Quin2 loading. In the samples used for measurement of $[Ca_i^{2+}]$ with quin2, PRP was incubated for 15 min with 5–10 μM quin2 acetoxymethyl ester to produce a final intracellular quin2 (acid form) concentration of 0.7–1.0 mM (mean: 0.86), as previously described (19). After the incubation, prostaglandin E_1 (PGE$_1$) (1 μM, final concentration) was added to the sample, which was then centrifuged at 432 g at 20° for 15 min. The supernatant was discarded and the pellet was resuspended in 1 ml of modified Hepes-Tyrode's buffer, prepared as described previously (19). The suspension was then gel-filtered through a 10-ml bed volume Sepharose 2B column preequilibrated with modified Hepes-Tyrode's buffer, containing EGTA (10 μM) and no added Ca^{2+}. The effluent platelet suspension was diluted with Hepes-Tyrode's buffer containing Ca^{2+} 1 mM to achieve a final platelet count of 1×10^8 platelets/ml and incubated for 30–45 min at 37°C before fluorescence measurements were begun. In experiments designed to demonstrate the effect of Ca^{2+} chelation by quin2 on aggregation and aequorin luminescence, platelets were loaded with higher concentrations of quin2 (mean: 2.9 mM) and the platelets were then loaded with aequorin, as described below. After being loaded with both aequorin and quin2, the platelets continued to retain over 90% of the total quin2 in the suspension, as assessed by addition of Mn^{2+} 30 μM (19). Agonist-induced changes in quin2 fluorescence were unchanged from those seen in platelets loaded with quin2 only. The concentration of intracellular quin2 was determined as described previously (19).

Aequorin loading. Our method for loading platelets with aequorin has been described and characterized in detail (19). In brief, a platelet pellet (prepared by centrifugation from PRP) was washed and resuspended in 500 μl of Hepes-Tyrode's buffer containing 10 mM EGTA and 1 μM PGE$_1$. After centrifugation, the platelets were resuspended in 300 μl of a solution containing EGTA (10 mM) MgCl$_2$ (2 mM), ATP (5 mM), and aequorin, 0.2 mg/ml (solution A; see Johnson et al. [19]). This suspension was incubated at 0°C for 60 min, and then recentrifuged. The platelets were resuspended in a solution containing a 0.1 mM EGTA-10 mM MgCl$_2$ (solution B) and reincubated for 60 min. CaCl$_2$ was added to the suspension (300 μM, final concentration); the platelets were warmed to room temperature and gel-filtered in Hepes-Tyrode's buffer containing Ca^{2+} 1 mM. The eluent was diluted with the same buffer, so that most studies were conducted with 5-ml aliquots containing 6×10^8 platelets (range 5×10^7 to 1×10^9). In some experiments, 6-carboxy fluorescein (1 mM) was added with solution A to the platelet suspension instead of aequorin, and the platelets were examined by fluorescence microscopy and by flow cytometry. In experiments involving selective digitonin lysis, 14C-labeled 5-hydroxy tryptamine was incubated with PRP before the platelets were loaded with aequorin. After selective digitonin lysis, a portion of the platelet supernatant was taken for lactic dehydrogenase determinations.

$[Ca_i^{2+}]$ *measurements (quin2).* Aliquots of 1.6 ml of platelet suspension containing 1×10^8 platelets/ml were placed into spectroscopy cuvettes (Evergreen, Los Angeles, CA) which were inserted into a temperature-controlled (37°) spectrophotofluorimeter (Aminco-Bowman, model J4-8911A American Instrument Co., Silver Springs, MD) with a magnetic stirrer. Agonists were added through a light-tight porthole above the sample, and signals were recorded on a Hewlett-Packard 7044 recorder (Hewlett-Packard Co., Palo Alto, CA) with a noise-reduction filter (Pomona Electronics, Pomona, CA). Calibration and determination of $[Ca_i^{2+}]$ levels were done as described by Tsien et al. (16, 18) using the ratio of platelet fluorescence (excitation 339 nm, emission 500 nm), to peak fluorescence (F_{max}) generated by Triton-induced cell lysis multiplied by the dissociation constant (K_d) of quin2 for Ca^{2+}. Changes in platelet autofluorescence produced by addition of agonists, Triton, or EGTA to non-quin2-loaded platelets were subtracted or added as appropriate to the fluorescent signal obtained in quin2-loaded platelets before $[Ca_i^{2+}]$ was calculated, as illustrated by Tsien et al. (20).

$[Ca_i^{2+}]$ *measurements (aequorin).* 5-ml aliquots of platelet suspension containing 5×10^7 to 1×10^9 platelets were placed in polystyrene petri dishes, which were then placed in a heated (37°C) light-tight holder with

a built-in stirrer, above an EMI refrigerated photomultiplier tube (model 8401-A). Agonists were added through a needle placed in a light-tight porthole above the sample. Resulting signals were recorded on a Fisher Recordall strip chart recorder. Calibration of light signals and determination of $[Ca_i^{2+}]$ were by a modification (19) of the method of Blinks et al. (21). The maximum luminescence obtained upon Triton-induced cell lysis in the presence of a saturating concentration of Ca^{2+} (Lmax) was divided into the luminescence obtained at rest or after agonist injection (L). The logarithm of the resultant quotient was compared to a calibration curve provided with each specific lot of aequorin, and the corresponding $[Ca_i^{2+}]$ was read from the curve calibrated for a Mg^{2+} concentration of 1.25 mM. Preliminary experiments showed no increased aequorin luminescence when in vitro aequorin was injected into cell-free buffer containing arachidonic acid, 1 mM, or other reagents used in this study. In some experiments, various concentrations of digitonin were used to lyse platelet membranes selectively, as described by Akkerman et al. (22).

Aggregation and ATP secretion. Studies of platelet aggregation and ATP secretion were performed using a combination aggregometer-photomultiplier tube (Lumiaggregometer, Chrono-Log Corp., Havertown, PA). Samples of 1×10^8 gel-filtered platelets (in 0.45 ml of 1 mM Ca^{2+}-containing Hepes-Tyrode's buffer) either loaded with aequorin or containing no indicator were heated to 37°C. For epinephrine and ADP-induced aggregation, purified human fibrinogen (400 μg/ml, kindly provided by Drs. Jack Lindon and Gerald McManama, Beth Israel Hospital, Boston, MA) was added before the agonist. The method of determining ATP secretion is based on the method of Feinman et al. (23). Firefly lantern extract (10 μl, Sigma Chemical Co., St. Louis, MO) was also added to the sample before the agonist. After each experiment, 1 μM ATP was added, and the resultant light spike provided calibration for assessment of the amount of ATP released during agonist-induced aggregation. Reliable measurements of ATP secretion could not be made in the presence of added ADP. The secretion of ATP produced by this agonist is therefore not reported. The extent of aggregation was measured as the changes in optical density in millimeters from the baseline at 30 and 90 s from agonist addition, and the difference (slope, millimeters/minute) was used to quantify aggregation. The lowest agonist concentration that produced a detectable change in $[Ca_i^{2+}]$, optical density, or ATP secretion from the preagonist baseline in each individual tracing was defined as the threshold concentration. For most agonists the initial response in optical density was an increase, indicating platelet shape change, followed by aggregation. For epinephrine the initial response was a decrease in optical density, indicating aggregation without preceding shape change.

Reagents. Aequorin was purchased from Dr. John Blinks (Mayo Clinic, Rochester, MN). Quin2 was obtained from Amersham Corp. (Arlington Heights, IL). The remainder of the agonists were obtained from commercial sources. All reagents were of enzyme grade.

Data analysis. Data as presented in the figures represent the mean of three to eight separate experiments. The threshold concentrations for aequorin and quin2 $[Ca_i^{2+}]$ rises, aggregation and secretion are presented in Table I. The influence of EGTA, aspirin, both, or neither on $[Ca_i^{2+}]$ and aggregation slope in response to each agonist was compared by parametric multiple sample testing (Newman-Keuls test). A P value of 0.01 or less was considered to be significant.

Results

Preliminary experiments were performed to explore further certain aspects of the mechanism by which aequorin is loaded into platelets. When a high concentration of 6-carboxy-fluorescein was substituted for aequorin in solution A, uniform-appearing fluorescence was noted in all platelets by fluorescence microscopy. Only trace amounts of fluorescence were seen in platelets to which the compound was added after the platelets were removed from solution A. Furthermore, when the platelets were separated on the basis of fluorescence by flow cytometry, 83.6%

Table I. Threshold Concentrations for Elevation of Cytoplasmic $[Ca_i^{2+}]$, Shape Change, Aggregation, and ATP Secretion in 1 mM Ca^{2+} Media after Addition of EGTA, Aspirin, Both, or Neither

Agonist	1 mM Ca²⁺				EGTA				Aspirin				EGTA/Aspirin			
	Aq	Q2	SC/Agg	Sec	Aq	Q2	SC/Agg	Sec	Aq	Q2	SC/Agg	Sec	Aq	Q2	SC/Agg	Sec
ADP, μM	0.5	0.5	0.5	*	5.0	5.0	5.0	*	0.5	0.5	0.5	*	5.0	5.0	5.0	*
Epinephrine, μM	0.5	>20	0.5	0.5	>20	>20	>20	>20	0.5	>20	0.5	20	>20	>20	>20	>20
Collagen, μg/ml	0.5	0.5	0.5	0.5	1.0	5.0	1.0	0.5	1.0	>20	1.0	20	5	>20	10	20
A23187, nM	5	20	10	10	50	50	50	100	50	50	50	100	50	50	50	100
Thrombin, mU/ml	1	5	5	5	5	5	5	5	5	5	5	5	10	10	10	10

Abbreviations: Aq, aequorin-induced [Ca²⁺] increase; Q2, quin2-indicated [Ca²⁺] increase; SC/Agg, shape change or aggregation; sec, secretion of ATP. * Unable to measure secretion of ATP in the presence of ADP.

of the platelets were loaded with the compound. This suggests that molecular species other than aequorin can be loaded into platelets by this technique, and that substances loaded by this method are contained in a high percentage of the platelets in the suspension.

Digitonin was used to lyse selectively the plasma membranes of aequorin-loaded platelets suspended in 1 mM Ca²⁺-containing media and the resultant luminescent spike was compared with that produced by subsequent addition of Triton X-100 to produce complete lysis of platelet organelles. Digitonin (20 μM) released 95% of the total aequorin in the sample; concentrations of 50 μM or greater released 100% of the aequorin. These concentrations of digitonin are much below those that lysed platelet granules or membranes of the dense tubular system (200 μM) (22), indicating that active aequorin does not enter these structures either during or after the loading procedure. At digitonin concentrations of 50 μM, 30% of the total amount of lactic dehydrogenase, a cytoplasmic enzyme, was released, suggesting that the aequorin is located in the cytoplasm. However, we cannot rule out the possibility that some portion of the aequorin is concentrated in a compartment of the platelet that is highly sensitive to digitonin (perhaps just under the plasma membrane).

Typical [Ca²⁺] signals in response to thrombin (0.01 U/ml) with platelets loaded with aequorin or quin2 are seen in Fig. 1. The [Ca²⁺] signals seen in response to agonists differed both qualitatively and quantitatively in aequorin- and quin2-loaded platelets. The aequorin signals displayed a more rapid rise in luminescence to a peak followed by a fall to baseline that may represent either decline in [Ca²⁺] or aequorin consumption (19). Quin2, on the other hand, demonstrated a more gradual rise and plateau of fluorescence. In addition, both the resting and stimulated [Ca²⁺] indicated by aequorin were higher (1-12 μM) than those reported by quin2 (65-780 nM). The values shown in the subsequent figures represent the peak level of [Ca²⁺] after stimulation. It should be noted that the apparently high basal [Ca²⁺] level in resting aequorin-loaded platelets is probably not indicative of the true mean [Ca²⁺], in that it may result from the existence of local zones of elevated [Ca²⁺], the response of extracellular aequorin that has leaked back out of the platelet (except in experiments [see below] in which EGTA is added to the external medium), or the luminescent response of a few damaged platelets in the cell suspension. Another possible cause for the apparently high [Ca²⁺] values is related to certain assumptions made about the cytoplasmic Mg²⁺ concentration. A

higher cytoplasmic Mg²⁺ concentration produces a shift in the calibration curve for aequorin, so that a given L/Lmax indicates a higher [Ca²⁺] (21). The free cytoplasmic Mg²⁺ has been esti-
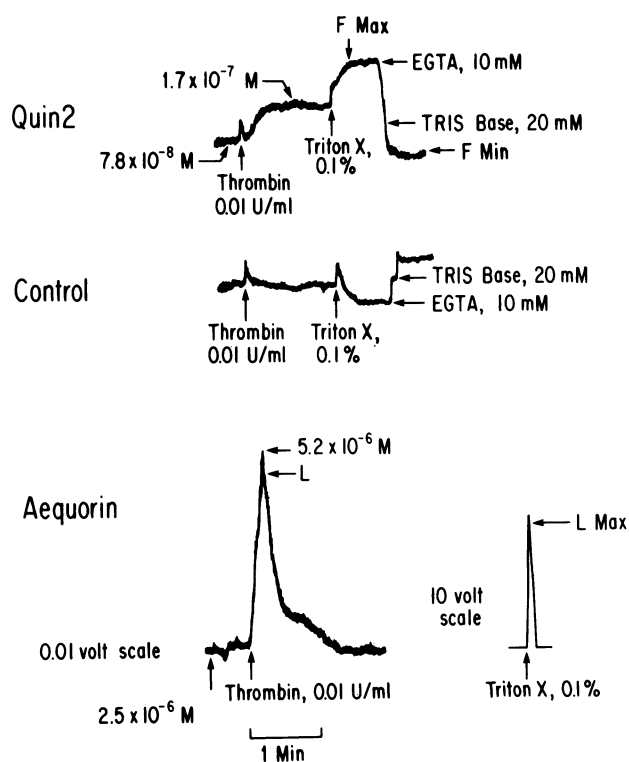

Figure 1. Typical thrombin-induced signals seen using quin2 (*top*) and aequorin (*bottom*) as [Ca²⁺] indicators. Calibration of the quin2 signal is performed by lysing the platelets with Triton X to obtain maximum fluorescence (Fmax), followed by quenching the quin2-associated fluorescence with EGTA and Tris base to obtain minimal fluorescence (Fmin). The fluorescent changes in control (no quin2) platelets (*middle tracing*) are subtracted from the quin2 tracing. The peak aequorin signal is calibrated by lysis of a known number of aequorin-loaded platelets to obtain maximum luminescence (Lmax), and dividing the Lmax into the light signal obtained by stimulation (L). See text for details. Note differences in both the magnitude of resting and stimulated [Ca²⁺] levels indicated by quin2 and aequorin, and the configuration of the signal. Both experiments were performed in media containing 1 mM Ca²⁺.

mated to be between 1 and 5 mM in most mammalian cells, and little or no concentration gradient exists across the cell membrane (24); however, this value has not been determined in platelets. We have arbitrarily used the aequorin calibration curve that assumes a Mg^{2+} concentration of 1.25 mM, which is approximately the value determined in lymphocytes by several methods (25). However, if the free Mg^{2+} concentration in platelets were actually as low as that reported in erythrocytes (0.4 mM [26]), both resting and stimulated $[Ca_i^{2+}]$ would be reduced significantly, possibly by as much as 70%. This adjustment would be roughly proportional, and would not alter substantially the relationships of the curves in Figs. 2–7.

In general, addition of all agonists except epinephrine (thrombin, A23187, ADP, collagen) to platelets suspended in media containing 1 mM Ca^{2+} led to a concentration-dependent increase in $[Ca_i^{2+}]$ indicated by both quin2 and aequorin. Addition of EGTA or prior treatment with aspirin eliminated $[Ca_i^{2+}]$ responses, or decreased them to a variable extent, depending on the agonist. The lowest agonist concentrations associated with $[Ca_i^{2+}]$ responses, shape change, and/or aggregation or ATP secretion (threshold concentrations) are shown in Table I. Secretion of ATP occurred at the same or higher concentration as aggregation, and generally had similar concentration/response characteristics. No shape change, aggregation or secretion was produced by any of the agonists in the absence of an aequorin-indicated $[Ca_i^{2+}]$ increase, but the quin2 signal was not always correlated with functional platelet response.

Concentration–response curves for aggregation and peak $[Ca_i^{2+}]$ levels as indicated by aequorin and quin2 in response to ADP are seen in Fig. 2. The curves shown in this and subsequent figures represent measurements obtained from platelets in media containing 1 mM Ca^{2+}, and with the addition of 2 mM EGTA, 1 mM aspirin, or both. In the presence of 1 mM Ca^{2+}, the $[Ca_i^{2+}]$ levels increased in a concentration-dependent manner in both aequorin- and quin2-loaded platelets.

Quin2 signals, aequorin signals, and platelet shape change were seen with ADP concentrations of 0.5 μM and above. Addition of EGTA to the media raised the threshold ADP concentration to 5 μM for both shape change and $[Ca_i^{2+}]$ signals in quin2- and aequorin-loaded platelets. Primary (reversible) aggregation in aspirin-treated platelets in Ca^{2+}-containing media was first seen at 0.5 μM ADP and was accompanied by $[Ca_i^{2+}]$ signals in both quin2- and aequorin-loaded platelets. Small $[Ca_i^{2+}]$ rises accompanied by platelet shape change were also obtained with both indicators in platelets stimulated by ADP in media containing both EGTA and aspirin. In general, chelation of extracellular Ca^{2+} limited both the rise in $[Ca_i^{2+}]$ and the functional response; addition of aspirin affected $[Ca_i^{2+}]$ rise only modestly. Although the slopes of primary aggregation with and without aspirin were similar, ADP did not result in secondary (irreversible) aggregation in the presence of aspirin. The $[Ca_i^{2+}]$ as indicated by quin2 was not significantly altered by aspirin.

Platelet responses to increasing concentrations of epinephrine are shown in Fig. 3. No rise in $[Ca_i^{2+}]$ was reported by quin2 in any instance. However, at or above epinephrine concentrations of 0.5 μM, $[Ca_i^{2+}]$ indicated by aequorin rose in both aspirin-treated and non-aspirin-treated platelets in Ca^{2+}-containing media, but not after addition of EGTA. The aequorin-indicated $[Ca_i^{2+}]$ correlated with aggregation and ATP secretion; neither could be obtained in the presence of EGTA, indicating a functional dependence on external Ca^{2+}. As with ADP, only primary aggregation was obtained with aspirin-treated platelets.
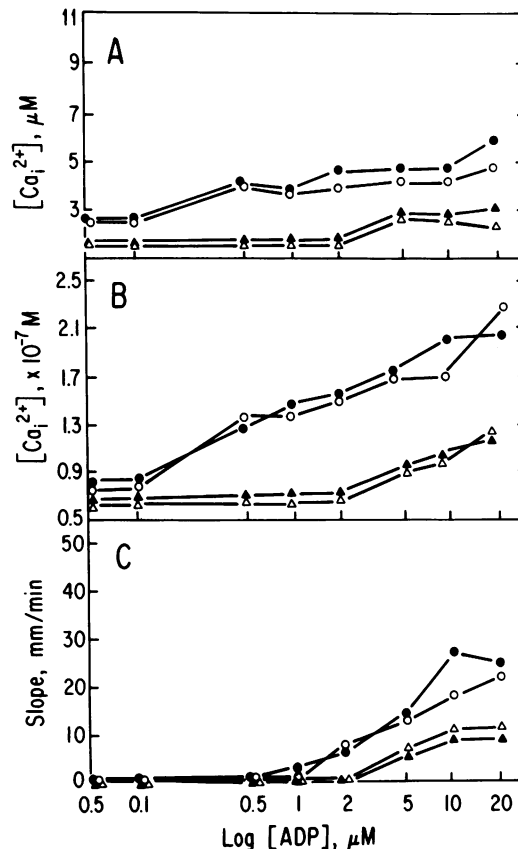

Figure 2. Peak $[Ca_i^{2+}]$ signals as reported by aequorin (A) and quin2 (B), and slope of aggregation as defined in Methods (C), in response to increasing concentrations of ADP. (●, ▲) Values obtained in media containing 1 mM Ca^{2+} and $<10^{-8}$ M Ca^{2+} (EGTA, 2 mM), respectively. (○, △) Values obtained with aspirin-treated platelets in media containing 1 mM Ca^{2+} and $<10^{-8}$ Ca^{2+}, respectively. The threshold concentration for shape change was 0.5 μM for both aspirin-treated and non-aspirin treated platelets; there was no significant aggregation at this agonist concentration. Agonist concentrations are considered "subthreshold" if addition of agonist produces no change in $[Ca_i^{2+}]$ signal (with quin2 or aequorin) above the basal level in unstimulated platelets. As noted in the text and in Johnson et al. (19), the basal level of $[Ca_i^{2+}]$ reported by aequorin is probably not indicative of the true mean $[Ca_i^{2+}]$.

In Fig. 4, both quin2 and aequorin responses and the functional correlates in response to increasing collagen concentrations are seen. Shape change and $[Ca_i^{2+}]$ signals with both indicators were seen at 0.5 μg/ml collagen in Ca^{2+}-containing media. After the addition of EGTA, only the aequorin-indicated $[Ca_i^{2+}]$ correlated with platelet shape change and aggregation. Furthermore, in aspirin-treated platelets, quin2 did not indicate a $[Ca_i^{2+}]$ elevation at concentrations of 20 μg/ml or less of collagen. With EGTA added, no quin2 signal was obtained from aspirin-treated platelets; both aequorin signals and aggregation were present, but significantly diminished. Both external Ca^{2+} and cyclooxygenase products contributed to the aequorin-indicated $[Ca_i^{2+}]$ rise and full aggregation; elimination of either decreased both the aequorin signal and aggregation, and markedly inhibited the quin2 indicated $[Ca_i^{2+}]$ rise in response to collagen.

In response to ionophore A23187 (Fig. 5), both aequorin and quin2 reported $[Ca_i^{2+}]$ signals at agonist concentrations of 5–20 nM in Ca^{2+}-containing media, but not until 50 nM after
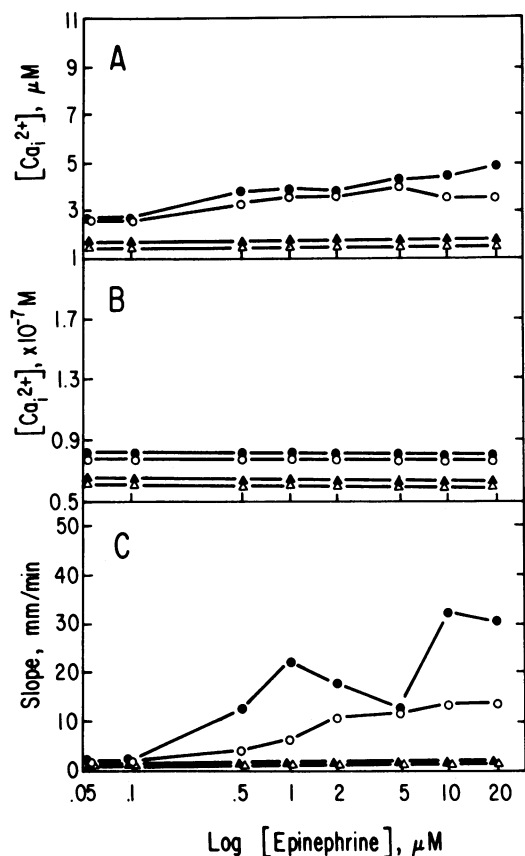
Figure 3. Peak [Ca$_i^{2+}$] signals as reported by aequorin (*A*) and slope of aggregation (*B*) in response to increasing concentrations of epinephrine. Symbols are as in Fig. 2. No elevation of [Ca$_i^{2+}$] was indicated by quin2 in response to epinephrine. No increase in [Ca$_i^{2+}$] or aggregation of aequorin-loaded platelets was seen in response to epinephrine with 2 mM EGTA added to the medium.

Figure 4. Peak [Ca$_i^{2+}$] signals as reported by aequorin (*A*) and quin2 (*B*), and aggregation slope (*C*) in response to increasing concentrations of collagen. Symbols are as in Fig. 2. No increases in [Ca$_i^{2+}$] with low concentrations of collagen were seen in quin2-loaded platelets with EGTA, aspirin or both, although shape change, aggregation, and [Ca$_i^{2+}$] responses were noted in aequorin-loaded platelets.

addition of EGTA or aspirin. These concentrations corresponded to those at which shape change or aggregation were first seen. EGTA and aspirin had similar inhibitory effects on both the aequorin-indicated [Ca$_i^{2+}$] rise, and the aggregation slope. In quin2-loaded platelets, addition of EGTA significantly decreased the peak [Ca$_i^{2+}$] values, but aspirin treatment did not produce signals significantly different from control platelets in 1 mM Ca^{2+}-containing media. As appears to be the case with collagen, both external Ca^{2+} and cyclooxygenase products contribute to platelet activation and the rise in [Ca$_i^{2+}$] by A23187, but neither is absolutely required for platelet shape change and aggregation.

The relationship of [Ca$_i^{2+}$] and aggregation in response to thrombin is seen in Fig. 6. Low concentrations (0.001 U/ml) of thrombin produced relatively large aequorin-indicated [Ca$_i^{2+}$] rises (4.65±0.8 μM) in Ca^{2+}-containing media. Platelet shape change and aggregation were produced by thrombin concentrations of 0.005 U/ml or greater. Aspirin treatment decreased quin2-indicated [Ca$_i^{2+}$] responses, but not the aggregation slope. Addition of EGTA significantly decreased [Ca$_i^{2+}$] signals as shown with either indicator. [Ca$_i^{2+}$] signals and aggregation with both indicators were obtained with aspirin-treated platelets in EGTA but were significantly less than those seen with either EGTA or aspirin alone.

As noted above, there were several instances in which platelet aggregation was accompanied by an increase in aequorin-indi-
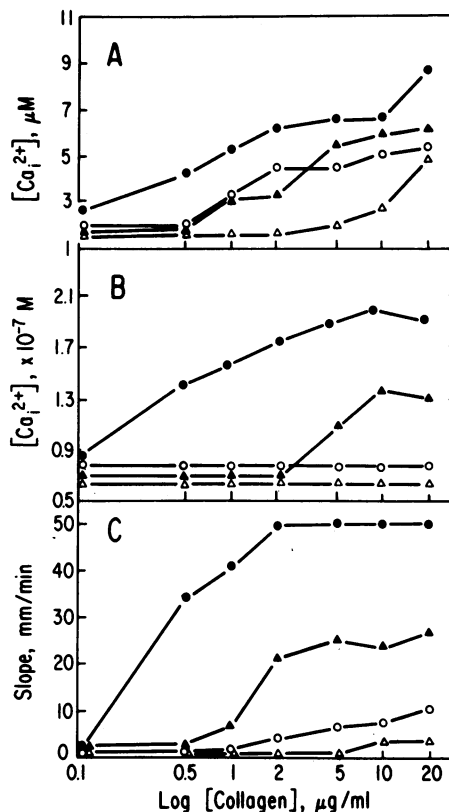
cated [Ca$_i^{2+}$], but not by an increase in [Ca$_i^{2+}$] shown by quin2. This suggests that either quin2 buffered the agonist-induced rise in [Ca$_i^{2+}$] sufficiently to prevent its producing a rise in fluorescence, or that aequorin and quin2 detect different pools of [Ca$_i^{2+}$] within the platelet. To distinguish between these possibilities, platelets loaded with both aequorin and high concentrations of quin2 (2.9 mM) were treated with epinephrine 10 μM or collagen 10 μg/ml; aggregation and aequorin-indicated [Ca$_i^{2+}$] rises are shown in Fig. 7. Addition of high concentrations of quin2 delayed and decreased aequorin-indicated peak [Ca$_i^{2+}$] and aggregation, but did not eliminate either; therefore, the lack of a quin2 rise under these circumstances is not solely due to the dye blunting the [Ca$_i^{2+}$] transient resulting from agonist addition. The basal [Ca$_i^{2+}$] as measured by aequorin was not markedly affected by quin2, as we have noted previously (19). Aequorin-indicated [Ca$_i^{2+}$] closely paralleled the platelet functional response, which suggests that [Ca$_i^{2+}$] rises seen by aequorin are of functional importance.

Discussion

In this study, an increase in platelet [Ca$_i^{2+}$] indicated by aequorin always occurred at an agonist concentration the same as or lower than that which produced shape change, aggregation, or ATP secretion. This relationship applied in both aspirin-treated and untreated platelets and in both 1 mM Ca^{2+} and <10^{-8} M Ca^{2+}
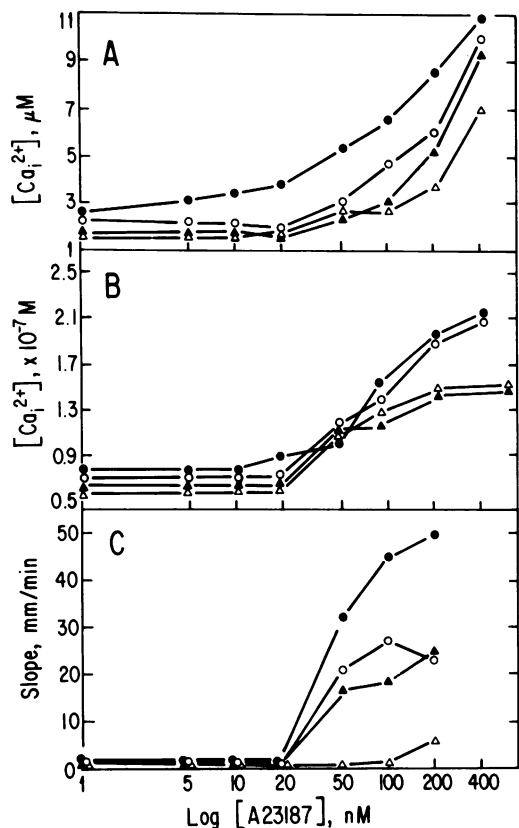
Figure 5. Peak [Ca$_i^{2+}$] signals as reported by aequorin (*A*) and quin2 (*B*), and aggregation slope (*C*) in response to ionophore A23187. Symbols are as in Fig. 2. Platelet shape change occurred without significant aggregation at 10 nM A23187 in 1 mM Ca^{2+} medium.

Figure 6. Peak [Ca$_i^{2+}$] signals as reported by aequorin (*A*) and quin2 (*B*), and slope of aggregation (*C*) in response to increasing concentrations of thrombin. Symbols are as in Fig. 2.

(in EGTA). Quin2-indicated [Ca$_i^{2+}$] elevations usually accompanied functional changes as well, but, in several instances (collagen and epinephrine), aggregation and secretion occurred without elevation of the quin2 signal. This latter finding has been reported by others (15, 17, 27) and has been offered as evidence of the existence of a "Ca^{2+}-independent" pathway to platelet activation (15, 17, 28). However, as we have suggested, certain features of quin2 (high Ca^{2+} buffering capacity, low K_d, relative insensitivity to [Ca$_i^{2+}$] change above 1–2 μM) may prevent detection of localized rises in [Ca$_i^{2+}$] if these exist. Such local changes might, however, produce a prominent aequorin signal owing to its exponential (2.5 power) relationship of luminescence to [Ca$_i^{2+}$] (19). That such local zones of [Ca$_i^{2+}$] elevation might exist has been suggested from work done with *Limulus* photoreceptors (29), insect salivary glands (30), rat mast cells, and macrophages during phagocytosis (31, 32). It seems possible that platelet activation induced by epinephrine and, in certain circumstances, collagen is associated with an increase in [Ca$_i^{2+}$] too localized for quin2 to detect, or in an area of the platelet cytoplasm that quin2 does not sample. This observation does not, of course, rule out the existence of a "Ca^{2+}-independent" pathway; the increases in [Ca$_i^{2+}$] associated with all of the agonists may result directly from the agonist or agonist-receptor coupling or may be secondary to the breakdown of phosphatidyl inositol-4,5-bisphosphate or derivatives and subsequent formation of inositol triphosphate or phosphatidic acid, both of which have been reported to abet Ca^{2+} mobilization (11, 13). Comparative studies examining the temporal relationship of
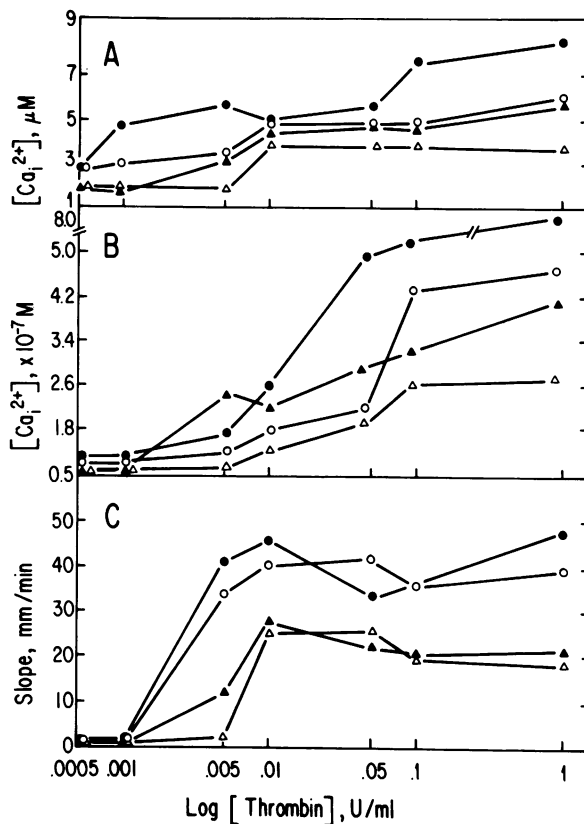
[Ca$_i^{2+}$] transients to markers of phosphoinositide metabolism might clarify this issue. In particular, other investigators have found evidence of agonist-induced activation of protein kinase C in platelets, and have suggested that this pathway has synergistic effects with [Ca$_i^{2+}$] mobilization on platelet response (12). The aequorin-indicated [Ca$_i^{2+}$] also supports this view, because a simple correlation between the [Ca$_i^{2+}$] generated by agonists and the functional response cannot be demonstrated. For instance, thrombin [0.001 U/ml] generates [Ca$_i^{2+}$] levels of 4.5 μM in the absence of aggregation, while a similar [Ca$_i^{2+}$] generated by 10 μM epinephrine is associated with full aggregation and secretion. This suggests that other factors in addition to a rise in [Ca$_i^{2+}$] mediate platelet response to agonists or that the rise in [Ca$_i^{2+}$] induced by different agonists occurs in different locations in the cell.

In previous comparisons of intraplatelet quin2 and aequorin, we reported that peak [Ca$_i^{2+}$] indicated by the two techniques were considerably different; peak [Ca$_i^{2+}$] indicated by quin2 are in the 100–700 nM range, whereas aequorin-indicated [Ca$_i^{2+}$] levels in response to agonists are in the 2–12 μM range (19). A possible explanation is that aequorin's luminescent response permits detection of local zones of micromolar levels of [Ca$_i^{2+}$], as stated above. Quin2, on the other hand, tends to indicate an "average" of the [Ca$_i^{2+}$] values emanating from both high and low [Ca$_i^{2+}$] regions of the platelet. However, this is not an inherent property of aequorin loaded into cells by means other than microinjection; Snowdowne and Borle (33) found that aequorin loaded into cultured renal cells by hypoosmotic shock indicated a resting [Ca$_i^{2+}$] of 50–100 nM, which is in the
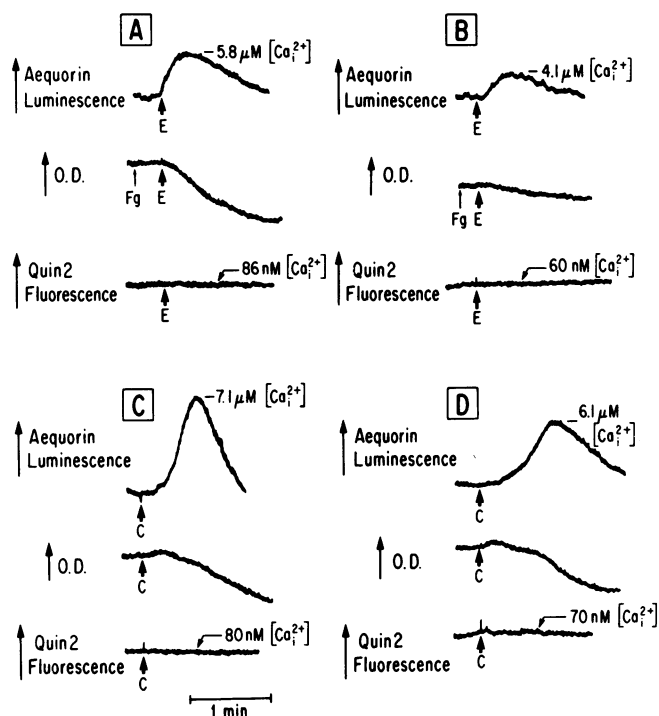
Figure 7. [Ca$_i^{2+}$] signals and aggregation in platelets loaded with aequorin, quin2, or both indicators. All tracings were obtained from platelets suspended in media containing Ca^{2+}, 1 mM. In A, the top and middle tracings represent the response of aequorin-loaded platelets to epinephrine (E), 10 μM; a parallel sample of platelets loaded with quin2 (0.85 mM) is seen on the bottom. Fibrinogen (Fg) 400 μg/ml was added to the platelet sample before epinephrine. In B, similar tracings were obtained in platelets loaded with both aequorin and quin2 (2.9 mM). Addition of very high quin2 concentrations delayed and decreased, but did not eliminate, rises in aequorin-indicated [Ca$_i^{2+}$] and aggregation. In neither A nor B was a rise in quin2 fluorescence seen. In C, the top and middle tracings were produced by stimulation of aspirin-treated aequorin-loaded platelets by collagen (C), 10 μg/ml; the bottom tracing represents a parallel sample of aspirin-treated quin2 (0.78 mM)-loaded platelets. Similar tracings obtained with aspirin-treated platelets loaded with both aequorin and quin2 (2.9 mM) are seen in D. Aequorin-indicated [Ca$_i^{2+}$] signals and aggregation were delayed and/or decreased, but were not eliminated by high concentrations of quin2. No increase in quin2 fluorescence was induced by epinephrine at either low or high quin2 concentrations.

range of that reported by quin2. Elevation of the resting [Ca$_i^{2+}$] is also not an inherent property of the EGTA-ATP method of loading aequorin, as DeFeo and Morgan (34), using a method very similar to ours and assuming an intracellular Mg^{2+} concentration of 0.5 mM, have recently reported a resting [Ca$_i^{2+}$] of 180 nM in ferret vascular smooth muscle, which is in the range estimated by Ca^{2+}-sensitive electrodes in skeletal muscle. Significant differences in aequorin-indicated basal [Ca$_i^{2+}$] may exist between different cell types, as Snowdowne et al. (35) found micromolar resting [Ca$_i^{2+}$] in rat ventricular myocytes loaded with aequorin using hypoosmotic shock, although quin2-indicated [Ca$_i^{2+}$] in rat myocytes appears to be in the 180 nM range (36). Snowdowne et al. (35) speculated that the elevated resting [Ca$_i^{2+}$] seen with aequorin was due to a population of myocytes that were either spontaneously contracting or permeable to extracellular Ca^{2+}; the possibility that aequorin was simply "overrepresenting" a localized zone of high [Ca$_i^{2+}$] was not considered.

Other possibilities for the apparently elevated basal [Ca$_i^{2+}$] seen with aequorin were considered previously, including the possibility that some of the aequorin is located in platelet granules, which have a much higher [Ca^{2+}], or that elevated [Ca$_i^{2+}$] results from aequorin discharge in a few damaged, and hence permeable, cells (19). Although we still cannot discard these possibilities, the results from the experiments in which plasma membranes were selectively lysed with digitonin, as described by Akkerman et al. (22), suggest that platelet granules do not contain a measurable quantity of undischarged aequorin. This does not necessarily establish that aequorin is freely diffusable in the platelet cytoplasm; in fact, the very low concentrations of digitonin required to release >90% of the aequorin might suggest that it is located superficially, perhaps just under the plasma membrane, which may fortuitously be an area of high [Ca$_i^{2+}$] concentration (37). The experiments in which 6-carboxy-fluorescein was uniformly loaded into platelets suggest that, if one assumes that aequorin is loaded by a process similar to this compound, the vast majority of platelets are at least capable of contributing to the aequorin-indicated [Ca$_i^{2+}$] signal. Further work is necessary before these possible artifacts can be confidently discarded.

When EGTA was added to chelate external Ca^{2+}, all agonists (except epinephrine) continued to generate concentration-dependent increases in aequorin luminescence. However, aequorin responses generated by thrombin, ADP, A23187, and collagen were less in EGTA than in Ca^{2+} media, indicating that increased permeability to extracellular Ca^{2+} may be one component of platelet activation by these agonists. Alternatively, other investigators have found that EGTA inhibits phospholipase C activity (38), which could limit platelet activation by some agonists, presumably by decreasing formation of metabolites with ionophoric properties and, indirectly, arachidonic acid (39). The finding that all of the agonists (except epinephrine) could mobilize intracellular Ca^{2+} in aspirin-treated platelets, and that these [Ca$_i^{2+}$] increases correlated with functional changes, offers support to the findings of Packham et al. (40), who found evidence for a dominant third pathway to platelet activation by thrombin and A23187 unassociated with cyclooxygenase metabolites. The present study suggests that this third pathway may be closely linked to mobilization of intracellular Ca^{2+}.

The [Ca$_i^{2+}$] signals reported by aequorin in response to ADP were much lower than those generated by moderate concentrations of collagen or thrombin, which correlates with their comparative effects on aggregation and phosphoinositide turnover (41). Both EGTA and aspirin decreased the aequorin-indicated [Ca$_i^{2+}$] response. Influx of extracellular Ca^{2+} may be crucial only during secondary aggregation due to ADP, as predicted by ^{45}Ca^{2+} studies (7, 42); however, the binding of extracellular Ca^{2+} to the platelet surface appears to be necessary for the earliest stages of platelet activation by ADP or epinephrine (43). Chelation of this extracellular Ca^{2+} by EGTA might explain the diminished aggregation and aequorin-induced Ca^{2+} signal. In sum, initial platelet activation by ADP is closely linked to an influx or internal redistribution of Ca^{2+}. Irreversible aggregation appears to require cyclooxygenase products as well; the peak [Ca$_i^{2+}$] is not significantly higher in non-aspirin-treated platelets, and therefore is not the sole cause of irreversible aggregation.

The finding that epinephrine-induced [Ca$_i^{2+}$] increase and platelet activation are highly dependent on extracellular Ca^{2+} is consistent with the hypothesis (7, 42) that the chief mode of action of epinephrine is to make the external platelet membrane permeable to extracellular Ca^{2+}. No quin2 signals were seen fol-

lowing epinephrine, confirming previous studies (27). $[Ca_i^{2+}]$ increase and primary aggregation (but not secretion) occurred in aspirin-treated platelets, indicating further that cyclooxygenase products are not required for influx of Ca^{2+} (42). In contrast to ADP, epinephrine-induced primary aggregation is not accompanied by shape change. Comparison of the $[Ca_i^{2+}]$ levels achieved with epinephrine (3.6–5.3 μM) and ADP (2.6–4.5 μM) does not explain the lack of shape change with epinephrine on the basis of an insufficient rise in $[Ca_i^{2+}]$. A difference between ADP and epinephrine in intracellular location of the rise in $[Ca_i^{2+}]$ might account for this phenomenon, but evidence for this is lacking.

In experiments using collagen as an agonist, there was a marked discrepancy between concentrations at which aequorin and quin2 signals were produced in the absence of extracellular Ca^{2+} or with aspirin (Fig. 4). Platelet aggregation and secretion occurred in the presence of aspirin, although much less than that seen without aspirin, and were associated with rises in $[Ca_i^{2+}]$, as indicated by aequorin. Comparison of the aspirin and EGTA curves with the corresponding curve in 1 mM Ca^{2+} indicates that collagen can employ either external Ca^{2+} or thromboxane A_2 for platelet activation at low agonist concentrations, but depends on neither to generate $[Ca_i^{2+}]$ rise or primary aggregation at higher concentrations. For full platelet response, cyclooxygenase products appear to be required, as $[Ca_i^{2+}]$ of 5 μM were generated in the presence of aspirin with only small amounts of aggregation; thus, aequorin-indicated $[Ca_i^{2+}]$ is not the sole determinant of platelet response. An explanation has recently been offered by Lapetina and Siess (44), who suggested that collagen-induced activation of phospholipase C, which is relatively insensitive to increased $[Ca_i^{2+}]$, is dependent on cyclooxygenase products. The discrepancy between the $[Ca_i^{2+}]$ increases as indicated by aequorin and quin2 was not simply due to aequorin's greater sensitivity, as aequorin-indicated $[Ca_i^{2+}]$ of similar magnitude but obtained under different experimental conditions (e.g., with aspirin or EGTA) were associated with large changes in the quin2-indicated $[Ca_i^{2+}]$.

The differences between quin2 and aequorin-indicated $[Ca_i^{2+}]$ in collagen and epinephrine-treated platelets were further investigated in experiments using platelets loaded with both indicators, with a much higher quin2 concentration than was used in the other experiments so that the effect of increased Ca^{2+} chelation could be assessed (Fig. 7). Addition of quin2 delayed and decreased both aggregation and the aequorin-indicated peak $[Ca_i^{2+}]$, but did not eliminate either, despite the lack of a rise in quin2 fluorescence. This suggests that aequorin and quin2 detect different pools of $[Ca_i^{2+}]$ within the platelet, although there are some aspects of $[Ca_i^{2+}]$ homeostasis reflected by both.

The curves obtained using ionophore A23187 indicate that extracellular Ca^{2+} participates in generation of the aequorin $[Ca_i^{2+}]$ signal. However, $[Ca_i^{2+}]$ mobilization occurred in the presence of EGTA as well, although less than that seen in Ca^{2+}-containing media. Aggregation and secretion were also less in EGTA, but did occur; this suggests that Ca^{2+} originating extracellularly is not essential for platelet activation by A23187 and supports the studies of secretion in response to A23187 reported by Holmsen and Dangelmaier (45). These authors concluded that EGTA did not inhibit platelet response to A23187 in non-aggregating platelets. Aequorin signals and platelet aggregation were significantly decreased by aspirin, but quin2 signals were unchanged. One hundredfold greater concentration of A23187 was necessary to produce secretion in aspirin-treated platelets;

secretion in this circumstance may result totally from aggregation rather than as a direct effect of A23187 (46).

As with A23187, it is evident that thrombin can elevate $[Ca_i^{2+}]$ associated with aggregation and secretion in the absence of arachidonic acid metabolites or extracellular Ca^{2+}. At thrombin concentrations of 0.001 U/ml, a large aequorin signal, representing 4.5 μM $[Ca_i^{2+}]$, was observed, although shape change and secretion did not occur until the thrombin concentration reached 0.005 U/ml. This was not observed with the other agonists tested and only occurred in the presence of extracellular Ca^{2+}. Low concentrations of thrombin might increase cellular permeability to extracellular Ca^{2+}, leading to a localized submembranous accumulation of Ca^{2+} of no apparent functional importance. Perhaps such a localized increase in $[Ca_i^{2+}]$ participates in thrombin-induced expression of fibrinogen receptors (47), which also occurs at low thrombin concentrations and is closely linked to further platelet activation and aggregation. It would be of interest to compare thrombin-induced localized $[Ca_i^{2+}]$ transients and expression of fibrinogen-binding sites or other functional changes not detected by aggregation or secretion measurements.

In conclusion, this study suggests that aequorin reflects different aspects of $[Ca_i^{2+}]$ homeostasis than does quin2. Aequorin-indicated $[Ca_i^{2+}]$ rises occur at agonist concentrations similar to those that produce platelet shape change, aggregation, and secretion whereas this is not always true with quin2. The data in this study do not support the existence of a pathway to platelet activation totally independent of intracellular Ca^{2+} mobilization, but do suggest that $[Ca_i^{2+}]$ is not the sole determinant of platelet response.

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