Biochimica et Biophysica Acta, 436 (1976) 448–459

BBA 77331

THE EFFECT OF EXTERNAL CALCIUM AND LANTHANUM ON PLATELET CALCIUM CONTENT AND ON THE RELEASE REACTION

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(Received November 25th, 1975)

SUMMARY

Calcium compartments in calf platelets were studied using a lanthanum washout procedure to distinguish between surface-bound calcium and intracellular calcium. The calcium content of calf platelets ranges from 20 to 60 nmol/10^9 platelets and is sensitive to the calcium concentration of the suspending medium. With 1 mM calcium in the medium, calcium uptake is rapid and reaches steady state within 1–2 min. Results obtained with the lanthanum procedure indicate that it is the surface compartment which is most affected by the extracellular calcium concentration. The surface compartment appears to be saturable and is highly exchangeable. Although the total calcium as well as the calcium content of the surface and internal compartments are variable, the ratio of calcium in either compartment to the total saturated calcium is quite constant. The data indicate that 68–85 % of the platelet calcium is located internally. Thrombin produces an immediate release of platelet calcium and labeled serotonin and an increase in the $^{45}Ca^{2+}$ uptake of both the surface and internal compartments. The release reaction is not dependent upon exogenous calcium or an influx of exogenous calcium since it occurs even in the presence of ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid. Lanthanum, however, inhibits the release reaction possibly by blocking surface calcium site and reducing the mobility of endogenous platelet calcium.

INTRODUCTION

A requirement for divalent cations, particularly calcium, is highly specific for the general biological phenomena of secretion [1] and contraction [2]; however, the source of the activating calcium, namely, extracellular, surface bound or stored, varies with different tissues. Platelets are generally considered to manifest both contractile [3, 4] and secretory [5] activities as part of the shape change, release reaction and aggregation. The evidence is increasing that endogenous calcium plays a significant role in initiating some of the platelet contractile and secretory activities [6–9].

Abbreviation: EGTA, ethyleneglycol-bis-(β-amino-ethylether)-*N*,*N*′-tetraacetic acid.

A large part of endogenous platelet calcium is stored within granules [10–12] and is released as part of the release reaction [13–15]. Other calcium compartments representing potential sources of activating calcium may be located in the dense tubular system, the mitochondria and the platelet surface. Isolated platelet membranes have a calcium transport activity similar to that of muscle sarcoplasmic reticulum [16, 17]. In platelets, this activity may perform the dual function of maintaining a low cytoplasmic calcium concentration and contributing to an intracellular storage pool of calcium.

In this paper calcium flux in both unstimulated and stimulated platelets is examined to elucidate further the compartmentalization of calcium within platelets and the possible significance of these compartments in platelet physiology. $LaCl_3$ is used in many of the experiments to distinguish the surface-bound calcium from intracellular calcium compartments. Lanthanum has a higher affinity for calcium binding sites than calcium [18] and displaces surface-bound calcium [19]. In addition, lanthanum blocks the movement of calcium across artificial lipid membranes and prevents the outward movement of calcium from internal sites [19, 20].

MATERIALS AND METHODS

(*1*) *Isolation of platelets.* Calf blood, obtained at the slaughter house, is collected directly into polyethylene bags containing 3.8 % trisodium citrate, care being taken to avoid collecting the initial flow of blood. The blood is mixed by slow inversion of the bags after which they are placed on ice. Blood from each animal is kept separate until platelet number and condition are assessed by phase microscopy. Those with a low platelet number, microscopic evidence of fibrin formation, or activated platelets, e.g. with pseudopod formation or microaggregates are discarded. All preparative procedures are performed at 4 °C. Red cells are sedimented at 500 × *g*, the platelet-rich plasma is decanted and centrifuged at 1800 × *g* for 15 min. The platelet pellets are rinsed with buffered saline (0.02 M Tris · HCl, pH 7.4; 0.15 M NaCl; 0.5 % glucose, 0.1 % albumin) and then resuspended in buffered saline to a final platelet count of 10^9 platelets/ml. All experiments are done within 30–60 min after isolation of the platelets from plasma.

(*2*) *Effect of extra-cellular calcium on platelet calcium content.* After equilibration at room temperature, the platelet suspension is mixed with an equal volume of buffered saline containing various concentrations of $CaCl_2$ and incubated at room temperature. For determinations of calcium uptake or exchange, a trace amount of $^{45}Ca^{2+}$ (4 μCi/ml) is included in the mixture. At various time intervals, 0.5-ml aliquots of the platelet suspensions are centrifuged through silicone oil (see part 4, Materials and Methods). The supernatant and silicone oil are removed with a Pasteur pipette. The platelet pellet is resuspended in 0.15 M NaCl and the calcium is extracted into atomic absorption medium as detailed in part 5.

In experiments utilizing a lanthanum wash of platelets to distinguish between extracellular and intracellular calcium compartments, the silicone oil is omitted. $LaCl_3$(1–5 mM) is added directly to the platelet suspension, the platelets are centrifuged for 30 s at 7000 × *g*, resuspended in buffered saline containing $LaCl_3$, and centrifuged again.

(*3*) *Thrombin-induced release.* One volume of a platelet suspension is added to

nine volumes of incubation medium (buffered saline containing various concentrations of $CaCl_2$, $LaCl_3$ or ethyleneglycol-bis-(β-aminoethylether)-*N*,*N'*-tetraacetic acid (EGTA). The suspension is layered on silicone oil, thrombin (1 unit/10^9 platelets) is added with gently stirring and the platelets are centrifuged at 7000 $\times$ *g* through silicone oil. The supernatant is decanted and reserved. The silicone oil is removed and the underlying platelet pellet analyzed according to the experiment being performed.

For measurement of 5-hydroxytryptamine release, platelet suspensions from part 1 are incubated at 37 °C with 0.5 μCi/ml 5-hydroxy[^{14}C]-tryptamine binoxalate (27.5 Ci/mol, New England Nuclear Corp.) for 30–60 min prior to the addition of thrombin. This suspension is used directly in release experiments without prior washing of the platelets to remove unincorporated label. For the determination of 5-hydroxytryptamine release, the amount of label in the supernatant is compared to that in the complete suspension.

(*4*) *Silicone oil centrifugation.* Some of the calcium measurements are made on platelets that are centrifuged out of a suspending medium containing added calcium. Consequently it is necessary to minimize the entrapment of suspending medium within the platelet pellet. The silicone oil method of Feinberg et al. [21] is an effective means of accomplishing this; moreover, it provides the added advantage of separating the platelets rapidly from the incubation medium. An aliquot of platelet suspension is layered on silicone oil in 1 ml polyethylene centrifuge tubes and centrifuged at 7000 $\times$ *g* in a Fisher Model 59 microfuge. Maximum speed is reached in 5–10 s thereby separating the platelets from the surrounding medium and pelleting them beneath the silicone oil layer. The effectiveness of this method in excluding the incubation medium from the platelet pellet is indicated by the results of [^{14}C]inulin labeling: platelets centrifuged through silicone oil have a [^{14}C]inulin "space" ranging from 0.07 to 0.29 μl/10^9 platelets whereas platelets centrifuged without silicone oil have an inulin space of 2.26–4.16 μl/10^9 platelets.

(*5*) *Atomic absorption spectrophotometry.* The general procedure, modified from Willis' [22] method for serum calcium is as follows: Platelet suspensions are mixed with an equal volume of ice-cold 10 % trichloroacetic acid containing 1 % La_2O_3 and allowed to remain at 4 °C overnight to ensure complete extraction of calcium. The precipitated protein is removed by centrifugation and the amount of calcium in the supernatant is measured on a Perkin-Elmer Model 306 atomic absorption spectrophotometer by comparison with standard concentrations of calcium prepared in the same matrix as the experimental samples. For the determination of the initial calcium content of platelets an aliquot of the platelet suspension (as described in part 1) is precipitated with 10 % trichloroacetic acid/1 % La_2O_3. In this way all the calcium that is originally in the platelets is accounted for. Platelets which have been subjected to various experimental conditions are first separated from the experimental medium by centrifugation, resuspended in 0.15 M NaCl, and precipitated with 10 % trichloroacetic acid/1 % La_2O_3.

(*6*) *Measurement of radioactivity.* In experiments requiring the determination of $^{45}Ca^{2+}$ or [^{14}C]inulin in platelets, the tip of the centrifuge tube containing the platelet pellet is cut off and placed in a scintillation vial with 1 ml Protosol (New England Nuclear Corp.). After overnight solubilization of the pellet at 55 °C, 10 ml Liquifluor scintillation fluid (New England Nuclear) is added and the radioactivity measured on a Beckman liquid scintillation counter, Model LS-230. When radio-

activity is measured in the supernatant, a 100 μl aliquot is counted in Scintiverse (Fisher Chemical Co.).

(7) *Chemicals.* La_2O_3 (low calcium) is obtained from Alfa Products, Ventron Corporation, Beverly, Mass. (Cat No. 87808); Dow Corning 560 silicone fluid is obtained from Dow Corning Corp., Midland, Michigan. All other chemicals are reagent grade.

Thrombin (Parke-Davis Topical Thrombin) is prepared as a stock solution of 200 units/ml in 0.02 M Tris · HCl, pH 7.0, and dialyzed overnight against the same buffer.

RESULTS

Calcium content of calf platelets

Calf platelets, isolated from citrated plasma by centrifugation, have a variable calcium content ranging from 20 to 60 nmol calcium/10^9 platelets. The mean value for platelets from 12 animals is 51.5 ± 14.7 nmol/10^9 platelets. This value is obtained from measurements of the initial platelet suspension in calcium-free medium (without further centrifugation) and therefore is believed to be a measure of all the calcium associated with the platelets, except for losses that may occur during separation of the platelets from plasma. We observe a similar variability in the calcium content of human platelets prepared in the same way, but the amount of calcium is considerably higher (mean calcium content for platelets from eight subjects is 141.4 ± 34.5 nmol/10^9 platelets). Comparison of this data with that from other laboratories [14, 23, 24] suggest that species differences and methods of preparing platelets may be significant factors when comparing platelet calcium content. Mürer and Holme [14] found that human platelets, washed three times with EDTA, contained 50 nmol calcium/mg platelet protein. This value is equivalent to 80–90 nmol/10^9 platelets using the approximation that 10^9 platelets contain 1.6–1.8 mg protein. Extrapolation of their data on unwashed human platelets gives a value of 80 nmol/calcium mg protein or 128–144 nmol/10^9 platelets. Lages et al. [23] found that unwashed human platelets isolated by centrifugation of platelet-rich plasma contain 260 ± 62 nmol calcium/10^9 platelets; those isolated by gel filtration contain 243 ± 44–284 ± 53 nmol/10^9 platelets depending on the elution medium. Kinlough-Rathbone et al. [24] reported a value of 51 ± 2.6 nmol calcium/10^9 platelets for pig platelets washed with EGTA.

Effect of external calcium and lanthanum on platelet calcium content

Centrifugation of the initial platelet suspension in Ca^{2+}-free medium results in a slight decrease in platelet calcium content. This is shown in the data of Table I which compares platelet calcium content in calcium-free medium before and after centrifugation. The decrease is believed to reflect calcium lost from platelets during the incubation in calcium-free medium and is constant for incubation periods ranging from 5 min to 2 h. The ability of calf platelets to maintain a stable calcium content in calcium-free medium is similar to what Lages et al. [23] observed using human platelets. If calcium is added to the platelet suspension, the platelet calcium content increases, and the rate of increase depends upon the concentration of calcium added (Fig. 1). In the presence of 1 mM calcium, there is an immediate increase in platelet calcium content which continues until a steady state is reached within 2 min. With

TABLE I

CALCIUM CONTENT OF CALF PLATELETS IN CALCIUM-FREE MEDIUM

Platelet calcium content is expressed in nmol/10^9 platelets $\pm$S.E. ($n = 3$). Platelets are suspended in calcium-free buffered saline as described in Materials and Methods. The calcium content of an aliquot of the suspension is determined by atomic absorption spectrophotometry and corrected for the background calcium content of the suspending medium. An aliquot of equal volume is centrifuged through silicone oil and the calcium content of the pellet is determined after extraction of the calcium as described in Materials and Methods.

Experiment	Platelet calcium content	
	In suspension	After centrifugation
1	21.0±1.0	18.0±0.5
2	31.5±1.5	29.5±0.7
3	31.5±1.5	28.0±0.5

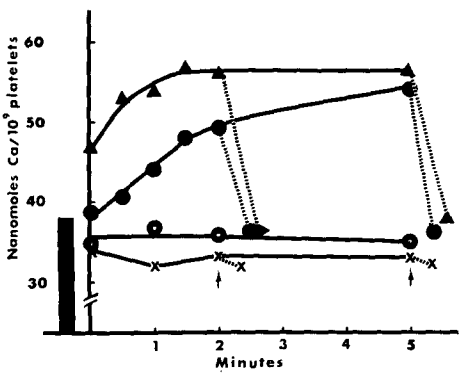

Fig. 1. The effect of calcium and lanthanum on platelet calcium content. The solid bar represents the initial calcium content of the platelet suspension in calcium-free buffered saline. After equilibration of 0.5 ml aliquots of the suspension at room temperature (5–10 min), Ca^{2+} or La^{3+} is added. The points represent the calcium content of platelets centrifuged at various times after the addition of Ca^{2+} or La^{3+} to the suspension. The "zero" time point is obtained by centrifuging the suspension immediately after the addition is made. Dotted lines indicate the effect of washing platelets with 5 mM La^{3+} at times indicated by the arrows. ×, no additions; ●, 0.1 mM Ca^{2+}; ▲, 1.0 mM Ca^{2+}; ○, 5.0 mM La^{3+}.

0.1 mM calcium, the rate of increase is slower; the calcium content is still increasing after 5 min but is approaching the level observed with 1 mM external calcium. These results indicate that the platelet calcium content is influenced by the external calcium concentration but that it is saturable and after reaching a maximum level, remains stable. Kinlough-Rathbone et al. [24] also observed a higher calcium content in pig platelets when calcium is included in the suspending medium, but the increase was much greater. However, they started with EGTA-washed platelets whose calcium content may have been reduced to a greater extent because of the efficiency of the chelator in removing calcium.

In order to determine whether the observed increase in platelet calcium was taking place at the platelet surface or by incorporation of calcium into an intracellular compartment, the effect of adding lanthanum to the platelet suspension was examined. This effect is also shown in Fig. 1. If lanthanum is added to the initial platelet sus-

TABLE II

$^{45}Ca^{2+}$ UPTAKE BY PLATELETS AND ITS DISPLACEMENT BY LANTHANUM

(a) and (b) Platelets are incubated in buffered saline containing 0.1 or 1.0 mM $CaCl_2$ with a trace amount of $^{45}Ca^{2+}$. After the indicated time interval three equal aliquots are centrifuged through silicone oil. The $^{45}Ca^{2+}$ content is determined on one of the pellets. The other two pellets are resuspended in buffered saline or buffered saline containing 5 mM $LaCl_3$ and centrifuged again. The $^{45}Ca^{2+}$ content is determined on the washed pellets. (c) Platelets are incubated in buffered saline containing 1.0 mM $CaCl_2$ for 30 min before the addition of a trace amount of $^{45}Ca^{2+}$. 1 min after adding $^{45}Ca^{2+}$, the suspension is centrifuged through silicone oil and the $^{45}Ca^{2+}$ content of the pellet determined. A second aliquot of a platelet suspension is incubated for 1 min in buffered saline containing 1.0 mM $CaCl_2$ with the tracer $^{45}Ca^{2+}$ present from the start. Both incubations are done with the same concentration of $^{45}Ca^{2+}$.

	Incubation conditions	$^{45}Ca^{2+}$ uptake (cpm/10^9 platelets)	^{45}Ca removed (%)
(a)	0.1 mM $CaCl_2$, 5 min	16225	
	Washed saline only	4802	70.4
	Washed saline + $LaCl_3$	3944	75.6
(b)	1.0 mM $CaCl_2$, 5 min	10769	
	Washed saline only	1792	83.4
	Washed saline + $LaCl_3$	1713	84.1
(c)	Preincubated 30 min in unlabelled Ca^{2+} medium before addition of $^{45}Ca^{2+}$	6526	
	$^{45}Ca^{2+}$ present from start of incubation	6688	

pension in calcium-free medium, the calcium content of the platelets decreases slightly, similar to what is observed in calcium-free medium alone. The addition of lanthanum to platelets whose calcium content is increased by preincubation with external calcium causes a decrease in calcium to the same level observed in lanthanum medium alone.

We interpret these results in terms of the model of lanthanum action proposed by van Breemen et al. [19, 20] and Weiss and Goodman [25], i.e. lanthanum displaces calcium from surface sites and prevents leakage of internal calcium. According to this model, these data provide an indication that the increase in platelet calcium content in calcium medium is the result of an increase in the calcium content of the surface calcium compartment. If the intracellular calcium compartment were being increased by the external calcium, subsequent washing of the platelets with lanthanum would prevent its loss.

To test this hypothesis further, similar experiments were done with $^{45}Ca^{2+}$ included in the suspending medium. The results of several experiments are summarized in Table II. A major part of the labeled calcium is removed by a saline wash and by lanthanum, supporting the concept that exogenous added calcium is added on at the platelet surface. The remaining $^{45}Ca^{2+}$ which resists removal by lanthanum may be located intracellularly. In an exchange experiment (Table II, Experiment c), platelets preincubated in 1 mM calcium medium before the addition of $^{45}Ca^{2+}$ have the same $^{45}Ca^{2+}$ content as those incubated with labeled calcium initially, indicating the calcium at the surface exchanges rapidly with calcium in the suspending medium.

Distribution of endogenous platelet calcium between surface and intracellular compartments

The above results indicate that the surface calcium compartment of calf

TABLE III

DISTRIBUTION OF PLATELET CALCIUM BETWEEN SURFACE AND INTRACELLULAR COMPARTMENTS

Platelet calcium content is expressed as nmol/10^9 platelets ±S.E. ($n = 3$). Platelets are incubated for 5 min in buffered saline containing 1 mM $CaCl_2$ (for determination of total saturated calcium content) or 5 mM $LaCl_3$ (for determination of intracellular calcium content). At the end of the incubation period, the platelets are centrifuged through silicone oil, and the calcium content of the platelet pellets determined by atomic absorption spectrophotometry as described in Materials and Methods.

Experiment	Calcium content		Internal Ca^{2+}
	In 1 mM Ca^{2+}	In 5 mM La^{3+}	Total Ca^{2+}
1	25.3 ± 0.4	18.0 ± 0.5	0.711
2	44.0 ± 1.0	30.0 ± 0.4	0.682
3	35.5 ± 0.7	30.0 ± 0.3	0.845
4	57.0 ± 0.5	39.0 ± 1.0	0.684
5	39.0 ± 1.0	28.5 ± 0.5	0.730

platelets can be saturated by incubation of the platelets in medium containing 1 mM calcium, and that this calcium is removed by lanthanum. Consequently it is possible to consider the calcium content of lanthanum-washed platelets to be an estimate of the intracellular calcium. The amount of surface calcium can be calculated from the relationship: surface calcium = total saturated calcium content minus calcium content after a lanthanum wash. Data obtained on platelets from five animals are shown in Table III. The calcium content of both the surface and intracellular calcium compartments is variable, as is the total saturated calcium content. However, the calculated ratio of the intracellular calcium to the total saturated calcium is fairly constant with 68–84 % of it located internally. This distribution is markedly different from that observed in Hela cells [26] or smooth muscle cells [27] where 70–100 % of the cell calcium appears to be superficially bound and highly exchangeable.

The effect of external calcium and lanthanum on the release reaction

Calf platelets exposed to thrombin release calcium, labeled serotonin and adenine nucleotides [7] similarly to what is reported for human platelets [5, 6, 13–15]. However, the actual amount of calcium released (4–10 nmol/10^9 platelets) and the percentage of total calcium which that amount represents (17–38 %) is considerably less than the amount of calcium released by human platelets (Table IV). In human platelets, approx. 68–85 % of the total calcium may be released in a thrombin-induced release reaction [10, 13]. We have obtained similar results with human platelets in our laboratory. The release of calcium from calf platelets is unaffected by the presence or absence of calcium in the suspending medium and is not inhibited by EGTA. In this respect, calf platelets are similar to human platelets, but are different from pig platelets where thrombin induces a loss of calcium only in the absence of external calcium. In the presence of external calcium, thrombin causes an increase in pig platelet calcium content [24].

The release of labeled 5-hydroxytryptamine is similar to that observed for human platelets [3, 5, 9] with 60–80 % of the label being released in the suspending

TABLE IV

THROMBIN-INDUCED RELEASE OF CALCIUM FROM CALF PLATELETS

Platelets are incubated in Ca^{2+}-free buffered saline containing the indicated additions for 5 min. 0.5-ml aliquots of these incubation mixtures are layered on silicone oil, 10 μl thrombin is added (final concentration = 1 unit/10^9 platelets), and 1 min later the platelets are centrifuged through silicone oil. Control aliquots of platelets are centrifuged through silicone oil after the addition of 10 μl saline. Ca^{2+} content is expressed as nmol $Ca^{2+}/10^9$ platelets.

	Incubation medium	Ca^{2+} content of platelets		Ca^{2+} released	Released (%)
		Control	Thrombin		
1	No additions	32.0	23.5	8.5	26.6
2	No additions	24.0	15.0	9.0	37.5
3	No additions	31.2	22.2	9.0	29.0
4	No additions	20.0	16.0	4.0	20.0
5	No additions	36.0	27.5	8.5	23.6
6a	No additions	29.8	24.6	5.2	17.4
6b	EGTA, 2 mM	30.1	24.5	5.6	18.6
7a	No additions	19.5	15.0	4.5	23.0
7b	Ca^{2+}, 1 mM	27.5	23.0	4.5	19.6

medium. The appearance of 22–27 % of 5-hydroxy[^{14}C]tryptamine in the supernatant from control platelets is probably the result of incomplete incorporation of 5-hydroxytryptamine by the platelets rather than release, since only 6–8 % of the platelet adenine nucleotides are found extracellularly in control platelets. The extent of 5-hydroxytryptamine release, like that of calcium release, is not affected by the external calcium concentration or by EGTA. Lanthanum, however, produces a definite inhibition of 5-hydroxytryptamine release at both concentrations tested (Table V).

TABLE V

THE EFFECT OF CALCIUM CONCENTRATION ON THE RELEASE OF 5-HYDROXY-[^{14}C]TRYPTAMINE BY CALF PLATELETS

Platelets are preincubated in buffered saline containing EGTA, Ca^{2+}, or La^{3+} for 5 min prior to the addition of thrombin. 1 min after adding thrombin (1 unit/10^9 platelets), the platelets are centrifuged through silicone oil and the supernatant analyzed for 5-hydroxy[^{14}C]tryptamine

Incubation medium	5-Hydroxy[^{14}C]tryptamine in supernatant	
	Control	Thrombin treated
EGTA, 2.0 mM	25.6	67.9
Ca^{2+} free	24.8	66.9
Ca^{2+}, 0.05 mM	23.8	76.0
Ca^{2+}, 0.1 mM	24.8	68.7
Ca^{2+}, 1.0 mM	27.8	73.2
Ca^{2+}, 2.0 mM	24.0	63.8
La^{3+}, 1.0 mM	23.7	37.1
La^{3+}, 2.0 mM	26.4	25.5

TABLE VI

DISPLACEMENT OF $^{45}Ca^{2+}$ BY LANTHANUM IN THROMBIN-TREATED PLATELETS

Platelet suspensions in $^{45}Ca^{2+}$ buffered saline (see Materials and Methods) (0.1 mM Ca^{2+}) are incubated with various concentrations of thrombin for 1 min. Platelets are centrifuged through silicone oil to determine total $^{45}Ca^{2+}$ uptake. For washed samples, the platelet suspensions are centrifuged without silicone oil; the pellets are resuspended in buffered saline or buffered saline containing 2.0 mM $LaCl_3$ and centrifuged again.

Thrombin concentration (units/10^9 platelets)	$^{45}Ca^{2+}$ uptake (nM/10^9 platelets)		
	Total	After saline wash	After La^{3+} wash
None	0.334	0.041	0.036
0.015	0.334	0.053	0.042
0.03	0.371	0.069	0.060
0.30	0.812	0.130	0.095
1.50	1.587	0.239	0.140
3.0	2.151	0.447	0.297
6.0	2.629	0.245	0.220
12.0	2.598	0.428	0.431

Thrombin-induced $^{45}Ca^{2+}$ uptake by platelets

In addition to causing the release of endogenous platelet calcium, thrombin also causes an increased $^{45}Ca^{2+}$ uptake by calf platelets [7] and by human platelets [9, 14]. In a previous paper [7] we postulated that most of the observed "uptake" took place at the platelet surface and did not represent an actual influx of calcium into the intracellular compartment. The lanthanum wash procedure is used in the present study to determine how much of the thrombin-induced $^{45}Ca^{2+}$ uptake might be due to increased surface binding of exogenous calcium. The results of a typical experiment are shown in Table VI. Total $^{45}Ca^{2+}$ uptake increases with increasing thrombin concentration. Lanthanum removes most of the labeled calcium that is taken up as a result of thrombin treatment indicating that most of it is located at the platelet surface. However, the thrombin-treated platelets retain more labeled calcium after the lanthanum wash than untreated platelets; and the amount that resists removal by lanthanum, and may be internalized, increases with increasing thrombin concentration.

DISCUSSION

We have attempted to determine which compartment of platelet calcium is affected by changes in extracellular calcium concentration by using a modification of the lanthanum method described by van Breemen et al. [19, 20]. Lanthanum displaces surface-bound calcium and blocks the outward transport of internally located calcium. Therefore, calcium remaining in the platelets after a lanthanum wash is believed to be located intracellularly. The method does not distinguish between various intracellular compartments, e.g. granules, mitochondria, or dense tubular system, but provides information about the entire intracellular calcium content. The validity of the lanthanum method in delineating a surface calcium compartment depends upon its action being confined to the cell surface. Most of the evidence ob-

tained from calcium efflux studies in the giant squid axon [19], aortic smooth muscle [19, 20], intestinal smooth muscle [25], and cardiac muscle [28, 29] indicates that lanthanum displaces mainly extracellular and superficial surface-bound calcium in these tissues. However, Hodgson et al. [30] observed lanthanum uptake in uterine smooth muscle with accumulation of lanthanum in a mitochondrial fraction and concluded that in uterine muscle, the use of lanthanum as a probe for surface calcium would be inappropriate. The permeability of the platelet membrane to the La^{3+} is unknown, but some of the data in this study support the assumption that the observed action of lanthanum is primarily at the cell surface. The effect of lanthanum in decreasing the total cell calcium and in displacing labeled calcium from calf platelets is immediate and the resulting calcium concentration remains constant for a relatively long time. If lanthanum were displacing calcium from an internal compartment as well as from a surface compartment, one might expect a biphasic form of calcium efflux.

The present application of the lanthanum method permits the delineation of two compartments of platelet calcium: a surface compartment in rapid equilibrium with the extracellular calcium, and an internal compartment which is permeated to a lesser extent by endogenous calcium. The observation of a multicompartment distribution of calcium with different exchange rates is in agreement with the results obtained by Steiner and Tateishi [31]. Of particular interest is the nearly constant proportionality between each of the two major calcium compartments and the total maximum calcium content of calf platelets at saturation. Additional study, using platelets from other species, is necessary to determine if this relationship holds true for platelets in general. If so, it may be more useful to compare this ratio between groups of platelets than to compare total calcium, a quantity which is highly variable.

The high percentage of internal calcium observed by the lanthanum method it in general agreement with estimates of the releasable, granule calcium compartment of human platelets obtained by other methods [10, 13]. However only 17–38 % of the platelet calcium is released by calf platelets stimulated by thrombin. This may be an indication that some release has already occurred during the isolation of the platelets. On the other hand, the disparity between the amount of calcium released and the observed internal: total calcium ratio may indicate the presence of other calcium compartments in calf platelets which are not released by thrombin. The identification of other calcium compartments is beyond the scope of this paper. However, Salganicoff et al. [32] observed that half of the particulate calcium in sub-cellular fractions of pig platelets is located in granules distinct from serotonin storage granules; a membrane system of both human and calf platelets is reported to have calcium sequestering ability [16, 17]; and the calcium-accumulating ability of mitochondria is well documented. All of these organelles in addition to the serotonin granules are likely to contribute to the intracellular calcium compartment measured by the lanthanum.

The platelet release reaction is generally considered to be analogous to secretion with calcium being involved in stimulus-secretion coupling [33] as it is in other tissues [1]. Exogenous calcium seems to be required for a thrombin-induced release reaction, particularly when low concentrations of thrombin are used, in pig platelets [24] and in rat platelets [34]. In these species, the model of a stimulus provoked influx of calcium to raise the cytoplasmic calcium concentration, believed necessary to elicit the secretory response of release, is a reasonable hypothesis. However, in human platelets [6, 14, 35] and in the study of calf platelets, release occurs even in

the absence of chelation or extracellular calcium. Detwiler and Feinman [6] on the basis of kinetic studies of thrombin-induced release in human platelets, proposed a model where thrombin might bring about an increase in cytoplasmic calcium concentration from endogenous calcium stores, and not necessarily from an influx of external calcium.

We find, in calf platelets, that the surface calcium compartment but not the intracellular compartment is affected by the external calcium concentration. We also find that release occurs in calcium-free medium, and in medium containing EGTA, both of which might be expected to reduce the calcium content of the surface compartment. It therefore appears that surface calcium, per se, may not be essential in initiating the release reaction. However, lanthanum, which seems to displace calcium from the surface, inhibits release. The inhibition of release by lanthanum and not by EGTA may appear contradictory unless one considers other possible effects of lanthanum. One of the observed effects of lanthanum on calcium fluxes in other tissues is the blockade of surface calcium sites so that calcium efflux is prevented [19]. Detwiler and Feinman [6] observed two stages of calcium release in human platelets, one of which begins prior to the release of adenine nucleotides. They propose that the early release of calcium is related in some manner to the mobilization of calcium within the cytoplasm which in turn is involved in initiating the release of other platelet components. In order for them to observe this early stage of release, the calcium has to leave the platelet and enter the extracellular medium. A lanthanum blockade of surface calcium sites might conceivably block this initial efflux of calcium, and in so doing, interfere with an intracellular sequence of events leading to release. Weiss and Goodman [25] found that lanthanum displacement of superficially located calcium in intestinal smooth muscle altered the mobility of calcium located in other cellular stores and thereby inhibited contractile responses. Alternate explanations for lanthanum inhibition of release are: (1) lanthanum may interfere with the initial interaction of thrombin with platelets, or (2) it may alter the platelet membrane in some non-specific way not related to calcium flux. However, the observed effects of lanthanum on total platelet calcium and $^{45}Ca^{2+}$ labeling suggest that in calf platelets, there is a lanthanum-calcium competition. Lanthanum also has been observed to inhibit aggregation induced by ADP [36] and by the ionophore A23187 [8]. It appears possible that lanthanum inhibition of release and aggregation may be related to the blockade of calcium sites at the platelet surface. Further investigation of lanthanum-calcium interactions in platelets may help elucidate calcium-dependent mechanisms in platelet physiology.

ACKNOWLEDGEMENT

This work was supported by National Institutes of Health Grant: HL 15503-02, HL-16714-01.

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