Effect of Ionic Calcium and Magnesium on Human Platelet Aggregation (34997)

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It is well-known that calcium is necessary for platelet aggregation. Hovig (1) observed that neither ADP nor collagen produced aggregation of rabbit platelets when the calcium concentration was less than 1.1–1.9 $\times$ 10^{-4} *M*. When the calcium concentration was 0.3–4.0 $\times$ 10^{-3} *M* maximal aggregation resulted. He arrived at these values by estimation of the available calcium after the addition of known quantities of a chelating agent. Born and Cross (2) obtained similar results and in addition observed that a high calcium concentration, 1.5 $\times$ 10^{-2} *M*, inhibited aggregation.

Most investigators working with platelets *in vitro* employ citrate as anticoagulant. Citrate chelates both calcium and magnesium equally (3). At a citrate concentration of 1.29 $\times$ 10^{-2} *M* near maximal platelet aggregation still occurs, but sufficient calcium has been complexed to prevent fibrin formation.

We are interested in the ionic calcium requirement for platelet aggregation and this communication presents some of our observations.

Materials and Methods. Human blood samples were obtained fresh at the same time each morning by forearm venipuncture. A 30-ml plastic disposable syringe and a thin-walled disposable 19G needle were employed. Nine volumes of the blood sample were immediately added to 1 vol of anticoagulant solution in a siliconized 40-ml centrifuge tube. The anticoagulants used were saline solutions of: heparin (14.6 μ/ml), sodium citrate (1.29 $\times$ 10^{-1} *M*), EDTA (1.2 $\times$ 10^{-2} *M*), and EGTA (1.2 $\times$ 10^{-2} *M*). The tube was capped with Parafilm and gently mixed by inverting four times. Platelet-rich plasma (PRP) was prepared by centrifugation at 100*g* for 20 min and kept at room temperature for use within 3 hr.

Platelet aggregation was measured by the optical method of Born and Cross (4) as described previously (5). The reaction volume was held constant at 0.65 ml (0.50 ml of PRP, 0.05 ml of a collagen suspension, and the remaining 0.10 ml was made up of either saline, chelating agent, $MgCl_2$ or $CaCl_2$ solution in saline). The stock collagen suspension was prepared as described previously (5) with a slight modification. Saline was used in place of Tyrode's solution, and centrifugation was replaced by straining twice through a 12-ply, 3 $\times$ 3-in. J & J gauze sponge. Each batch of PRP was tested against serial dilutions of this stock suspension, and the dilution giving just maximal aggregation was used in subsequent testing. The ADP was obtained from CalBiochem. and added to give a final concentration of 3.9 $\times$ 10^{-6} *M*.

The heparinized PRP (PRP-H) contained 1.46 U of heparin per ml. It was found, in a separate experiment, that 36.5, 73, 146, and 292 U of heparin per ml decreased the ionic calcium concentration [Ca^{2+}] from a control concentration of 10 $\times$ 10^{-4} *M* to 9.4 $\times$ 10^{-4}, 8.5 $\times$ 10^{-4}, 7 $\times$ 10^{-4}, and 5.12 $\times$ 10, respectively. Thus, the 1.46 U per ml used in this work was considered to have a negligible effect on ionic calcium concentration [Ca^{2+}]. The citrate, EDTA, and EGTA solutions added to the reaction mixture were 1.29 $\times$ 10^{-2}, 2.4 $\times$ 10^{-2}, and 1.2 $\times$ 10^{-2} *M* in saline, respectively.

The [Ca^{2+}] was measured directly in each reaction mixture after the induction of platelet aggregation. Hence, the actual [Ca^{2+}] could be directly correlated with the aggregation reaction. For the [Ca^{2+}] measurement we used the Orion calcium ion-activity system, Model 99-20, coupled to the Orion digital pH/mV meter, Model 801. We found that the lowest level of [Ca^{2+}] which could be precisely measured was 1×10^{-5} M, but concentration as low as 5×10^{-6} M could be measured with reasonable reliability.

The extent of aggregation was determined by measuring the slope of a line drawn through the point at which the aggregating agent was added and tangent to the optical-density curve. Results are reported as percentage of control. Platelet aggregation was induced with either ADP or collagen suspension. All the results reported are those obtained using collagen since the experiments with ADP gave similar results.

Results and Discussion. Citrate PRP exhibited near maximal aggregation even though the [Ca^{2+}] was well below 5×10^{-6} M. In PRP anticoagulated with either EDTA or EGTA addition of $CaCl_2$ was required before the platelets would aggregate.

As the [Ca^{2+}] was increased, by adding $CaCl_2$ to the reaction mixture, fibrin formation began to interfere with the assessment of platelet aggregation so heparin was added. It was found in 12 experiments that the average [Ca^{2+}] at which fibrin formed within 60 and 10 min was 2.4×10^{-4} and 5.3×10^{-4} M, respectively. Heparin, therefore, was employed as anticoagulant to circumvent the problem of thrombin interference, and either EDTA or EGTA was added in successively increasing amounts until aggregation was markedly reduced. In each case [Ca^{2+}] was measured and plotted vs the calculated final molar concentration of added chelator. The percentage change in aggregation rate from control was also plotted vs the measured [Ca^{2+}], (Figs. 1 and 2). The curves of chelator concentrations vs [Ca^{2+}] are similar; however, the curves of aggregation rate vs [Ca^{2+}] show a striking difference. With EGTA the reduction in aggregation rate occurred at a much higher [Ca^{2+}] than the EDTA. Since the affinity of EDTA for calcium is 100 times that for magnesium and EGTA binds calcium 100,000 times more avidly (1) than it binds magnesium, the Ca^{2+}/Mg^{2+} ratio decreases more rapidly with increasing amounts of EGTA than with increasing amounts of EDTA. Similarly, addition of excess magnesium to PRP-H with the [Ca^{2+}] remaining constant results in in-

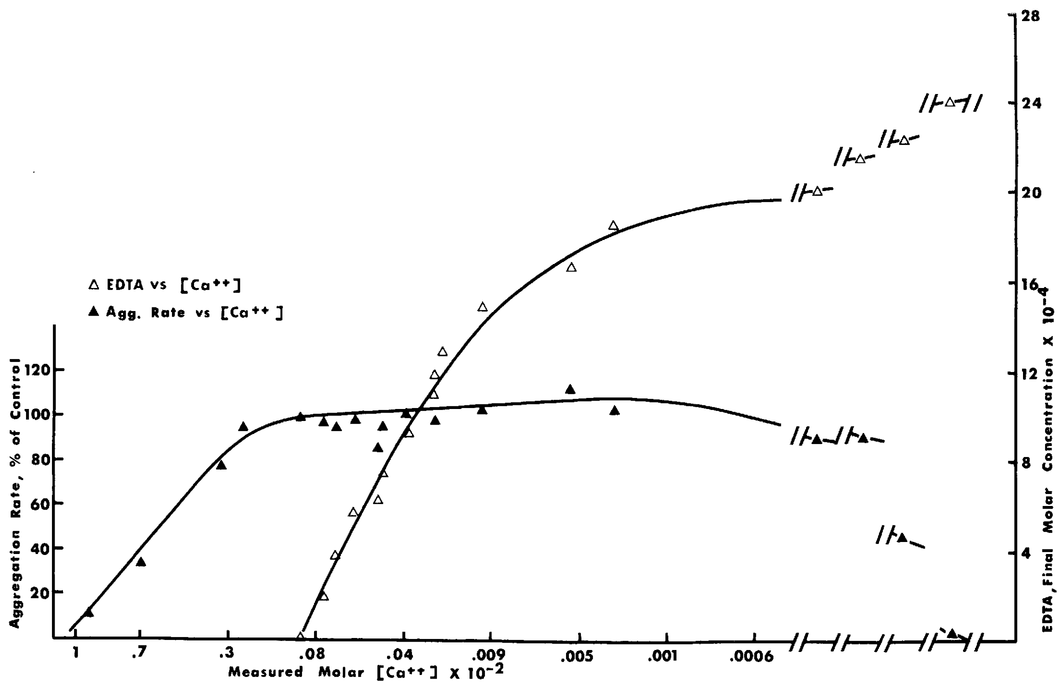

FIG. 1. Effect of EDTA on platelet aggregation and ionic calcium concentration.

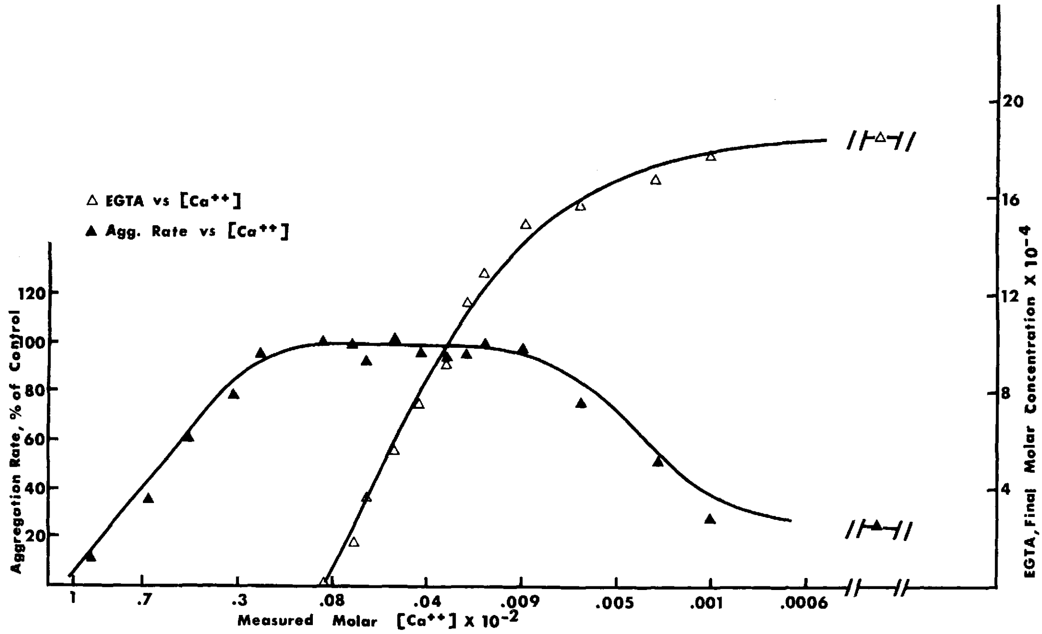

FIG. 2. Effect of EGTA on platelet aggregation and ionic calcium concentration.

hibition of aggregation, Table I. On the other hand, addition of excess calcium to PRP-H with the [Mg^{2+}] remaining constant also leads to inhibition of aggregation (Table I and Figs. 1 and 2). This suggests that maximal aggregation occurs when the ratio of [Ca^{2+}] to [Mg^{2+}] is within certain limits

TABLE I. Effect of Excess Ionic Calcium or Magnesium on Aggregation Rate of Human Platelets.

Molar [Ca^{2+}]	Molar [Mg^{2+}]	Aggregation rate (% of control)
0.87×10^{-3} [a]	0.415×10^{-3} [a] (5)	100
2.1×10^{-3}	0.415×10^{-3}	95
3.3×10^{-3}	0.415×10^{-3}	78
5.3×10^{-3}	0.415×10^{-3}	60
7.0×10^{-3}	0.415×10^{-3}	35
9.3×10^{-3}	0.415×10^{-3}	12
0.87×10^{-3}	1.54×10^{-3}	92
0.87×10^{-3}	6.2×10^{-3}	50
0.87×10^{-3}	9.3×10^{-3}	40
0.87×10^{-3}	12.3×10^{-3}	16

[a] Measured [Ca^{2+}] in reaction mixture of 0.5 ml PRP-H + 0.1 ml saline + 0.05 ml collagen suspension; calculated [Mg^{2+}] in reaction mixture from normal value (6) in plasma.

and that values of this ratio above or below these limits results in diminished platelet response.

The value of this ratio in normal plasma seems to be near the upper limit, since the aggregation rate falls off sharply when either the [Ca^{2+}] or [Mg^{2+}] is increased above the normal level, (Table I and Fig. 1). On the other hand, at the lower limit the results indicate that very minute amounts of Ca^{2+} and Mg^{2+} are required to allow platelets to aggregate provided the Ca^{2+}/Mg^{2+} ratio remains within the optimal limits.

Ardlie *et al.* (6) using washed-platelet suspensions found that the [Ca^{2+}/Mg^{2+}] ratio was of importance with respect to the degree of platelet aggregation and deaggregation induced by ADP. They proposed that Ca^{2+} was necessary for aggregation and Mg^{2+} for deaggregation.

In an endeavor to ascertain the validity of the postulate relating to the [Ca^{2+}/Mg^{2+}] ratio we attempted to restore the aggregation rate depressed with either excess Ca^{2+} or Mg^{2+} by adding the other ion in equivalent amounts. The practicality of this approach using PRP was limited to the left-hand side of the aggregation curves, Figs. 1 and 2.

TABLE II. Effect of Ionic (Calcium/Magnesium) Ratio on Platelet Aggregation in Heparinized PRP.

$[Ca^{2+}]/[Mg^{2+}]$	Aggregation rate (% of control)	$[Ca^{2+}]$[a] calculated $\times 10^{-4}M$	$[Mg^{2+}]$ calculated $\times 10^{-4}M$
2.05	100	8.6[b]	4.2[b]
0.20	47	8.6	42.6
0.44	71	8.6	19.5
0.58	29	47.1	81.0
1.05	15	85.4	81.0
1.23	60	23.9	19.5
2.00	20	85.4	42.6
2.41	51	47.1	19.5
4.37	42	85.4	19.5
11.21	73	47.1	4.2
20.31	45	85.4	4.2

[a] The $[Ca^{2+}]$ measured was found to be 87.6% (average of 15 experiments) of the concentration calculated from the amount of $CaCl_2$ solution added.

[b] Normal Ca and Mg ionic concentrations in reaction mixture.

Heparinized platelet-rich plasma was prepared, and aggregation was inhibited upon addition of either $MgCl_2$ or $CaCl_2$ solution. Addition of equivalent amounts of the other solution did not restore aggregation but, in fact, produced further inhibition (Table II). These results seem to refute the importance of $[Ca^{2+}/Mg^{2+}]$ ratio in platelet aggregation. However, there may be a difference in this requirement at the minimal ionic limits necessary for platelet aggregation.

The possibility remained that Ca–chelator complex was itself inhibitiory. This was tested by addition of either Ca–EDTA or Ca–EGTA. At concentrations as high as 3.9 $\times 10^{-3}$ *M* neither complex inhibited platelet aggregation.

Summary. The ionic calcium requirement for platelet aggregation is much lower than that required for fibrin formation. Maximal aggregation can still take place with the $[Ca^{2+}]$ well below 5×10^{-6} *M*. Chelation of Ca^{2+} with EGTA results in a decrease in aggregation at a higher $[Ca^{2+}]$ than when EDTA is employed. This seems to indicate that the $[Ca^{2+}]/[Mg^{2+}]$ ratio may be of importance at low ionic concentrations. However, excess $[Ca^{2+}]$ and/or $[Mg^{2+}]$ above the normal levels inhibit aggregation. In fact, when both of these ions are added in excess of the normal levels their inhibitory effects are additive, indicating that the $[Ca^{2+}]/[Mg^{2+}]$ ratio is of little consequence under these conditions.

Platelet aggregation is not inhibited by the calcium complex of either EDTA or EGTA.

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