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# DISTRIBUTION AND TRANSPORT OF CALCIUM IN HUMAN PLATELETS

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## SUMMARY

The total content, the distribution and the uptake characteristics of $Ca^{2+}$ in human platelets were studied. Platelets contained a mean of 57.5 nmoles $Ca^{2+}$ per mg platelet protein. In intact platelets most of the cation (98 %) was bound to protein while only small amounts were ligated by lipids. In contrast a considerable portion (21.1 %) of the platelet membrane-bound $Ca^{2+}$ was ligated by lipids. When platelets were disrupted by the glycerol lysis technique and their cellular contents fractionated by interrupted sucrose density gradient centrifugation platelet granules exhibited the highest $Ca^{2+}$ content per mg protein, while the cytosol portion contained almost two-thirds of the platelet $Ca^{2+}$. The uptake of $Ca^{2+}$ which was investigated by the use of $^{45}Ca^{2+}$ was found to be temperature dependent, inhibited by $Mg^{2+}$ and $Fe^{2+}$ and showed a sharp pH optimum at 6.4–6.6. The monovalent cations $K^+$ and $Na^+$ had opposite effects on the uptake of $Ca^{2+}$, the former increasing and the latter decreasing uptake. Exchange of platelet $Ca^{2+}$ with the medium did occur. The efflux of $Ca^{2+}$ from platelets was potentiated by the presence of glucose in the medium. Of the aggregating agents studied only thrombin and to a lesser degree collagen induced a release of platelet $Ca^{2+}$.

## INTRODUCTION

The importance of calcium in controlling structure, deformability, adhesiveness and permeability of cells and in regulating cellular activity has been well established [1–3]. In platelets this divalent cation plays a significant role in a variety of physiological functions relating to hemostasis: in general the release reaction has been found to require $Ca^{2+}$ [4], as has clot retraction [5] and phagocytosis [6]. Lack of inhibition by EDTA of the release reaction induced by thrombin and latex particles has been claimed by some authors [7, 8]. Platelet adherence to glass has also been reported to be inhibited by chelation of calcium [9] but the evidence remains inconclusive. In spite of the apparent importance of this cation for platelets and their function few studies have investigated the characteristics of uptake, distribution and exchangeability of calcium in this cell. Platelets are unique among cells in that they

have a very high calcium content which is five to six times greater than that in tissues such as skeletal muscle, smooth muscle, connective tissue, brain, heart, kidney, skin and cornea [1]. Previous investigations on uptake and exchangeability of calcium in platelets have given conflicting results albeit in quite different experimental settings [10, 11].

We report here the results of a series of experiments which were undertaken to characterize uptake, distribution and efflux of calcium in platelets and examine some of the factors influencing these processes. All our investigations were carried out on carefully washed human platelets utilizing either radioactive calcium as a tracer or determining total calcium concentrations by atomic absorption spectroscopy.

## METHODS

### *Collection of blood and preparation of platelets*

Blood was collected from normal, healthy male volunteers into plastic bags (Blood Pack JA-2C, Fenwal Laboratories, Ill.) containing acid–citrate–dextrose anticoagulant (U.S.P. Formula "A"). Platelets were isolated by differential centrifugation at 15 °C as described previously [12]. Red and white cell contamination was reduced by brief centrifugation and was always less than 1 per 10 000 and 1 per 30 000 platelets, respectively. Platelets were washed twice, once with 0.15 M NaCl containing 1 mM EDTA and buffered with 0.01 M Tris–HCl, pH 7.5 and once with 0.154 M NaCl. Unless otherwise indicated washed platelets were suspended in 0.15 M NaCl buffered with Tris–maleate, pH 6.4 (1 vol. of 0.2 M Tris–maleate buffer+9 vol. of NaCl (0.154 M)–Tris–maleate buffer) at a concentration of $2 \cdot 10^9$ platelets/ml.

The water used for all solutions was rendered $Ca^{2+}$ free by passage through an ion-exchanger, distillation in a Pyrex-glass distillation system and demineralization in a mixed-bed resin Barnstead demineralizer. All solutions were stored in plastic (Nalgene) containers which were at first rinsed with the purified water described above.

### *$^{45}Ca^{2+}$ uptake studies*

A stock solution of $^{45}CaCl_2$ was prepared by diluting $^{45}CaCl_2$ of spec. act. 729 Ci/mole to $2 \cdot 10^{-4}$ M $Ca^{2+}$ using 10 mM Tris–HCl buffer, pH 7.4. The desired concentrations of $Ca^{2+}$ were obtained by dilution with Tris–HCl buffer or by addition of appropriate amounts of solid $CaCl_2$.

In general 1-ml aliquots of the platelet suspension were incubated with varying concentrations of $CaCl_2$ containing $2 \cdot 10^{-7}$ M $^{45}CaCl_2$ at 37 °C in a water-bath with gentle agitation. The incubation was terminated by addition of 10 ml ice-cold Tris–maleate buffer followed by immediate centrifugation. The platelets were washed twice with 10 ml aliquots of the Tris–maleate buffer and were finally suspended in 1 ml 0.154 M NaCl. After addition of 0.5 vol. 0.3 M KOH, aliquots were taken for measurement of protein content and determination of radioactivity. The latter was measured with a Tri-Carb liquid scintillation spectrometer (Packard Instrum. Co., Downers Grove, Ill.) using 2,5-diphenyloxazole as the primary fluor and 1,4-bis(2-(4-methyl-5-phenyloxazolyl))-benzene as the secondary fluor both dissolved in toluene. Bio-Solv (Beckman Instrum. Inc., Fullerton, Calif.) served as solubilizer.

Isolated platelet membranes were prepared as described previously [12] and suspended in the Tris–maleate buffer at a concentration of 1 mg/ml. After incubation with varying concentrations of $^{45}Ca^{2+}$ the membrane vesicles were separated and washed twice with the Tris–maleate buffer. Radioactivity was then determined.

Total $Ca^{2+}$ in platelets was determined by atomic absorption spectroscopy. All experiments were performed in duplicate or triplicate.

*Separation of subcellular platelet fractions*

Platelets were lysed essentially according to Barber and Jamieson [13]. The platelet lysate was layered onto an interrupted sucrose density gradient made of equal portions of 27, 35 and 40 % sucrose solutions. This gradient was centrifuged for 180 min at 63 500 × *g* at 4 °C. Each fraction was washed twice with the Tris–maleate buffer and then suspended in this buffer.

*Characterization of subcellular platelet fractions*

5-Hydroxy-[$^{14}$C]tryptamine was utilized to label the dense granules of platelets. About 200 ml of platelet-rich plasma (approximately 40 · $10^9$ platelets) were incubated with 40 μg of 5-hydroxy-[$^{14}$C]tryptamine dissolved in 4 ml of 0.154 M NaCl at 37 °C for 20 min with gentle agitation. The platelets were then isolated by centrifugation, washed twice with 1 mM EDTA–Tris–maleate buffer. Subcellular fractions of these platelets were prepared as described above. Radioactivity and absolute $Ca^{2+}$ concentration was determined in aliquots of each fraction by liquid scintillation and atomic absorption spectrometry.

β-Glucuronidase (EC 3.2.1.31) was assayed by the method of Fishman [14] and isocitrate dehydrogenase (EC 1.1.1.41) was measured according to Green et al. [15]. The protein concentration in this and all other experiments was measured by the method of Lowry et al. [16] with crystalline bovine serum albumin as a reference standard.

*Lipid extraction of platelets and platelet membranes*

Platelets or platelet membranes were initially labeled with $^{45}Ca^{2+}$ as described above. The washed platelets or membrane vesicles were extracted with chloroform–methanol (1 : 1; v/v) and protein residue, aqueous and lipid phase were then separated according to Forstner and Manery [17]. The major lipid classes were separated by thin-layer chromatography as described previously [18]. For determination of radioactivity, the spots on the thin-layer chromatography plates corresponding to the individual lipids were scraped into scintillation vials, suspended in 5 % Cab-O-Sil in toluene containing 0.4 % 2,5-diphenyloxazole–0.05 % *p*-bis[2-(5-phenyloxazolyl)]-benzene and counted in a liquid scintillation spectrometer.

The phosphorus of each clearly identified major phospholipid was determined [19]. Corrections were made for the absorbance of corresponding areas of blank lanes.

Phosphoinositides held as contaminants in the insoluble protein residue resulting from the above neutral solvent extraction were extracted and acid hydrolysed according to Eichberg and Hauser [20]. The water-soluble hydrolysis products were then dissolved in a small volume of 5 mM EDTA (sodium salt) at neutral pH and spotted together with appropriate standards of mono-, di- and triphosphoinositides on silica gel NH prepared according to Gonzalez-Sastre and Folch-Pi [21] using chloro-

form–methanol–4 N $NH_4OH$ (9 : 7 : 2; v/v) as the solvent system. After exposure to iodine vapor the identified spots were scraped and phosphorus analyzed [19].

*Aggregation studies*

Platelets prelabeled with 5-hydroxy-[$^{14}$C]tryptamine (see above) were resuspended at a concentration of $1 \cdot 10^9$–$1.25 \cdot 10^9$ platelets/ml Tris–maleate buffer, pH 7.4 containing 5.5 mM D-glucose–0.5 mM $Ca^{2+}$–1 mg/ml human fibrinogen. The fibrinogen obtained from commercial sources was purified according to Blombäck and Blombäck [22]. Platelet aggregation was induced by addition of 1 unit/ml, thrombin, 100 $\mu$M ADP, 0.45 $\mu$mole epinephrine /ml or 110 $\mu$g soluble collagen/ml. Experimental and control platelet suspensions were incubated with constant agitation (magnetically stirred) for 10 min at 37 °C. Aggregation was easily verified by inspection. The platelets were then separated by centrifugation at room temperature, the supernatant was removed and set aside for counting. The platelets were washed two times with the Tris–maleate buffer and solubilized in 0.3 M KOH. Aliquots of both the alkali-digests of platelets and the supernatants of the aggregated platelets were counted in a liquid scintillation spectrometer utilizing the solubilizer and scintillation system described above. The $Ca^{2+}$ content of control and aggregated platelets was determined on another aliquot of the alkali-digests by atomic absorption spectroscopy. Protein concentration was also measured.

## MATERIALS

$^{45}CaCl_2$ (spec. act. 729 Ci/mole) was obtained from International Chemical and Nuclear Corp., Irvine, Calif. and 5-hydroxy-[2-$^{14}$C]tryptamine creatinine sulphate (spec. act. 58 Ci/mole) from Amersham/Searle Corp., Arlington Heights, Ill. Bovine thrombin was purchased from Upjohn Co., Kalamazoo, Mich. and was used without further purification. All lipids used as reference standards for thin-layer chromatography were purchased from Mann Research Laboratories, New York, N.Y., as was crystalline bovine serum albumin. Human fibrinogen was a product of Merck, Sharp and Dohme, West Point, Pa. and soluble skin collagen was obtained from Worthington Biochemical Corp., Freehold, N. J. Silica gel N-HR was obtained from Brinkmann Instruments, Westbury, N. Y. Cab-O-Sil was purchased from the Cabot Corp., Boston, Mass.

## RESULTS

*Content and distribution of calcium in platelets*

The calcium concentration of washed human platelets was found to vary considerably from donor to donor. In eight normal subjects it ranged from 48–74 nmoles/mg platelet proteins with a mean of ($57.5 \pm 11.0$) nmoles/mg protein ($\pm 1$ S.D.). The distribution of $^{45}Ca^{2+}$ between lipid, aqueous phase and protein residue was measured in platelets of three different donors. Virtually all the radioactive counts were recovered in the protein residue, i.e. 98.3 % of the total radioactivity. Lipid and aqueous phase contained $0.8\% \pm 0.2$ and $0.9\% \pm 0.15$ (mean $\pm 1$ S.E.; four experiments) of the $^{45}Ca^{2+}$. The possibility of a change in the pattern of distribution with varying concentrations of $^{45}Ca^{2+}$ was investigated. In the range

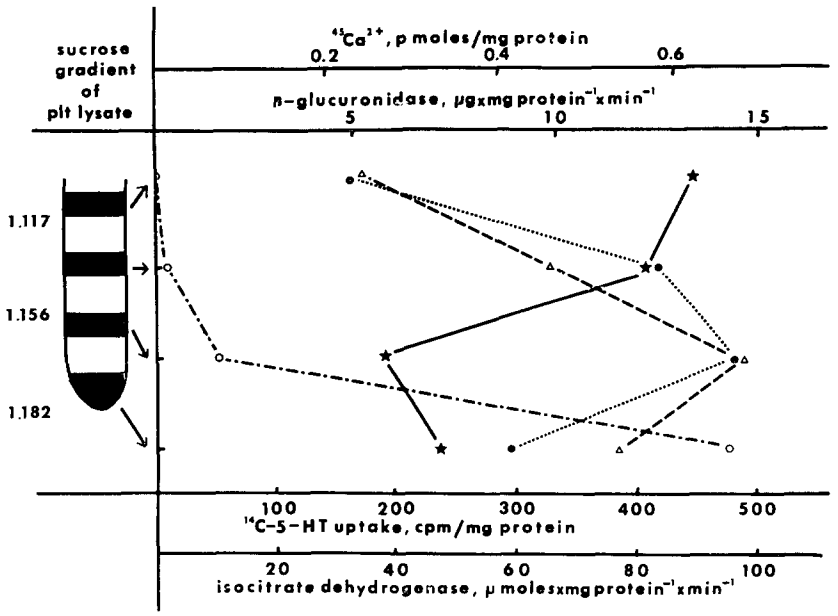


Fig. 1. Calcium binding by subcellular platelet fractions. Platelets (Plt) suspended in Tris-maleate buffer were incubated with $^{45}Ca^{2+}$ ($2 \cdot 10^{-7}$ M) at 37 °C for 120 min. After disruption of platelets by the glycerol lysis technique the lysate was layered onto an interrupted sucrose gradient. The three bands of the gradient were located in the 27, 35 and 40 % sucrose layer with a substantial sediment at the bottom. The densities of the different sucrose layers is indicated on the left hand side of the figure. Specific activities for $^{45}Ca^{2+}$ uptake (*); 5-hydroxy-[$^{14}$C]tryptamine($^{14}$C-5-HT) uptake (●), β-glucuronidase (△) and isocitrate dehydrogenase activity (○) are indicated for each subcellular fraction. This experiment is representative of the three that were performed.

from $2 \cdot 10^{-2}$–$2 \cdot 10^{-7}$ M $^{45}Ca^{2+}$ no significant change in distribution was found. The distribution of non-radioactive $Ca^{2+}$ between these three phases was determine-in platelets from three donors. The protein residue was the principal $Ca^{2+}$-containing fraction (97.8 %), followed by lipid (1.3 %) and aqueous phase (0.9 %) (all values are means). The distribution of $^{45}Ca^{2+}$ was also measured in isolated platelet membranes which were incubated with $1 \cdot 10^{-2}$ M $^{45}CaCl_2$. In these vesicles protein residue retained 69.4 % and lipids 21.1 % of the radioactivity, the remainder 9.5 % was recovered in the aqueous phase.

The subcellular distribution of calcium in platelets was studied in fractions of platelet lysate isolated from an interrupted sucrose gradient (Fig. 1 and Table I). The recovered fractions were identified by measuring specific activity of certain marker enzymes or functions. The uppermost band representing relatively pure membrane vesicles showed the highest $^{45}Ca^{2+}$ binding activity. The dense α-granules which could not be clearly separated from lysosomal granules were concentrated in the 35 and 40 % sucrose layer (densities 1.156 and 1.182, respectively). While the marker enzyme activity of β-glucuronidase and 5-hydroxy-[$^{14}$C]tryptamine uptake showed considerable overlap in these two sucrose bands, calcium was found in much higher specific activity in the 35 % sucrose fraction (density 1.156). The bottom sediment, composed primarily of mitochondria and non-disrupted platelets, had a specific uptake of $^{45}Ca^{2+}$ similar to that of the 40 % sucrose band (density 1.182).

TABLE I

DISTRIBUTION OF $Ca^{2+}$ AMONG SUBCELLULAR PLATELET FRACTIONS

$80 \cdot 10^9$–$100 \cdot 10^9$ washed platelets were disrupted by the glycerol lysis technique of Barber and Jamieson [13]. The platelet lysate was layered onto an interrupted sucrose gradient consisting of equal volumes of 27, 35 and 40 % sucrose. After centrifugation at $64\,000 \times g$ for a minimum of 180 min. The individual layers and bands were removed, fractions containing particulate matter were resedimented and after resuspension in a small volume of Tris–maleate buffer, $Ca^{2+}$ content was measured by atomic absorption spectroscopy. Protein concentration [16] was determined on an aliquot to which an equal volume of 0.6 M KOH had been added. The recovery of $Ca^{2+}$ varied from 92–101 %.

| | $Ca^{2+}$ ($\mu$g/mg protein) | $Ca^{2+}$ (% of total) |
|---|---|---|
| Cytosol fraction | 2.9 | 61.0 |
| Plasma membranes | 4.1 | 5.6 |
| Sucrose band (35 %) | 10.4 | 3.8 |
| Sucrose band (40 %) | 15.7 | 9.6 |
| Sediment (mitochondria, non-disrupted platelets) | 2.7 | 20.0 |

The greatest portion of $^{45}Ca^{2+}$ was recovered in the fraction containing the soluble proteins of the platelet lysate. Because of technical problems in the separation of this fraction from that of the 27 % sucrose band (density 1.117) no specific activities are given but more than 80 % of the total radioactivity could be recovered in the fraction representing the cytosol portion of the platelets.

The distribution of non-radioactive $Ca^{2+}$ among these subcellular platelet fractions is shown in Table I. The highest $Ca^{2+}$ content per mg protein was present in the bands containing platelet granules. The discrepancy in the specific activities of radioactive and non-radioactive $Ca^{2+}$ was most marked in these fractions indicating that the newly acquired $^{45}Ca^{2+}$ mixed imperfectly with the $Ca^{2+}$ in these platelet granules.

*Calcium ligand activity of platelet lipids*

As only about 1 % of the $^{45}Ca^{2+}$ taken up by platelets was bound by lipids it was necessary to utilize isolated platelet membranes in which organic-soluble material ligated a much higher fraction of the total calcium than in intact platelets. Thin-layer chromatographic separation of the major lipid classes made it possible to identify the major ligands (Table II). More than 90 % of the lipid–calcium was recovered in sphingomyelin and phosphatidylcholine. Phosphatidylserine, phospatidylinositol and phosphatidylethanolamine ligated only a minor fraction of the lipid–calcium.

Lipid extraction of platelets or platelet membranes by the standard method employed in these studies invariably yielded a protein residue which contained a small amount of lipid phosphorus. It was considered possible that some of the protein-bound $Ca^{2+}$ might be associated with highly acidic phosphoinositides contaminating the protein residue. We, therefore, extracted the protein residue remaining after lipid extraction as described in Methods to remove phosphoinositides. From 90 mg platelet protein 0.68 $\mu$mole of phospholipid phosphorus were extracted. Under optimal binding conditions, i.e. assuming a molar ratio of Ca/P of 1 : 2 [17] these lipids could

TABLE II

CALCIUM DISTRIBUTION IN PLATELET MEMBRANE LIPIDS

Platelet membrane vesicles suspended in 10 mM Tris-maleate buffer, pH 6.4, containing 10 mM $^{45}Ca^{2+}$ were incubated for 60 min at 37 °C. Membranes were then separated by centrifugation and lipids extracted as described in Methods. Phosphorus and $^{45}Ca^{2+}$ were determined on each identifiable spot on thin-layer chromatography. Each value is the mean of three separate experiments. All determinations were carried out in triplicate. Recovery of $Ca^{2+}$ and phosphorus from thin-layer chromatography varied between 92 and 102 %.

| Lipid class | $Ca^{2+}$ (% of total) | Phosphorus (% of total) | $Ca^{2+}$/P (molar ratio) |
|---|---|---|---|
| Sphingomyelin | 54.7 | 13.6 | 1 : 10 |
| Phosphatidylcholine | 26.6 | 45.6 | 1 : 70.3 |
| Phosphatidylinositol, phosphatidylserine | 16.0 | 7.3 | 1 : 18.6 |
| Phosphatidylethanolamine | 2.4 | 32.0 | 1 : 535 |
| Neutral lipids ceramides, cerebrosides | 0.3 | 1.5 | 1 : 207 |

maximally bind 8 % of the $Ca^{2+}$ found in the protein residue. Thus most of the $^{45}Ca^{2+}$ in the protein fraction was indeed bound to protein.

*Characteristics of binding of calcium by platelets*

Owing to the centrifugation technique employed to separate cells from medium, the shortest incubation time interval investigated was 5 min. Equilibration of $Ca^{2+}$ between platelets and medium was reached only after more than 120 min. A marked temperature dependence of the calcium uptake was noted. Assuming that first-order kinetics are operative throughout the first 15 min of incubation the apparent energy of activation, $E_a$ for calcium binding is 6900 cal/mole.

The correlation between the amount of $Ca^{2+}$ taken up by the platelets and their concentration expressed in mg of protein was found to be linear in the range tested, i.e. from 1–6 mg platelet protein.

Calcium uptake by platelets was markedly influenced by the $H^+$ concentration in the medium (Fig. 2). Our studies were confined to a pH range in which platelets were not significantly damaged or subjected to spontaneous aggregation. A sharply defined peak of $Ca^{2+}$ binding was noted at pH 6.4–6.6. Because of this finding all experiments were conducted at this optimal pH.

The rate of $Ca^{2+}$ uptake in platelets was investigated over a wide range of $Ca^{2+}$ concentrations in the medium (Fig. 3). At 30 mM concentration $Ca^{2+}$ binding had distinctly declined compared to low extracellular concentrations of the cation but saturation of its uptake mechanism had not yet been attained. For comparison we examined also the ligand activity of isolated platelet membrane vesicles (Fig. 3). With these structures saturation of all the possible binding sites was achieved at an extracellular concentration of 10 mM $Ca^{2+}$.

The effect of divalent cations on $Ca^{2+}$ uptake by platelets was examined at three different concentrations of the respective cations, 0.01, 0.001 and 0.0002 mM. Only $Mg^{2+}$ and $Fe^{2+}$ exerted a significant effect on the $Ca^{2+}$ uptake of platelets.

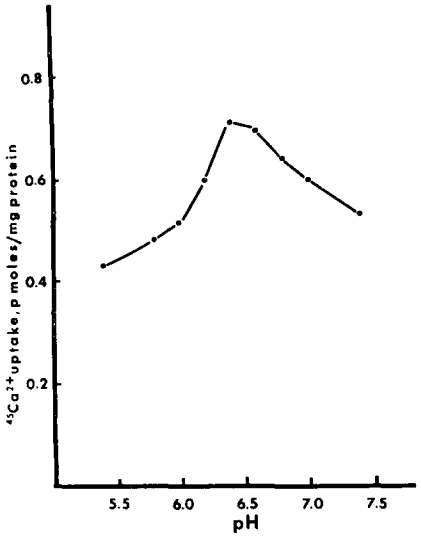


Fig. 2. Effect of pH on $Ca^{2+}$ binding. Platelets were suspended in Tris–maleate buffer of varying pH and incubated with $2 \cdot 10^{-7}$ M $^{45}Ca^{2+}$ for 120 min.

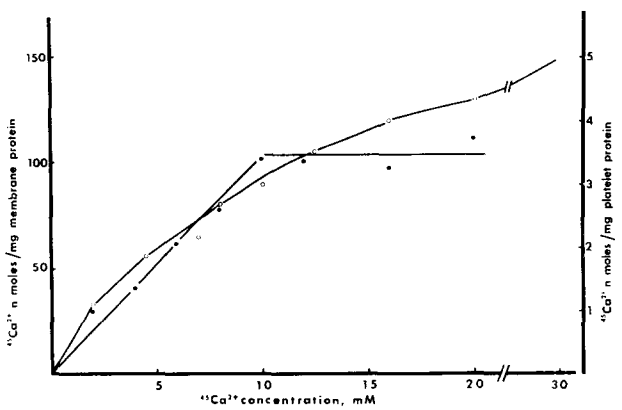


Fig. 3. $Ca^{2+}$ uptake by intact platelets and $Ca^{2+}$ binding by isolated platelet membranes as a function of the $Ca^{2+}$ concentration in the medium. Platelets suspended in Tris–maleate buffer containing $^{45}Ca^{2+}$, ranging from 2–30 mM, were incubated for 90 min at 37 °C (○, right hand ordinate). Suspensions of platelet membrane vesicles (1 mg/ml) were incubated with varying $^{45}Ca^{2+}$ concentrations, ranging from 2–20 mM, for 60 min at 37 °C (●, left hand ordinate). The membranes were washed twice with Tris–maleate buffer and their radioactivity was then determined. Each point is the mean of three experiments.

$Mg^{2+}$ at 0.01 mM decreased the $^{45}Ca^{2+}$ uptake of platelets to 85 % of the control whereas $Fe^{2+}$ at the same concentration reduced it to 65 % of that observed in controls.

Monovalent cations have been shown to influence calcium binding in a variety of cells [1]. Our studies revealed marked depression of $Ca^{2+}$ uptake as the concentration of $Na^{+}$ in the medium was increased (Fig. 4). Conversely, an increase in the

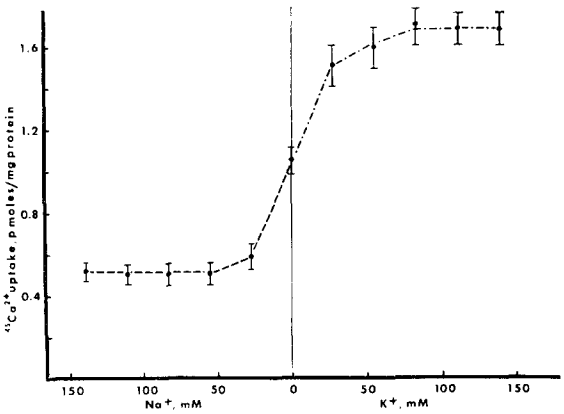


Fig. 4. Effect of monovalent cations on $^{45}Ca^{2+}$ uptake by platelets. Platelets were suspended in 20 mM Tris–maleate buffer, pH 6.4, containing $Na^+$ or $K^+$ at the indicated concentrations. Isotonicity was restored in each incubation medium by addition of sucrose. Incubation was carried out at 37 °C for 120 min. Each point represents the mean of three separate experiments, each bar indicates 2 S.E.

extracellular $K^+$ concentration resulted in a progressive rise in the rate of $Ca^{2+}$ binding. For both electrolytes this effect was proportional to their concentration in the medium in the range from 0–55 mM. At concentrations exceeding this level no further change in $Ca^{2+}$ binding was noted. The stimulatory effect of $K^+$ on the $Ca^{2+}$ binding activity of platelets was shown to depend also on the level of $Ca^{2+}$ in the suspending medium. At low extracellular $Ca^{2+}$ concentration the effect of the electrolyte was very marked while at high concentrations ($> 5$ mM) no acceleration of $Ca^{2+}$ uptake by $K^+$ could be observed.

*Exchange of calcium*

The exchange of $Ca^{2+}$ between platelets and environment was studied by suspending platelets prelabeled with $^{45}Ca^{2+}$ in media containing concentrations of non-radioactive $Ca^{2+}$ ranging from 2 $\mu$M –20mM (Fig. 5). The amount of $^{45}Ca^{2+}$ remaining in platelets suspended in these media decreased hyperbolically towards an equilibrium level which varied inversely with the extracellular $Ca^{2+}$ concentration. The time required to reach the new steady state exceeded 120 min in those platelets which were resuspended in solutions of high $Ca^{2+}$ concentrations, i.e. $> 0.2$ mM but was less than 60 min for platelets which were reincubated in media of low $Ca^{2+}$ concentration, i.e. $< 20$ $\mu$M. Graphic analysis of the curve depicting the change in $^{45}Ca^{2+}$ in platelets by a sum of exponential terms with constant coefficients [23] suggested a multicompartmental distribution of this divalent cation in platelets. Applying this method to the release of $^{45}Ca^{2+}$ from platelets a minimum of two exponential terms were estimated with $t_{\frac{1}{2}}$ of 4 and 16 min, respectively, for the two slopes.

Measurement of the absolute amounts of $Ca^{2+}$ in platelets incubated for up to 120 min in media of varying $Ca^{2+}$ concentration (up to 20 mM) showed only a very slight rise in platelet $Ca^{2+}$ at 15 min and return to preincubation levels at 30 min

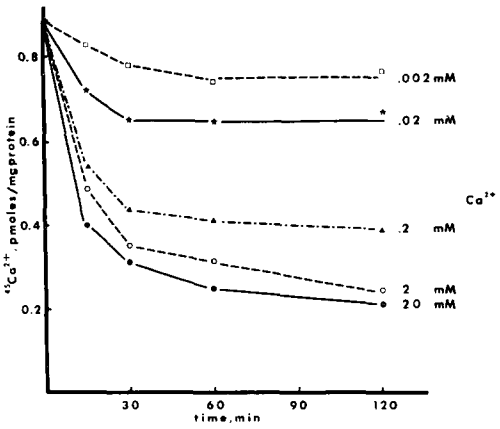


Fig. 5. Release of $^{45}Ca^{2+}$ from platelets. Platelets suspended in Tris–maleate buffer were incubated with $2 \cdot 10^{-7}$ M $^{45}Ca^{2+}$ for 120 min at 37 °C following which non-radioactive $CaCl_2$ was added to give the indicated concentrations in the medium and incubation continued. The $^{45}Ca^{2+}$ remaining in platelets after the time periods indicated in the figure was measured. Each point signifies the mean of three experiments.

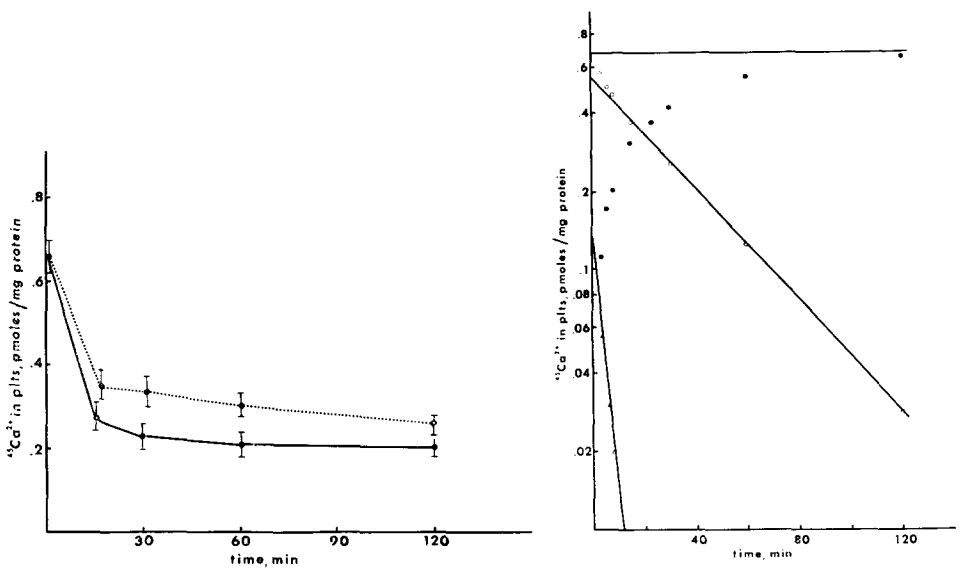


Fig. 6. Effect of glucose in the medium on the release of $^{45}Ca^{2+}$ from platelets. Platelets (Plts) suspended in Tris–maleate buffer with or without 5.5 mM D-glucose were incubated with $2 \cdot 10^{-7}$ M $^{45}Ca^{2+}$ for 60 min at 37 °C. The $Ca^{2+}$ concentration was then adjusted to 2 mM and incubation continued for the time periods shown in the figure. Each value represents the mean of four separate experiments. The extent of 2 S.E. is indicated by the horizontal bars.

Fig. 7. Graphic analysis of $Ca^{2+}$ uptake data by the method of Solomon [23]. Platelets (Plts) were incubated as described in the text. The constant term was found to be 0.67 pmole $^{45}Ca^{2+}$/mg protein. ●, observed values; ○, calculated second-rate values; △, first-rate values.

incubation. The magnitude of the transient increase in platelet $Ca^{2+}$ was dependent on the concentration of this cation in the medium.

$Ca^{2+}$ exchange between platelets and suspending medium was also studied with respect to the effect of glucose in the medium. The exchange was significantly greater in platelets suspended in a glucose-containing medium than in solutions lacking it (Fig. 6).

TABLE III

EFFECT OF AGGREGATING AGENTS ON PLATELET $Ca^{2+}$

Change in platelet $Ca^{2+}$, each value represents the mean of four experiments ± 1 S.E. Procedural details are described in Methods.

| Aggregating agent | Concn | Change in platelet $Ca^{2+}$ (% of control) | 5-Hydroxy-[$^{14}$C]-tryptamine release (% of total platelet activity) |
|---|---|---|---|
| Control | — | 100 | 0 |
| ADP | 100 $\mu$M | 97.6±4.5 | 72.4±5.7 |
| Collagen | 110 $\mu$g/ml | 59.8±5.1 | 76.2±6.3 |
| Epinephrine | 0.45 $\mu$mole/ml | 96.8±4.7 | 74.3±5.9 |
| Thrombin | 1 unit/ml | 20.2±3.2 | 88.4±5.8 |

*Effect of aggregating agents on platelet calcium*

Thrombin, a powerful platelet aggregator, has been shown to cause a release of $Ca^{2+}$ from platelets [24]. Almost 80% of the platelet $Ca^{2+}$ was released into the medium when platelets were incubated with thrombin at a concentration of 1 unit/ml (Table III). This effect was specific for $Ca^{2+}$, the $Mg^{2+}$ concentration in the platelet did not significantly change. Of the other aggregating agents tested only collagen produced a release of $Ca^{2+}$ from platelets. The release response was however only a fraction of that induced by thrombin.

*Analysis of $^{45}Ca^{2+}$ uptake*

Experiments were carried out in which platelets were preincubated with 0.2 $\mu$M non-radioactive $Ca^{2+}$ containing incubation buffer for 90 min following which a tracer dose of $^{45}Ca^{2+}$ was added. Based on our time experiments of $^{45}Ca^{2+}$ uptake by platelets a condition close to steady state was known to exist at such a time interval.

As the volume of the incubation medium was so much larger than the platelet volume the radioactive concentration in the medium was virtually constant throughout the experiment. The $^{45}Ca^{2+}$ uptake data were analyzed graphically according to Solomon [23] as a sum of exponential terms (Fig. 7). The equation describing the $^{45}Ca^{2+}$ uptake was estimated employing a minimum number of exponential terms which turned out to be two for human platelets.

$$C = 0.126\, e^{-0.225t} - 0.539\, e^{-0.025t} + 0.665$$

whereby $C$ is the $^{45}Ca^{2+}$ concentration in the platelet and $t$ is the time in minutes. As the equation of uptake by a system of $n$ compartments is generally represented by a

linear differential equation of order $n-1$ with constant coefficients, the above equation of $Ca^{2+}$ uptake by platelets should correspond to a three compartment system.

Based on our results we propose a three compartment system for the platelet including a surface compartment, an intracellular and a non-exchangeable space. We have assumed that the $Ca^{2+}$ in the medium equilibrates first with the surface compartment before significant amounts are taken up by the intracellular space. It should be pointed out that the designations of the conjectured compartments are not intended to correspond to definite morphologically identifiable platelet structures. Based on the afore-mentioned assumption the uptake of $Ca^{2+}$ into the platelet compartments is described by the following equations:

$$S = -0.126\, e^{-0.225\, t} + 0.126$$
$$I = -0.539\, e^{-0.025\, t} + 0.539$$

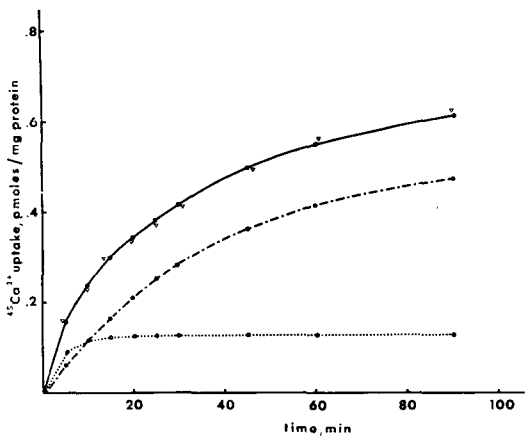


Fig. 8. Calculated $^{45}Ca^{2+}$ uptake curve of platelets (●–●–●); △, observed values. ·····, the calculated uptake curve of the surface space; – · – · –, the calculated uptake curve of the intracellular space.

whereby $S$ signifies the surface and $I$ the intracellular space. From the exponents and coefficients of these equations apparent transfer rate constants could be calculated. The constant for the influx into the surface compartment was $k_1 = 0.028\ min^{-1}$ whereas that for the intracellular space was found to be $k_3 = 0.0168\ min^{-1}$. The efflux constants were $k_2 = 0.197\ min^{-1}$ and $k_4 = 0.009\ min^{-1}$. The effective surface compartment with which the extracellular $Ca^{2+}$ equilibrates is 13.4 % and the intracellular compartment 57.2 % of the total platelet water space. The remainder constitutes a non-exchangeable $Ca^{2+}$ compartment.

## DISCUSSION

The calcium content of human platelets has been investigated by a number of authors. Our results fall into the range of values reported by Maupin [25] and by Morita and Asada [26]. The reason for the large discrepancy between our results and

those of Cousin and Caen [27] and Wallach *et al.* [10] who reported a $Ca^{2+}$ content of platelets twice to three and one-half times that found in this study is not completely clear. In the preparation of platelets we have included one washing with EDTA-containing buffer solution to remove loosely bound $Ca^{2+}$ on the platelet surface. It is possible that this procedural detail is responsible for some of the discrepancy of the calcium content of platelets.

The distribution of platelet calcium among lipid, protein and aqueous phase was unexpected as previous studies by Wallach et al. [10] have suggested that the major portion of $Ca^{2+}$ in platelets is bound to lipids. Our studies repeated numerous times on platelets from different donors gave very reproducible results which showed proteins to be the primary $Ca^{2+}$ ligands.

The distribution of $Ca^{2+}$ in isolated platelet membranes was very similar to that in erythrocyte ghosts [17]. As the greater part of the cellular lipids is present in membranes, virtually all the $Ca^{2+}$ within platelets must be bound to proteins. The ligand activity of individual lipid species in isolated platelet membranes differed from that observed in red cell ghosts in that the predominant $Ca^{2+}$ binders were sphingomyelin and phosphatidylcholine which in platelets constitute almost two-thirds of the lipid phosphorus [18]. Unlike in red cell membranes the $Ca^{2+}$/P ratio was highest in the fraction of sphingomyelin followed by phosphatidylserine and phosphatidylinositol. In none did it reach the ratio of 1 : 2 found in red cell ghosts by Forstner and Manery [17]. The small amount of $Ca^{2+}$ in the aqueous phase is probably present both in complexed and ionic form, with nucleotides the primary ligands among the former. While the technique utilized to separate platelet surface membranes will not produce a completely pure uncontaminated preparation, studies by other authors [13] and by Steiner and Tateishi [28] have shown that the membrane vesicles obtained are of high purity as judged by electron microscopy, assay of specific activities of marker enzymes and outside-inside orientation determined by lactoperoxidase-catalyzed iodination of surface membrane proteins.

Although the results of the studies on subcellular distribution of platelet $Ca^{2+}$ seem to indicate that almost two-thirds are localized in the cytosol portion of platelets caution must be exercised in the interpretation of these data. It is probable that during the fractionation procedure some of the $Ca^{2+}$ is released from the subcellular organelles and appears subsequently in the cytoplasmic pool. While this possibility must be considered in view of the recent report that a portion of the platelet granules contains most of the intracellular $Ca^{2+}$ [29] our finding that the $Ca^{2+}$ release response is much greater with thrombin than with the other aggregating agents used would seem to argue against this hypothesis. The $Ca^{2+}$ release effect of thrombin may be related to the release of a protein with the characteristics of thrombosthenin from platelets aggregated by this agent [30]. In the cytosol portion of platelets troponin appears to be the most likely candidate for a role in $Ca^{2+}$ binding. This protein together with tropomyosin has been shown to be a constituent of the platelet's contractile system [31].

While some aspects of the calcium ligand activity of platelets are similar to that in other mammalian cells the sharp pH optimum at 6.4 appears to be specific for platelets. Based on this observation the most likely receptor sites for $Ca^{2+}$ are carboxyl groups which generally have rather low p$K$ values, certainly those of aspartic and glutamic acid [32]. Sialic acid although a good potential $Ca^{2+}$ binding site and quite

abundant in platelet membranes has not been shown to be a significant ligand for the cation.

The effect of divalent cations was limited to a moderate inhibition by $Mg^{2+}$ at a concentration at least one order of magnitude higher than that of $Ca^{2+}$. Competitive inhibition of $Ca^{2+}$ binding by $Mg^{2+}$ has been shown in isolated sarcoplasmic reticulum of muscle [33] and in intact hepatic cells [34]. The reduction of $Ca^{2+}$ uptake in the presence of $Fe^{2+}$ was quite unexpected. In previous studies examining the transport of chromate to platelets a similar depressing effect of $Fe^{2+}$ was noted [35]. Most of the $Fe^{2+}$ was found attached to the platelet membrane. We, therefore, believe that this cation exerts an unspecific effect on the plasma membrane of platelets.

The effect of the monovalent cations $Na^+$ and $K^+$ on $Ca^{2+}$ flux and particularly the effect of extracellular $Ca^{2+}$ on the intracellular levels of these two electrolytes has been extensively studied both in intact cells [36, 37] and also in isolated membrane systems [33]. Our results are in general agreement with those of Niedergerke and Lüttgau [38] and Morrill and Robbins [37] whose data suggested competition of $Ca^{2+}$ and $Na^+$ at the cell membrane. The stimulatory effect of $K^+$ on $Ca^{2+}$ uptake which was limited to low extracellular $Ca^{2+}$ concentrations is difficult to explain. A direct influence of $Ca^{2+}$ ions on the physicochemical properties of the membrane has been postulated by Manery [1] to explain the $K^+$ depletion and $Na^+$ loading of cells in $Ca^{2+}$-free media. It is possible that at low extracellular $Ca^{2+}$ concentrations an effect of $K^+$ on membranes may express itself which at high $Ca^{2+}$ concentrations in the medium is overshadowed by the latters's influence on these structures.

The question whether platelet $Ca^{2+}$ exchanges with the medium or not has been quite controversial. While in vivo studies have led Odell and Upton [11] to conclude that $Ca^{2+}$ in platelets is in free equilibrium with plasma calcium, in vitro studies by Wallach and co-workers [10] and also by Mürer and Holme [39] have argued against an exchange. Our studies have established that an exchange of $Ca^{2+}$ between platelets and medium is possible. By using $^{45}Ca^{2+}$ and by measuring total $Ca^{2+}$ by atomic absorption spectroscopy we were able to show that at physiological $Ca^{2+}$ concentrations somewhat more than 5 % of the platelet $Ca^{2+}$ exchanges with the medium. Although the fitting of curves by the exponential method [23] may become quite difficult when the slopes of the curves are very steep, by using $^{45}Ca^{2+}$ we were limited to incubation times longer than 5 min and thus avoided the problem of fitting curves to the initial rates of $Ca^{2+}$ flux. The calculation of two fractions of exchangeable $Ca^{2+}$ in platelets with widely differing rates of turnover must thus be considered as a minimum estimate.

Although our data of course do not allow identification of structural location for the two $Ca^{2+}$ compartments, in analogy with other cells containing large quantities of contractile protein we suggest that the rapidly exchangeable $Ca^{2+}$ pool in platelets comprises the dense tubular system of canaliculi which has been likened to the sarcoplasmic reticulum of muscle cells [40].

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