Biochimica et Biophysica Acta, 772 (1984) 251–258
Elsevier

BBA 72053

CHARACTERIZATION OF CALCIUM LIBERATION FROM A HUMAN PLATELET MEMBRANE FRACTION

JOCELYNE ENOUF, RAYMONDE BREDOUX and SYLVIANE LÉVY-TOLEDANO

Unité de Recherches de Thrombose Expérimentale et Hémostase, INSERM U150, LA 334 CNRS, Hôpital Lariboisière, Université Paris VI, 6, Rue Guy Patin, Paris (France)

(Received August 12th, 1983)
(Revised manuscript received November 29th, 1983)

Key words: Ca^{2+} efflux; Ca^{2+} release; Internal membrane; (Human platelet)

Calcium efflux and EGTA-induced calcium release from an internal platelet membrane fraction have been studied after the oxalate-supported calcium uptake had reached steady state. Increasing external calcium concentrations stimulate the calcium efflux velocity, with an apparent half-maximal stimulation at about 5 μM outside calcium concentration and a maximal velocity of calcium efflux of 4.66 ± 2.32 nmol · min^{-1} · mg^{-1}. Moreover, the ratio of the liberated calcium on the loaded calcium seems to be independent of the increasing external calcium concentration. Increasing the calculated internal calcium concentration by varying the oxalate potassium concentration from 10 mM to 1 mM results in an increase of the liberated calcium from the membrane vesicles from 7.4% to 63%, respectively, without changing the calcium efflux velocity. Similar conclusions can be drawn from the observation of results from the calcium efflux and EGTA-induced calcium release methods. Moreover, calcium pump reversal does not seem to be responsible for the calcium efflux or calcium release. All these different points added to the previously described regulation of calcium efflux by the catalytic subunit of cAMP protein kinase suggest us that the mechanism of calcium liberation by the platelet membranes is different from the calcium uptake.

Introduction

It is generally considered that the intracellular movements of calcium ions play a decisive role in regulating physiologic and biochemical processes in platelets [1,2]. This is supported by the platelet aggregation and secretion induced by the calcium ionophore A23187, the regulation of many intracellular enzymes through a calmodulin intermediate such as the myosin light-chain kinase [3] or phospholipase A_2 [4] and by the fact that drugs which interfere with calcium transport block platelet function [5]. The mechanism of regulation of the calcium concentration is not clear, but both external and internal membranes seem to be involved.

The external membranes contain calcium associated with phospholipids, and Le Breton et al. [7] have demonstrated its intracellular liberation [6]. Vickers et al. [7] calculated that the calcium bound to phosphatidylinositol 4,5-diphosphate liberated after ADP stimulation provides the major source of the cytoplasmic calcium.

The intracellular membranes are able to incorporate and liberate calcium. This is achieved by the dense tubular system, a major component of the internal membranes which resembles to the

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N'*-tetracetic acid; Arsenazo III, 2,7-bis(arsonophenylazo)-1,8-dihydroxynaphthalene 3,6-disulfonic acid; $(Ca^{2+} + Mg^{2+})$-ATPase, calcium-activated, magnesium-dependent adenosine triphosphatase.

muscular sarcoplasmic reticulum. Furthermore, calcium from the external membranes might serve as a trigger for the liberation of calcium from storage organelles or dense tubular system. This is called the calcium-induced calcium release and has been shown in skinned cardiac cells [8].

A platelet membrane fraction which sequesters calcium has been described by Käser-Glanzmann and co-workers [9,10]. We have confirmed that calcium uptake is associated with platelet membranes and characterized the uptake system [5]. We were also interested in the liberation of calcium from these membrane vesicles this function being essential for platelet activation. Such liberation has been approached and appears to be regulated in a way different from that of calcium uptake. The catalytic subunit of the cAMP protein kinase increases the liberation, whereas this subunit has no effect on the calcium uptake [11]. This discrepancy suggests to us that the mechanism of calcium uptake and calcium liberation are not the same. The work reported here is designed to examine the various parameters which lead to a characterization of the calcium liberation from the membrane vesicles.

Methods

Preparation of the vesicular membrane fraction

A platelet membrane fraction which actively sequesters calcium was prepared using a modification of the method of Käser-Glanzmann and coworkers [9,10]. Blood was drawn from the antecubital vein of normal donors who had not ingested any drugs for 2 weeks and was anticoagulated with 1/10 vol. (15.2 mM citric acid/8.94 mM trisodium citrate/15 mM monosodium phosphate · $2H_2O$/128 mM glucose).

Platelet-rich plasma was separated by centrifugation at 120 × *g* for 15 min at room temperature. EDTA was then added to the platelet-rich plasma to a final concentration of 5 mM and the platelets were pelleted by centrifugation at 3000 × *g* 15 min at room temperature. They were then washed twice using a modified Tyrode's buffer containing 130 mM NaCl/5 mM KCl/1 mM NaH_2PO_4/24 mM $NaHCO_3$/2 mM Na_2 EDTA/10 mM glucose/12.5 mM saccharose/0.35% bovine serum albumin and left overnight at 4°C. The washed platelets were then centrifuged and resuspended in a homogenizing medium containing 100 mM KCl/2 mM $MgSO_4 \cdot 7H_2O$/25 mM NaCl/12 mM trisodium citrate/10 mM glucose/25 mM Hepes/5 mM ATP/0.35% bovine serum albumin (pH 7.0, 340 mosmol/l).

After this step all the following procedures were carried out at 4°C. Platelets were lysed by ultrasonication in a Branson sonifier B_{12} at 40 W. The ultrasonication was applied to 2 ml platelet fractions ($2 \cdot 10^9$ platelets/ml) four times, for 5 s. The resulting platelet lysate was centrifuged at 19000 × *g* during 25 min to eliminate granules, mitochondria and unlysed platelets. The supernatant was then centrifuged at 100000 × *g* for 60 min. The pellet membrane fraction was resuspended in an incubation medium comprising 10 mM potassium oxalate/100 mM KCl/10 mM $MgCl_2$/20 mM Hepes/5 mM ATP (pH 7.55) stored at 4°C and used within 48–72 h. Freezing at −20°C involves a loss of activity.

This calcium-accumulating membrane fraction has been characterized [5,11]: briefly, calcium uptake into the membranes is mediated by a (Ca^{2+} + Mg^{2+})-ATPase of 120 kDa with a high affinity towards calcium. Biochemical evaluation revealed a mixture of plasma membranes and internal membranes (data not shown), but calcium release came probably from internal membranes, as will be discussed. Moreover, studies of calcium fluxes in membrane vesicles would benefit from the availability of populations of uniform polarity. Such populations are now under investigation, but for the studies presented here, no attempt has been made to obtain vesicles of uniform polarity.

Determination of the volume of the membrane vesicles

5 mM of Arsenazo III was added to the platelet suspension during the lysis step. Then the platelet membrane fraction was obtained as described above. The 100000 × *g* pellet was resuspended and was applied to a Sephadex G-50 column in order to eliminate extravesicular Arsenazo III. The membrane vesicles were then lysed by adding Triton X-100. The concentration of Arsenazo III in the lysate was calculated from the 535 nm absorption with reference to a standard curve obtained using a range of Arsenazo III solutions of known

concentration. A dilution factor can be calculated by reference with the Arsenazo III solution used to enclose in the membrane vesicles.

Calcium efflux measurement

Membrane vesicles were loaded with calcium at 37°C essentially according to Martonosi and Feretos [12] in media containing 10 mM potassium oxalate/100 mM KCl/10 mM $MgCl_2$/20 mM Hepes (pH 7.55)/5 mM ATP/10 to 200 μM $^{45}CaCl_2$ (1000 cpm/nmol) (Amersham, France). Calcium uptake was initiated by addition of platelet membrane vesicles (up to 500 μg protein per ml). Samples (0.2 ml) were removed and filtered through Millipore HAWP filters (0.45 μm pore size) previously soaked in a 2 mg/ml bovine serum albumin solution. Filters were washed with 2 ml of 0.1 M $CaCl_2$ and counted in 10 ml of Unisolve scintillation liquid. Calcium efflux measurement was performed according to Kirchberger and Wong [13]. Briefly, platelet membrane vesicles were loaded with $^{45}CaCl_2$ as described before. A second set of reactions was run concomitantly under the same conditions except that non-radioactive $CaCl_2$ was used. When the maximal calcium content of vesicles was attained (about 120 min) as determined in a preliminary experiment, a small amount (1 μCi/ml) (0.45 μM) of tracer $^{45}CaCl_2$ was added to the unlabelled suspension in order to estimate the unidirectional influx of calcium into the membrane vesicles. Calcium efflux was calculated from the measured influx of ^{45}Ca under steady-state conditions during which efflux equals influx. The external calcium concentration at zero-time of the calcium efflux was estimated by the difference between calcium added and calcium sequestered at the steady state.

Measurement of EGTA-induced calcium release

Membrane fraction was incubated in the presence of different calcium concentration of $^{45}CaCl_2$ in the incubation medium [12]. After steady-state calcium fluxes had been reached, 2 mM EGTA was added to the mixture incubation to reduce the calcium concentration outside the membranes so that no calcium uptake occurred. Calcium release was calculated from the decrease in calcium content of the vesicles upon addition of EGTA.

Protein determination

The protein concentration of the membrane fraction was determined by the method of Bradford using gamma-globulins as standard [14].

Results

(a) Further characterization of the calcium uptake into platelet membranes

Characterization of the calcium uptake has been described previously [5,11]. 300 ± 100 nmol of calcium could be accumulated per mg of protein in the presence of 100 μM $CaCl_2$ (mean of ten experiments ± S.D.). The intravesicular calcium concentration can be calculated from the intravesicular calcium load at the steady state and the intravesicular volume. We have estimated the volume of the membrane vesicles which reached 2.85 μl per mg of protein. This value is similar to the value of membrane vesicles from reticulum volume which ranges from 1.3 μl/mg to 4.41 μl/mg of protein [15,16]. With an intravesicular

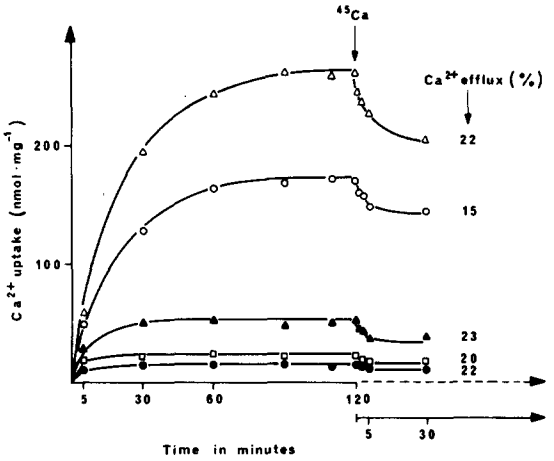

Fig. 1. Time-course of calcium uptake and calcium efflux of platelet membrane vesicles as a function of different external calcium concentrations. 180 μg/ml were incubated with the following concentrations of $CaCl_2$: △——△, 100 μM; ○——○, 50 μM; ▲——▲, 20 μM; □——□, 10 μM and ●——●, 5 μM. The outside calcium concentrations, $[Ca]_o$, after 120 min of incubation were estimated by parallel sets of experiments carried out in the presence of $^{45}CaCl_2$ as described under Methods: △——△, 58 μM; ○——○, 9.95 μM; ▲——▲, 4.3 μM; □——□, 1.35 μM and ●——●, 1.4 μM. Incubations were carried out in the presence of 10 mM potassium oxalate.

calcium load of 300 nmol/mg we obtained a calcium concentration of 105 mM. This value would represent a 1000-fold concentration gradient with respect to the medium, indicating that in addition to its high affinity for calcium, the calcium transport system has a high capacity.

(b) Effect of varying external calcium concentrations on the calcium efflux

The effect of extravesicular calcium concentration on the calcium efflux was studied by incubation of the membranes with variable concentrations of $CaCl_2$ in the medium in the presence of 10 mM potassium oxalate giving a fixed free internal calcium concentration.

Fig. 1 shows the time-course of calcium uptake as a function of various external calcium concentrations. It can be seen that the platelet membranes reached filling after 120 min of incubation for high calcium concentrations (50 to 100 μM) under the conditions used. The same figure shows that the time-course of the calcium efflux is rapid and a maximum degree of efflux is obtained within 10 to 15 min. The maximal efflux represents a percentage of liberated calcium from the vesicles which varied between 15 and 23% in this experiment. The ratio of the liberated calcium on the loaded calcium seems to be independent of the external calcium concentration calculated at zero-time of the efflux study.

There is variation from one preparation to another. The mean value obtained is $15 \pm 9\%$ for 12 experiments. Explanations for these variations are still uncertain, but the platelets, being from different donors might be a contributing factor.

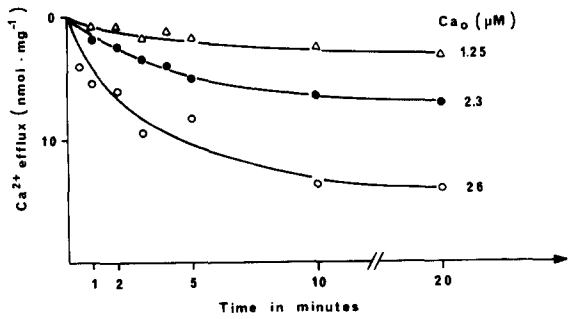

Fig. 2. Time-course of calcium efflux as a function of various outside calcium concentrations $[Ca]_o$ after 120 min of incubation.

Fig. 2 shows the dependence of calcium efflux velocity on the external calcium concentration. An increase of the external calcium concentration from 1.25 to 26 μM leads to an increase of the calcium efflux velocity. Using the double-reciprocal plots of the initial rate of calcium efflux as a function of outside calcium concentration at the zero-time of efflux, we can estimate an apparent K_m for calcium of 5 μM. The maximum velocity of calcium efflux is 4.66 ± 2.32 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} (mean of nine experiments $\pm$ S.D.) when using 100 μM calcium concentration in the incubation mixture.

From these results, an increase in the concentration of extravesicular calcium at zero-time of the efflux, involves an increase in the calcium permeability, the permeability coefficient being the ratio between the calcium efflux rate and the free internal calcium concentration.

(c) Effect of varying external calcium concentrations on the EGTA-induced calcium release

The addition of 2 mM EGTA to calcium-loaded vesicles when steady state has been reached pro-

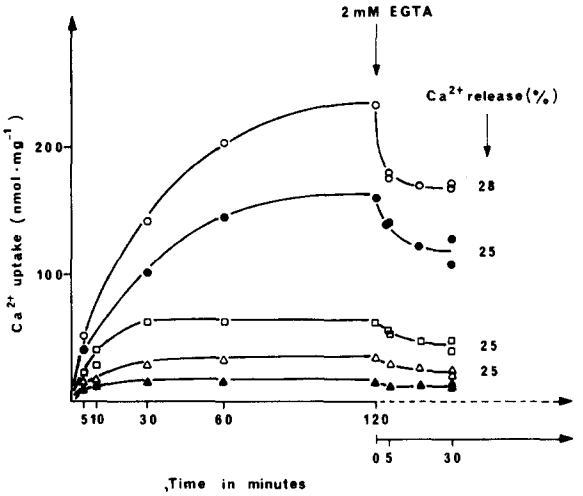

Fig. 3. Time-course of calcium uptake and EGTA-induced calcium release of platelet membrane vesicles as a function of different external calcium concentrations. Membranes (180 μg/ml) were incubated with the following concentrations of $^{45}CaCl_2$: ○——○, 100 μM; ●——●, 50 μM; □——□, 20 μM; △——△, 10 μM and ▲——▲, 5 μM. The estimated outside calcium concentrations $[Ca]_o$ after 120 min of incubation were: ○——○, 34 μM; ●——●, 7.5 μM; □——□, 6.4 μM; △——△, 3.5 μM and ▲——▲, 1.65 μM. Reactions were carried out as described under Methods with 10 mM potassium oxalate.

duces a release of stored calcium.

Fig. 3 shows the external calcium concentration dependence curves obtained by this method. Again, a decrease in external calcium concentration induces a decrease in the calcium efflux velocity, while the ratio of released calcium to loaded calcium remains constant (25–28%). This figure represents a typical experiment, but a mean value of the rate of calcium release has been calculated when 100 μM calcium had been used to load the membrane vesicles. This value is of 7.5 ± 2.8 nmol $\cdot \min^{-1} \cdot \mathrm{mg}^{-1}$ for three experiments. The corresponding mean value for the amount of liberated calcium is of $34 \pm 16\%$. Another way to obtain such information is to test the calcium release from different concentrations of membrane vesicles loaded with a given calcium concentration. When using 100 μM calcium and membrane vesicles varying from 0.57 mg/ml to 0.057 mg/ml, we realize a variation of external calcium concentration at the time of calcium release study and the results confirm the data previously concluded (data not shown). These values are higher than those determined by the method of calcium efflux. This is probably due to outside-bound calcium which was desorbed from membrane vesicles upon addition of EGTA. Dissociation of superficial ions occurs in the millisecond scale, so it would be too rapid to be detected by the Millipore technique used [17]. However, the calcium content, 30 s after adding EGTA can be considered as a measure of this parameter. This value is higher than the corresponding value determined by the method of calcium efflux.

(d) Effect of varying the internal calcium concentrations on calcium efflux and EGTA-induced calcium release

Calcium precipitating anions have been used in studies of Ca^{2+} uptake to enhance the amount of accumulated Ca^{2+} by lowering the intravesicular calcium activity [18]. In order to estimate the influence of this parameter, we compared results obtained from experiments carried out in the presence of different potassium oxalate concentrations. Assuming a solubility product of $4.6 \cdot 10^{-6}$ M^2 [19], one may calculate the internal calcium concentrations. The calcium concentration varied from 0.46 to 4.6 mM when potassium oxalate concentration varied from 10 to 1 mM, respectively.

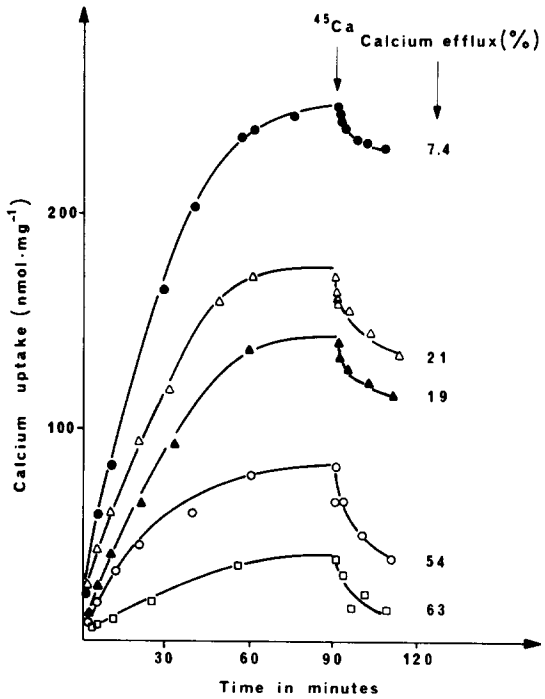

Fig. 4. Time-course of calcium uptake and calcium efflux as a function of various potassium oxalate concentrations: ●——●, 10 mM; △——△, 5 mM; ▲——▲, 3 mM; ○——○, 2 mM and □——□, 1 mM. Experiments were carried out as described in Methods.

Fig. 4 shows that the liberation of calcium from the vesicles varies from 7.4% to 63% when the internal free calcium concentration varies between 0.46 mM and 4.6 mM. This variation also appears when we compare the mean value obtained from eight different experiments in the presence of 4.6 mM internal calcium concentration: $37.5 \pm 19\%$ to the mean value of $15 \pm 9\%$ previously given when using 0.46 mM free internal calcium concentration. When the free internal calcium concentration is over 4.6 mM the percentage of liberated calcium remains unchanged. This last point is correlated with the fact that oxalate concentrations enhanced the calcium uptake in a concentration-dependent fashion only over 1 mM. If we consider the effect of the variation of internal calcium concentrations on the calcium efflux velocity, we do not obtain direct correlation (Table I). It seems that the variation is inversely proportional up to 2.5 mM oxalate concentration. If these results are expressed in

TABLE I

EFFECT OF VARIOUS INTERNAL CALCIUM CONCENTRATIONS ON THE CALCIUM EFFLUX RATE AND THE AMOUNT OF LIBERATED CALCIUM

Potassium oxalate (mM)	Internal calcium concentrations (mM)	Efflux rate ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Efflux (%)
10	0.46	6.11	7.5
5	0.92	4.05	8
2.5	1.8	2.64	12
1.25	3.6	3.05	40
1	4.6	2.55	60
0.5	9.2	1.92	56

terms of the permeability coefficient, this is found to decrease when the internal calcium concentration increases. Data obtained by the determination of EGTA-induced calcium release allow us the same conclusions (data not shown).

(e) Effect of acetyl phosphate on calcium efflux and EGTA-induced calcium release

In view of further characterization of the mechanism of calcium liberation, we tested the calcium fluxes when acetyl phosphate (Sigma) replaced ATP as the energy donor. In these conditions, ADP formation during the initial calcium uptake reaction is excluded and calcium fluxes cannot be coupled to the resynthesis of ATP. Thus, the reversal of the pump is not supported.

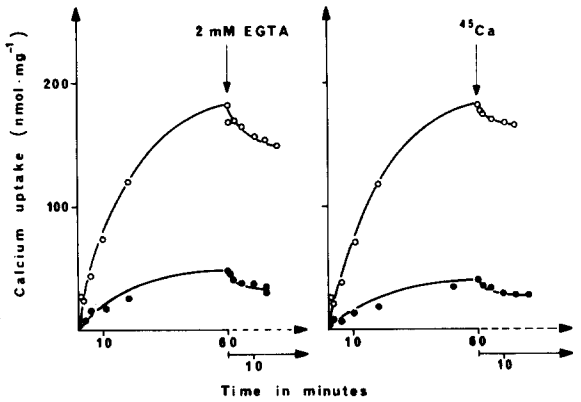

Fig. 5. Effect of ATP and acetyl phosphate on the calcium uptake, calcium efflux, and EGTA-induced calcium release. Experiments were carried out in the presence of 10 mM potassium oxalate and 5 mM ATP or acetyl phosphate and 100 μM $CaCl_2$: O——O, ATP; ●——●, acetyl phosphate.

Fig. 5 shows that the rate of calcium uptake decreases when 5 mM acetyl phosphate is used as energy donor and the calcium content at the steady state is reduced by 73%. It can be seen that liberation of calcium occurs when the pump does not run backward and that both methods gave similar results. The rate of the calcium efflux and calcium release are not affected by the acetyl phosphate and the amount of liberated calcium is about 30%. Moreover, the study of the effect of external calcium concentrations on the calcium efflux measured in the presence of acetyl phosphate leads to the same conclusions as in the presence of ATP. An increase in the external calcium concentration involves an increase in the efflux rate, but the amount of liberated calcium is not correlated (data not shown).

Discussion

This work describes the release of calcium from platelet membrane vesicles. The membrane fraction which is used for our studies consists of internal and external membranes [20]. A controlled ultrasonication of platelets is used in order to obtain maximal platelet lysis without degradation of internal structures [21]. Therefore, the membranes of the dense tubular system are supposed to be right-side-out vesicles. However, ultrasonication is known to yield a mixture of inside-out and right-side-out vesicles of the external membranes. The right-side-out vesicles formed from the dense tubular system can accumulate and release calcium, whereas there is some evidence to suggest that external membranes do not play a role in calcium release. Indeed, calcium uptake by right-side-out vesicles has been described [22–25], but this calcium uptake is not related to active calcium uptake which is performed in this work. In contrast, inside-out external membranes might interfere in the calcium uptake if an ATPase pump is localized in the inner leaflet of the external membranes as for other plasma membranes [26]. However, preliminary results suggest that internal platelet membranes show a higher affinity towards calcium than do external membranes [20].

Therefore, we conclude that the calcium liberation as measured with the mixture of internal and

external membranes is almost exclusively due to release of calcium from internal membrane vesicles.

Characterization of the calcium release is performed with comparison of results obtained with two distinct methods: the calcium efflux has the disadvantage of possibly including a contribution of calcium-calcium exchange. We therefore compare the EGTA-induced calcium release which allows us to measure the rate of calcium release under conditions where no influx via the pump is possible and effectively unmasking the efflux pathway [27].

Characterization of the calcium release was first studied with varying external calcium concentrations. The calcium release rate is stimulated by micromolar extravesicular calcium concentrations. Similar results have also been obtained for cardiac sarcoplasmic reticulum [13] and for rabbit skeletal muscle microsomes [28]. This phenomenon is described as the trans stimulation of carrier-mediated transport [13]. Interestingly Yamamoto and Kasai [29–31] found a similar Ca^{2+} dependence related to a Ca^{2+} channel which mediates the Ca^{2+}-induced calcium release in the rabbit skeletal muscle sarcoplasmic reticulum membrane vesicles. The activation of calcium liberation by external calcium is interesting with regard to the hypothesis of calcium-induced calcium release. This may explain the mechanism of the increase of cytoplasmic calcium concentration during platelet activation. Such a possible regulation of calcium liberation by calcium itself suggests that phosphatidic acid [32], and more specifically lysophosphatidic acid [33], is not the only mechanism responsible for the increase of intracellular calcium concentration during platelet activation.

Calcium release has also been characterized by its internal calcium concentration dependence. Variations of this parameter did not affect in proportion the calcium efflux velocity. This effect of internal calcium concentration on the calcium liberation rate may be compared to the effect of external calcium liberation on the calcium uptake rate. Results differ and have been interpreted as suggesting that calcium liberation is not mediated by a carrier with a single calcium-binding site that can move between the interior and exterior of the membrane [28]. Furthermore, an increasing internal calcium concentration results in an increase in the amount of liberated calcium. This probably reflects the constraints imposed by a fixed internal calcium concentration of the membranes loaded in the presence of a calcium-precipitating anion [34]. The release of calcium related to the amount of free calcium not trapped by oxalate is supported by the fact that calcium released by ionophore, A23187 is a function of the oxalate concentration [11].

We also conclude from our results that calcium efflux and calcium release can occur as for other systems [28,35], under conditions where the pump reversal is blocked.

Finally, all the characteristics of the calcium release suggest that this function is different from calcium uptake. The activation of calcium release by micromolar extravesicular calcium and the increasing calcium efflux under phosphorylation of a 23 kDa protein [11] can suggest the participation of a Ca^{2+} channel. If we apply the model of Glossmann et al. [36] to our studies, the phosphorylation of a gate on the exterior of the membrane vesicles can produce a calcium release. This model supposes that phosphorylation can be responsible for opening the Ca^{2+} channel. Such a system has been recently demonstrated where the phosphorylation of calciductin, a 23 kDa sarcolemmal protein is responsible for mediating the Ca^{2+} channel [37].

Further work will be directed towards investigating a Ca^{2+} channel in purified internal membranes which can regulate the calcium concentration in the platelets.

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