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REGULATION OF CALCIUM ACCUMULATION AND EFFLUX FROM PLATELET VESICLES

POSSIBLE ROLE FOR CYCLIC-AMP-DEPENDENT PHOSPHORYLATION AND CALMODULIN

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Calcium-accumulating vesicles were isolated by differential centrifugation of sonicated platelets. Such vesicles exhibit a $(Ca^{2+} + Mg^{2+})$-ATPase activity of about 10 nmol $(min \cdot mg)^{-1}$ and an ATP-dependent Ca^{2+} uptake of about 10 nmol $(min \cdot mg)^{-1}$. When incubated in the presence of Mg[γ-^{32}P]ATP, the pump is phosphorylated and the acyl phosphate bond is sensitive to hydroxylamine. The [^{32}P]phosphate-labeled Ca^{2+} pump exhibits a subunit molecular weight of 120000 when analyzed by lithium dodecyl sulfate-polyacrylamide gel electrophoresis. Platelet calcium-accumulating vesicles contain a 23 kDa membrane protein that is phosphorylatable by the catalytic subunit of cAMP-dependent protein kinase but not by protein kinase C. This phosphate acceptor is not phosphorylated when the vesicles are incubated in the presence of either Ca^{2+} or Ca^{2+} plus calmodulin. The latter protein is bound to the vesicles and represents 0.5% of the proteins present in the membrane fraction. Binding of ^{125}I-labeled calmodulin to this membrane fraction was of high affinity (16 nM), and the use of an overlay technique revealed four major calmodulin-binding proteins in the platelet cytosol (M_r = 94000, 87000, 60000 and 43000). Some minor calmodulin-binding proteins were enriched in the membrane fractions (M_r = 69000, 57000, 39000 and 37000). When the vesicles are phosphorylated in the presence of MgATP and of the catalytic subunit of cAMP-dependent protein kinase, the rate of Ca^{2+} uptake is essentially unaltered, while the Ca^{2+} capacity is diminished as a consequence of a doubling in the rate of Ca^{2+} efflux. Therefore, the inhibitory effect of cAMP on platelet function cannot be explained in such simple terms as an increased rate of Ca^{2+} removal from the cytosol. Calmodulin, on the other hand, was observed to have no effect on the initial rate of calcium efflux when added either in the absence or in the presence of the catalytic subunit of the cyclic AMP-dependent protein kinase, nor did the addition of 0.5 μM calmodulin result in increased levels of vesicle phosphorylation.

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Abbreviations: cAMP, cyclic adenosine 3′:5′-monophosphate; PMSF, phenylmethylsulfonyl fluoride; Hepes, *N*-2-hydroxyethyl-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; LDS, lithium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

Introduction

Platelets, anucleated fragments of the megakaryocytes, are able to respond to a number of stimuli by extensive shape changes from smooth disks to small spheres with fine filopodia. During these events, they also develop the capacity to aggregate and to secrete granules. Platelets are a very useful model for studying excitation-contraction and excitation-secretion coupling. Protein phosphorylations play a prime role in these couplings. Indeed it has been previously shown by Bennett et al. [1] and by Haslam and Lynham [2] that some platelet proteins become phosphorylated on exposure to physiological agonists such as thrombin and collagen.

One of these proteins of M_r 20000 has been identified as the regulatory light chain of myosin [3]. Phosphate incorporation in the myosin light chains is stimulated in the presence of calcium and calmodulin, the ubiquitous calcium-dependent activator, and results in a concomitant increase of the actin-activated myosin ATPase activity [4]. The resulting contraction probably mediates the release reaction, and it is established that mobilization of intracellular calcium ions plays a decisive role in platelet activation. On the other hand, physiological events that result in an increased level of cAMP within the cell were reported to prevent platelet activation [5,6].

In this paper, calcium accumulating vesicles are shown to exhibit a $(Ca^{2+} + Mg^{2+})$-ATPase of M_r 120000. The acylphosphate intermediate E ~ P is sensitive to hydroxylamine. Upon incubation with the catalytic subunit of cAMP-dependent proteinkinase, a membrane protein of M_r 23000 was phosphorylated with no effect on either the rate of Ca^{2+} uptake or the $(Ca^{2+} + Mg^{2+})$-ATPase activity. In contrast, the rate of Ca^{2+} efflux from the vesicles doubled upon cAMP-dependent phosphorylation of the 23 kDa protein.

Materials and Methods

Bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, were obtained from Sigma. Trypsin and soybean trypsin inhibitor were from Worthington. Carrier-free [^{32}P]orthophosphoric acid and $^{45}CaCl_2$ were purchased from NEN and the Radiochemical Centre, Amersham, respectively. All other chemicals were from Merck and were of analytical grade.

[γ-^{32}P]ATP (500–1000 Ci/mol) was synthesized according to Glynn and Chappell [7]. The catalytic subunit of cAMP-dependent protein kinase (type II from bovine heart) was prepared and stored as previously described [8]. The heat-stable protein kinase inhibitor was purified to homogeneity from rabbit skeletal muscle as described by Demaille et al. [9]. Calmodulin was isolated from ram testes according to Autric et al. [10]. Glycogen phosphorylase *b* kinase was prepared from rabbit skeletal muscle according to Cohen [11]. The cyclic nucleotide-independent protein kinase C, which is stimulated by calcium ions, phospholipids and unsaturated diacylglycerols, was partially purified from platelets as described by Inoue et al. [12]. Polyacrylamide gel electrophoresis reagents were from Bio-Rad and Serva.

Preparation of the calcium-accumulating vesicles was performed by using a modification of the method of Käser-Glanzmann et al. [13,14]. Blood was drawn from the antecubital vein of donors after obtaining informed consent according to the declaration of Helsinki. It was collected on 0.1 vol. of an anticoagulant composed of 0.32% citric acid, 2.63% trisodium citrate, 0.25% monosodium phosphate, 2 H_2O and 2.32% glucose (w/v).

Platelet-rich plasma was separated by centrifugation at $100 \times g$ for 15 min at room temperature. EDTA was then added to platelet-rich plasma to a final concentration of 5 mM, and platelets were pelleted by centrifugation for 15 min at $3000 \times g$. Then, they were washed twice using a modified Tyrode buffer, pH 7.5, containing 130 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 24 mM $NaHCO_3$, 2 mM Na_2EDTA, 10 mM glucose, 12.5 mM sucrose and 0.35% bovine serum albumin (w/v). Washed platelets were then centrifuged and the pellets resuspended at 4°C in 100 mM KCl, 2 mM $MgSO_4$, 25 mM NaCl, 12 mM sodium citrate, 10 mM glucose, 5 mM ATP, 0.35% bovine serum albumin, 25 mM Hepes buffer, pH 7.5. From this step all procedures were carried out at 4°C. Platelets (2-ml fractions containing $2 \cdot 10^9$ platelets/ml) were lyzed by ultrasonication in a Branson sonifier B12

at 40 watts, 4 times for 5 s. The lysate was centrifuged at 19000 × *g* for 25 min and the supernatant then centrifuged at 100000 × *g* for 60 min. The membrane pellet was resuspended in 10 mM potassium oxalate, 100 mM KCl, 10 mM $MgCl_2$, 20 mM Hepes buffer, pH 7.55, containing 5 mM ATP, 1 mM dithiothreitol and 0.35% bovine serum albumin. All buffers used were saturated with nitrogen.

The plasma membrane contamination was estimated by the phosphodiesterase assay according to Koerner and Sinsheimer [15] using bis-*p*-nitrophenylphosphate as a substrate. In addition, the plasma membrane surface proteins were ^{125}I-labeled using the lactoperoxidase-catalyzed iodination of intact platelets [16]. The ^{125}I-contamination of the 100000 × *g* pellet allowed the estimation of the amount of plasma membrane present in the preparation. The β-glucuronidase activity, which is indicative of lysosome contamination, was measured according to Talalay et al. [17]. Mitochondrial contamination was estimated by the sensitivity of the $(Ca^{2+} + Mg^{2+})$-ATPase activity to sodium azide (0.5 to 5 mM), and lactate dehydrogenase [18] activity was used as a marker of cytosol.

Protein concentrations were determined by the Coomassie blue technique [19]. Dodecyl sulfate (0.1%)-polyacrylamide (5–20% gradient) gel electrophoresis was performed according to Laemmli [20]. When radioactive samples were submitted to electrophoresis, gels were dried under vacuum after staining with Coomassie blue R 250 and destaining, before autoradiography.

Measurement of calcium flux. The rate of calcium uptake was determined at 30°C essentially according to Martonosi and Feretos [21] in media containing 5 mM oxalate, 150 mM KCl, 0.1 mM $^{45}CaCl_2$ (1300–1500 cpm/nmol), 5 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.35% bovine serum albumin, 20 mM Hepes buffer, pH 7.2 and 300–500 μg of platelet membrane protein per ml. Aliquots (0.2 ml) were withdrawn and filtered through 0.22 μm pore size Millipore GSWP filters previously soaked in a 2 mg/ml bovine serum albumin solution. Filters were washed twice with 1 ml of 0.1 M $CaCl_2$ and counted in 10 ml of dioxane-naphthalene scintillant. Calcium efflux measurements were performed in the following manner: Platelet membrane vesicles were loaded with $^{45}CaCl_2$ in the same medium as previously described. 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 the vesicles was attained (after 60 min, as determined in a preliminary experiment), a small amount (1 μCi/ml) of tracer $^{45}CaCl_2$ was added in order to estimate the unidirectional influx of calcium into the vesicles. Calcium efflux was calculated from the measured influx of ^{45}Ca under steady-state conditions during which efflux equals influx [22].

Characterization of $(Ca^{2+} + Mg^{2+})$-ATPase. The acyl-phosphate intermediate of the platelet $(Ca^{2+} + Mg^{2+})$-ATPase was detected by acid-polyacrylamide (10%) gel electrophoresis at low temperature (4°C) [23] in a citrate-phosphate buffer, pH 2.4, containing 0.1% lithium dodecyl sulfate. Pyronin Y was used as dye front marker.

The $(Ca^{2+} + Mg^{2+})$-ATPase activity of platelet membrane vesicles was measured in the presence of 0.1 mM Ca^{2+} or 2 mM EGTA by the liberation of orthophosphate at 30°C according to Reimann and Umfleet [24], utilizing [γ-^{32}P]ATP as the substrate of the $(Ca^{2+} + Mg^{2+})$-ATPase. [γ-^{32}P]Orthophosphate was bound to Whatman paper ET 31 in the presence of molybdate and triethylamine. Papers were dried and counted in 5.0 ml of toluene-scintillant.

Platelet vesicle phosphorylation was carried out at 20°C in a 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM NaF, 0.5 mM [γ-^{32}P]ATP (100–300 cpm/pmol), 50 mM sodium phosphate buffer, pH 7.0, in the presence of the catalytic subunit of cAMP-dependent protein kinase (enzyme/substrate = 0.01) or 10^{-5} M cAMP or 10^{-7} M protein kinase inhibitor, in order to check the specificity of protein phosphorylation by the cAMP-dependent protein kinase. In other experiments, trypsin-activated protein kinase C was used in a medium containing 0.3 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 20 mM Tris-HCl buffer, pH 7.5 and 1.5 mg/ml platelet vesicle protein. Ca^{2+}-calmodulin dependent phosphorylation was carried out at 20°C in 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP (100–300 cpm/pmol), 50 mM Tris-HCl buffer, pH 8.2, in the presence of 0.1 mM Ca^{2+} and 0.5 μM calmodulin.

Calmodulin determination and binding. To determine calmodulin-binding sites, calmodulin was iodinated using the iodogene technique [25]. In order to measure the binding of ^{125}I-labeled calmodulin to the platelet vesicle preparations, the membranes were first stripped of endogenous calmodulin by adding 15 mM EDTA to the resuspension solution, yielding a net EDTA concentration of 5 mM. Following centrifugation (1 h, 100 000 × *g*), the pelleted membranes were suspended into a solution containing 100 mM KCl, 20 mM Tris-Hepes, pH 7.4, 2.5 mM $MgCl_2$ and 100 μM $CaCl_2$. All further manipulations were performed at room temperature. Approx. 150 μg of membranes were incubated with quantities of iodinated calmodulin calculated to yield a binding curve between 10^{-9} M and 10^{-7} M calmodulin. After 10 min, an equal volume of 20% (w/v) poly(ethylene glycol) 20 000 (Serva) was added to precipitate the bound calmodulin. These manipulations were performed in Eppendorf microcentrifuge tubes. After a further incubation of 5 min, the mixture was centrifuged for 5 min at top speed in a Beckman microfuge B. The supernatant was removed by aspiration and the residual film soaked up with a cotton applicator. The radioactivity remaining in the tubes was counted in an MR 252 gamma counter. Nonspecific binding of the ^{125}I-calmodulin, appreciable at higher concentrations, was determined by including a 50-fold excess of unlabeled calmodulin in the assay procedure.

The calmodulin gel overlay technique was performed using a procedure modified from that of Carlin et al. [26]: Following SDS gel electrophoresis (150 μg protein per lane, 5–15% gradient [20], the gels were washed in a solution of 25% (v/v) isopropanol, 10% (v/v) acetic acid to remove SDS. The solution was changed four times in a 2 h period. Following two 5-min incubations in distilled water, the gels were soaked for 30 min in the renaturation buffer of 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 20% (v/v) glycerol, 1 mM $MgSO_4$, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mg/ml bovine serum albumin) supplemented with 6 M guanidine HCl.

The gels were then removed and incubated for at least 12 h in the renaturation buffer, with four changes. Prior to incubation with ^{125}I-labelled calmodulin, the gels were soaked for 6 h in 50 mM Tris-HCl, 0.15 M NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 1% bovine serum albumin. The gels were then incubated with ^{125}I-calmodulin (20 nM, 10^4–10^5 cpm/pmol, 50 ml per gel) in a sealed pouch, for 12 to 24 h, in the same solution subsequently used to wash the gels. Washes to remove non-specifically bound calmodulin were performed four times during a 12 to 24 h period, with a buffer containing 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM dithiothreitol. The gels were then dried and submitted to autoradiography. Control experiments were performed in which 5 mM EGTA or 1 mM chlorpromazine-HCl were included in the final wash solutions. To determine molecular weights, the lanes, containing appropriate standards, were cut from the rest of the gel immediately following electrophoresis, and stained and destained as usual. R_F values were used to assign molecular weights to the calmodulin-labeled bands.

Results

Characterization of the calcium-accumulating membrane fraction

The platelet membrane fraction which sedimented at 100 000 × *g* was found to consist primarily of empty membrane vesicles, when analyzed by electron microscopy (results not shown). Dense bodies, granules and mitochondria sedimented almost completely at 19 000 × *g*. However, biochemical evaluation did reveal a contamination by plasma membranes. ^{125}I-iodinated plasma membrane proteins were recovered in the 100 000 × *g* membrane fraction. The specific radioactivity ratio, relative to whole platelets, was 1.62 while the specific phosphodiesterase activity ratio reached 1.44. Lysosomal and cytoplasmic contaminations were small as suggested by the β-glucuronidase and lactate dehydrogenase activity ratios of 0.90 and 0.12, respectively. In addition, as shown in other experiments (see below), the ATPase activity was due mainly to $(Ca^{2+} + Mg^{2+})$-ATPase, suggesting that this fraction was enriched in intracellular membranes.

Calmodulin

Calmodulin, assayed by myosin light chain

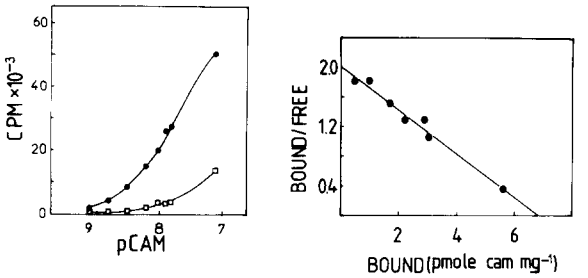

Fig. 1. (A) The binding of ^{125}I-calmodulin ($6.8 \cdot 10^7$ cpm·nmol^{-1}) by the platelet vesicle preparation (100000 × *g* pellet), as in Methods. ●, specific binding, representing the difference between total cpm and non-specific cpm values; □, non-specific binding, obtained by including a 50-fold-excess of unlabelled calmodulin at each point. (B) Scatchard plot of the binding data, yielding $K_d = 16$ nM and total calmodulin-binding capacity of 6.8 pmol·mg^{-1}.

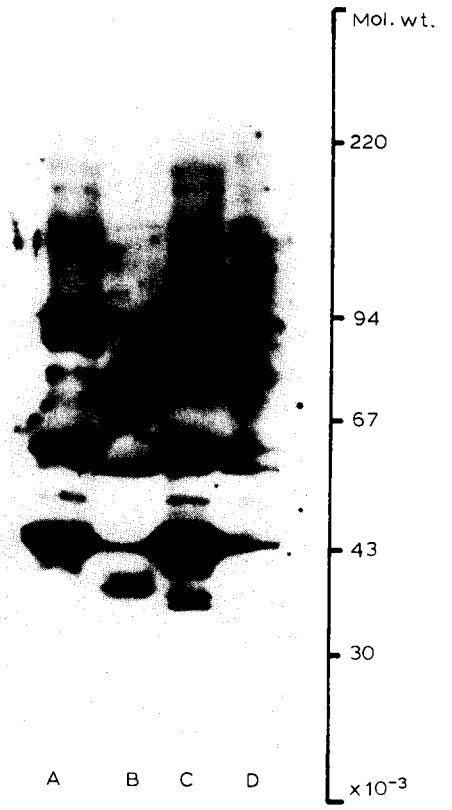

Fig. 2. Calmodulin gel overlay: lane A, whole platelets; B, 19000 × *g* pellet; C, 100000 × *g* supernatant; D, 100000 × *g* vesicle fraction. The positions of molecular weight standards are shown on the right edge. 150 μg of each fraction were utilized in this procedure, as described in Methods.

kinase activation, was found to represent 0.5% (w/w) of the protein in the 100000 × *g* platelet membrane fraction. The same value was obtained for the 100000 × *g* supernatant under our purification techniques.

Binding of iodinated calmodulin by the vesicle fraction indicated a single class of high affinity sites (Fig. 1 A and B). The Scatchard plot was linear and yielded an affinity of 16 nM, and a capacity for calmodulin binding of 6.8 pmol per mg membranes.

Calmodulin-overlay experiments (Fig. 2) revealed a large number of calmodulin binding proteins in platelets. Those proteins found in the greatest concentrations (M_r = 94000, 87000, 60000 and 43000) appear to be soluble proteins, almost totally absent in the membrane fractions. Some fainter high molecular weight bands (M_r = 250000, 230000 and 153000) also appear to be found solely at the level of the platelet cytosol, while other bands are definitely enriched in the membrane fractions (M_r = 69000, 57000, 39000 and 37000). When 5 mM EGTA or 1 mM chlorpromazine HCl were included in the wash medium, the bound ^{125}I-calmodulin was completely removed from all bands.

Phosphorylation of platelet vesicles

When 100000 × *g* platelet vesicles were incubated in the presence of 10 μM cAMP or in the presence of the purified catalytic subunit of cAMP-dependent protein kinase (enzyme/substrate = 0.01), phosphate was mostly incorporated into a polypeptide of M_r = 23000 (Fig. 3). This phosphorylation was more pronounced in the presence of the kinase (Fig. 3A) than in the presence of cAMP (Fig. 3B), pointing to the presence of very small amounts of endogenous cAMP-dependent protein kinase holoenzyme. Excess protein kinase inhibitor abolished phosphate incorporation (Fig. 3C). Addition of Triton X-100 to the samples prior to dodecyl sulfate gel electrophoresis did not alter the phosphorylation pattern. In the absence of cAMP or the catalytic subunit of cAMP-dependent protein kinase, addition of 0.5 μM calmodulin and/or 0.1 mM Ca^{2+} did not result in vesicle phosphorylation. The M_r 23000 polypeptide did not appear to be a substrate for protein kinase C since no phosphate incorporation

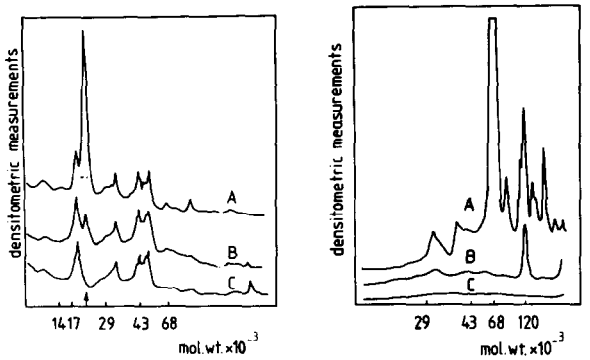

Fig. 3. Autoradiograms of SDS (0.1%)-polyacrylamide (5–20%) gradient gel electrophoretogram of the 100000 × *g* platelet fraction phosphorylated in the presence of (A) catalytic subunit of cAMP-dependent protein kinase (enzyme/substrate = 0.01); (B) 10^{-5} M cAMP; (C) 10^{-7} M protein kinase inhibitor. All other conditions as described under Methods.

Fig. 4. Detection of the $(Ca^{2+} + Mg^{2+})$-ATPase phosphorylated intermediate. LDS (0.1%) polyacrylamide (8%) gel electrophoresis at pH 2.4 of the 100000 × *g* platelet fraction incubated in the presence of Ca^{2+}, Mg^{2+} and [γ-^{32}P]ATP. Densitometry of the Coomassie blue-stained gel (A) and of the autoradiogram (B). In (C) the sample was treated with hydroxylamine prior to electrophoresis and autoradiography. All other conditions as described under Methods.

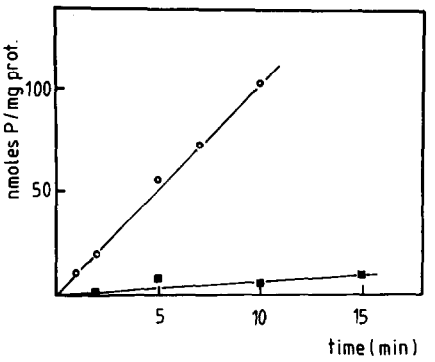

Fig. 5. Mg^{2+}-ATPase activity of the platelet vesicles incubated in the presence of 0.1 mM $CaCl_2$ (○) or in the presence of 2 mM EGTA (■).

was observed in the presence of the trypsin-activated kinase (Results not shown).

Characterization of the $(Ca^{2+} + Mg^{2+})$-ATPase

The formation of the ATPase phosphorylated intermediate occurred in a few seconds at 4°C. After 10 s incubation the reaction mixture containing the phosphorylated intermediate was submitted to an acidic polyacrylamide gel electrophoresis. Some samples were treated by 0.6 M hydroxylamine prior to electrophoresis. As shown in Fig. 4 the ATPase exhibited an M_r of 120000. The ATPase was no longer labeled by [^{32}P]phosphate after hydroxylamine treatment pointing to an acyl phosphate bond in the intermediate. The initial rate of ATP hydrolysis by the vesicular fraction at 30°C reached 10.2 nmol/min per mg protein. This reaction is almost totally inhibited by 2 mM EGTA, suggesting that more than 95% of the ATPases present in this membrane fraction are $(Ca^{2+} + Mg^{2+})$-ATPases (Fig. 5).

Ca^{2+} flux studies

Ca^{2+} uptake studies using an EGTA-buffered system indicated that the $(Ca^{2+} + Mg^{2+})$-ATPase has a very high affinity for calcium; a K_m of 0.6 μM was determined (data not shown). Vanadate ions were an efficient inhibitor of Ca^{2+} uptake; at calcium concentrations of 20 μM and 120 μM, the K_i was 27 μM (data not shown). The rate of calcium uptake (about 10.3 nmol Ca^{2+}/min per mg protein) was not altered by phosphorylation of the 100000 × *g* vesicles by the catalytic subunit of

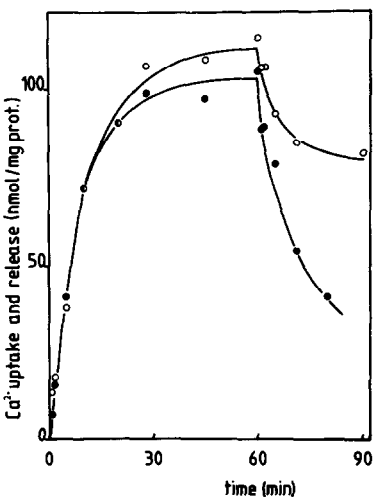

Fig. 6. Calcium fluxes (uptake and release) in the 100000 × *g* platelet vesicular fraction non-phosphorylated (○) or phosphorylated (●) by the catalytic subunit of cAMP-dependent protein kinase.

the cAMP-dependent protein kinase (Fig. 6), except for a slight decrease in the calcium capacity, i.e. the plateau level. In some experiments, however, a slight transient stimulation of the initial rate of Ca^{2+} uptake was observed in the first 2 to 5 min following addition of the catalytic subunit of cAMP-dependent protein kinase. Similar results were obtained at different Ca^{2+} concentrations.

The extent of ionophore A23187-induced Ca^{2+} release under these conditions depended on the oxalate and calcium concentrations. At 1 mM oxalate, 25 μM calcium, the ionophore induced 70–80% calcium release. However, only 20–30% of the sequestered calcium could be released at 10 mM oxalate and 50–100 μM calcium.

After about 60 min an equilibrium state is reached in which calcium efflux and influx are equal. The initial rate of calcium efflux measured as described under Methods was 3.5 nmol/min per mg protein (Fig. 6). Under the same experimental conditions, addition of the catalytic subunit of the cAMP-dependent protein kinase induced a 2-fold activation of the initial rate of Ca^{2+} efflux (6.7 nmol/min per mg protein). Again, similar results were obtained at different Ca^{2+} concentrations. No change was observed when calmodulin was added either in the absence or in the presence of the catalytic subunit of the cAMP-dependent protein kinase.

Discussion

Our initial experiments indicate that the catalytic subunit of cAMP-dependent protein kinase catalyzes the phosphorylation of a 23 kDa membrane protein in the 100000 × *g* vesicular fraction. Such phosphorylation was also described by Käser-Glanzmann et al. [13], who compared this polypeptide with phospholamban, the phosphorylatable activator of the $(Ca^{2+} + Mg^{2+})$-ATPase in cardiac sarcoplasmic reticulum. However, except for the similar molecular size, the platelet phosphoprotein differs strikingly from phospholamban: Upon treatment by non ionic detergents such as Triton X-100 prior to dodecyl sulfate gel electrophoresis, the phosphorylation pattern is not altered and the radioactivity still migrates with a velocity corresponding to M_r 23000 whereas phospholamban dissociates into 11 kDa monomers [27]. In addition, the platelet polypeptide is a good substrate for the catalytic subunit of cAMP-dependent protein kinase, but does not appear to be phosphorylated by a calcium-calmodulin-dependent protein kinase. In contrast, phospholamban has been shown to be phosphorylated by cAMP-dependent protein kinase [28], by a Ca^{2+}-calmodulin-dependent protein kinase [27] and also by the calcium-activated, phospholipid-dependent, protein kinase C [29]. The latter kinase, present at very high concentration in platelets [30], does not seem to phosphorylate the M_r 23000 platelet protein. As well, the M_r 23000 platelet protein shows no cross reactivity with phospholamban antibodies following transfer to nitrocellulose paper by electroblotting (Bio-Rad), under conditions which are extremely selective for phospholamban from canine cardiac sarcoplasmic reticulum (Rinaldi, M. and Hincke, M.T., unpublished observations). This is in good agreement with our inability to purify the phosphorylated M_r 23000 protein by acidic chloroform-methanol extraction, followed by HPLC on a silica column, conditions which have been specifically developed for phospholamban purification [31]. These negative results are very definite; no homology can be drawn between phospholamban and the M_r 23000 platelet protein.

The $(Ca^{2+} + Mg^{2+})$-ATPase was characterized as an M_r 120000 polypeptide. Similar molecular weights were reported for other $(Ca^{2+} + Mg^{2+})$-ATPases [32–34]. It exhibits the characteristic E ~ P hydroxylamine-sensitive phosphorylated intermediate. Also in common with $(Ca^{2+} + Mg^{2+})$-ATPases previously characterized, the platelet vesicle ATPase demonstrates a high affinity for Ca^{2+} and is inhibited by vanadate.

The important role of Ca^{2+} fluxes in platelet function is suggested by the large amount of calmodulin present. In agreement, the overlay results reveal the existence of a large number of proteins which bind calmodulin in a Ca^{2+}-dependent manner and presumably reflect the activation by calmodulin of many enzyme functions. Direct calmodulin binding measurements indicate the high-affinity nature of this interaction. The exact nature of these enzymes is more uncertain.

Our results differ somewhat from those of Grinstein and Furuya [35] whose overlay experiments revealed a single major calmodulin-binding pro-

tein of M_r 149000 in purified platelet plasma membranes. However this result is in agreement with our conclusion that the major calmodulin binding proteins are cytosolic in nature. An M_r 149000 band is not enriched in our platelet vesicle fraction, in agreement with the intracellular nature of this preparation.

In contrast to the erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase, where the M_r 145000 enzyme is activated by direct binding of calmodulin [36], no calmodulin binding band of M_r 120000 is revealed in the platelet membrane fractions, in agreement with the lack of an effect of calmodulin upon Ca^{2+} uptake in this preparation.

It must be emphasized that the overlay methodology only yields results of a positive nature. The absence of a band binding ^{125}I-calmodulin may simply reflect the incomplete regeneration of this function following SDS denaturation. In fact the quantity of calmodulin bound (which is proportional to the band intensity by autoradiography) is not strictly related to the quantity of protein present, since different calmodulin-binding proteins may renature with different efficiencies. Because of this, the conditions of renaturation have been carefully chosen to maximize regeneration of enzyme activities, and the overlay procedure has been modified from its original development [26,37]. The 6 M guanidine HCl step was incorporated because of its demonstrated ability to aid renaturation following SDS extraction [38]. Ingredients such as high salt, glycerol, dithiothreitol, EDTA, Mg^{2+} and bovine serum albumin have been shown to increase the degree of activity regeneration following SDS electrophoresis [39].

Under our experimental conditions, the Ca^{2+} uptake activity was not stimulated by cAMP-dependent phosphorylation of the M_r 23000 polypeptide. In this respect, our results are at variance with the stimulation of calcium uptake reported by Käser-Glanzmann et al. [13,14] to occur upon cAMP-dependent posphorylation. On the other hand, this phosphorylation doubles the rate of calcium efflux from the $100000 \times g$ platelet vesicles. The fact that under our experimental conditions alterations in the rate of efflux could be noted with little apparent alteration in the plateau levels of calcium uptake is explained by the low rate of efflux compared to uptake. Therefore even though efflux is increased 2-fold by cAMP-dependent phosphorylation this would produce a negligible change in the plateau level of calcium uptake.

The preparation used in these studies consisted mostly of reticulum membranes orientated cytoplasmic face outwards. In the intact platelet, stimulation of calcium efflux may result in a stimulation of platelet functions, e.g. activation of myosin light chain kinase [4] or phospholipases [40]. Physiological events which stimulate cAMP synthesis in platelets are however reported to prevent or reverse their activation [41]. Such a paradox cannot be explained by the plasma membrane contamination of our vesicle preparation shown by plasma membrane enzymatic and iodinated protein contamination. In fact, calcium fluxes which pass through the ATPase system are translocated from a 'compartment' containing MgATP and calcium to another 'compartment' where calcium ions are stored and which corresponds to either the extracellular space when plasma membrane vesicles are concerned or the intrareticular space for endoplasmic reticulum membranes. Calcium efflux stimulated by cAMP-dependent phosphorylation is in all cases directed toward the MgATP compartment, i.e. the cytosol. Moreover a recent study utilizing highly purified human platelet membranes of surface and intracellular origin has demonstrated that the ATP-dependent, Ca^{2+}-accumulating process is almost exclusively associated with the intracellular membrane fraction [42]. This implies that only the endoplasmic reticulum membranes in our preparation contributes to the observed Ca^{2+} uptake.

In fact, cAMP-dependent inhibition of platelet function is not always observed. For instance, when platelet activation is triggered by the Ca^{2+} ionophore A 23187 in the absence of extracellular Ca^{2+}, ^{14}C-labeled serotonin release and thromboxane B2 synthesis are not inhibited by the prostaglandin E_1-induced synthesis of cAMP [43]. Therefore, cAMP effects in platelets cannot be entirely accounted for by either a modulation of calcium fluxes [13,14] or by inhibition of calcium-calmodulin-dependent myosin light chain kinase through cAMP-dependent phosphorylation of the kinase [44].

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