Ca^{2+} binding and charge movements in membranes of platelets and sarcoplasmic reticulum

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The properties of the Ca^{2+}-pump system of platelet microsomes isolated without Ca^{2+}-precipitating anions are studied. Passive Ca^{2+} binding to the microsomes takes place in a noncooperative manner with $K_d = 0.7\mu M$. Half-maximal stimulation of ATP-dependent transport occurs at $0.4\mu M$ Ca^{2+}. The velocity of Ca^{2+} uptake, Ca^{2+} capacity and the level of phosphoprotein in platelet microsomes are significantly lower than in cardiac microsomes. Energization of platelet and muscle microsomes and activation of intact platelets result in opposite charge redistribution in hydrophobic regions of the membranes. It is concluded that these charge movements are caused by Ca^{2+} binding to and dissociation from nonpolar binding sites in the membranes.

Sarcoplasmic reticulum *Ca^{2+}-binding site* *Platelet activation* *Ca^{2+} transport* *Platelet microsome* *Charge movement*

1. INTRODUCTION

The crucial role played by Ca^{2+} in cellular metabolism and excitation-concentration coupling is ensured by the functioning of cationic pumps which maintain a more than 1000-fold gradient for Ca^{2+} concentration across surface membranes and membranes of its intracellular reservoirs. Previous results show that platelets contain a system removing Ca^{2+} from the cytoplasm in an ATP-dependent manner [1]. Platelet microsomes (PM) isolated in the presence of ATP and oxalate accumulate Ca^{2+} and catalyze formation of a phosphorylated product sensitive to hydroxylamine [2–5]. Immunological cross-reactivity between the phosphorylated component and Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) from skeletal muscle [5] indicates similarities in the structures of Ca^{2+}-pump proteins in muscle cells and platelets.

The mechanisms which underlie Ca^{2+} movements in platelets and charge redistribution during platelet activation are not fully understood. Experiments with a cyanine dye and a penetrating cation (tetraphenylphosphonium) have demonstrated a decrease in platelet transmembrane potential upon thrombin addition [6]. An attempt has been made to find a correlation between platelet activation and changes in the microenvironment of a membrane-bound fluorescent dye [7]. However, the quantum yield and spectrum of ANS^- fluorescence did not change in the presence of thrombin and collagen.

In this paper properties of the PM Ca^{2+}-pump system are described and compared to those of the SR Ca^{2+}-pump. Omission of Ca^{2+}-precipitating anions and ATP from the medium during PM isolation made it possible to investigate charge changes in the membranes induced by their ATP-dependent interaction with Ca^{2+}.

2. MATERIALS AND METHODS

[γ-^{32}P]ATP and $^{45}CaCl_2$ were obtained from Amersham, U46619 (15*S*-hydroxy-11α,9α-epoxymethano-5*Z*,13*E*-prostadienoic acid) from Upjohn (USA) and A23187 from Calbiochem. Adenylyl

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imidodiphosphate and PMSF (phenylmethylsulfonyl fluoride) were purchased from Serva. PCB^- (phenyldicarbaundecaborane) was a generous gift from Dr L.M. Tsofina (Institute of Problems of Information, Moscow).

PM were isolated in a medium containing 30% glycerol, 30 μM PMSF, 20 mM Tris–HCl (pH 7.0). NaCl-washed human platelets were diluted 5-fold with the ice-cold isolation mixture and sonicated twice for 15 s at maximal intensity of a Kerry cell disruptor. The suspension was centrifuged for 30 min at 21 000 × g. The supernatant obtained was centrifuged for 60 min at 100 000 × g. The pellet of PM was resedimented in the presence of 0.6 M KCl and suspended in the isolation medium from which PMSF was omitted.

SR fragments from rabbit skeletal muscle and guinea pig heart were isolated as in [8,9]. Calsequestrin was partially purified as in [10] with some modifications.

Phosphorylation of microsomes by [γ-^{32}P]ATP at 0°C and measurements of Ca^{2+} uptake were made as in [9]. Free [Ca^{2+}] at different pH values was calculated as in [11]. SDS–PAGE (10%) was performed as in [12].

Adsorption of a lipophilic anion on the membranes was estimated by changes in electrical potential across bilayer lipid membranes (BLM) which were used as selective electrodes to PCB^- [13]. BLM were prepared from a decane solution of phosphatidylcholine and cholesterol. The volume of each of the compartments separated by BLM was 1 ml. The PCB^- concentration in the back compartment of the cell was 0.1 μM (or 0.2 μM) and 1 μM (or 2 μM) in the front one. Aliquots of microsomal suspensions were added to the front compartment. Changes in [PCB^-] were calculated using a modified Nernst equation:

$$[PCB^-]_{bound} = [PCB^-]_{back} - [PCB^-]_{front} \times 10^{|\Delta\psi|/(57\pm2)}$$

3. RESULTS AND DISCUSSION

3.1. Characterization of microsomal fractions from human platelets

Major polypeptides in a crude fraction of PM are of 106, 100, 56 and 35 kDa. As seen in fig.1, the protein composition of PM differs from that of skeletal muscle and cardiac muscle SR where Ca^{2+}-ATPase (100 kDa), calsequestrin (72 kDa, its

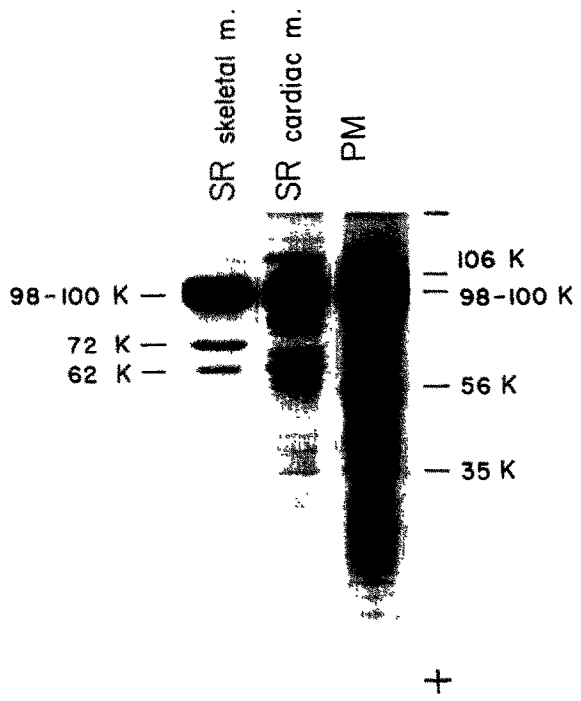

Fig.1. SDS–PAGE of PM and SR proteins.

apparent size varies depending on conditions of electrophoresis) [10] or 100- and 62-kDa polypeptides dominate, respectively.

PM accumulate Ca^{2+} in an ATP-dependent manner in the presence as well in the absence of oxalate (fig.2, 3A). The velocity of Ca^{2+} uptake by PM isolated in glycerol-containing medium (fig.3, curve b) is the same as that by PM isolated according to [3], in the presence of oxalate and ATP (curve c). Scatchard plot analysis of passive Ca^{2+} binding to PM, in the range 0.03–30 μM (fig.2A), revealed the existence of a single type of binding site (K_d = 0.7 μm). Half-maximal Ca^{2+}-pumping activity is at Ca_f^{2+} = 0.4 μM. It is therefore reasonable to suggest that identical high-affinity PM Ca^{2+}-binding sites participate in Ca^{2+} passive binding and energy-dependent transport.

The Ca^{2+} pump of PM is activated to a considerable extent at temperatures above 30°C (fig.2B). Increase in the temperature from 40 to 50°C leads to a sharp drop in ATP-dependent Ca^{2+} binding. At 1 μM free Ca^{2+}, the maximal velocity of Ca^{2+} transport is at pH 7.5 (not shown). As shown in fig.3A, the velocity of Ca^{2+} transport and the Ca^{2+}

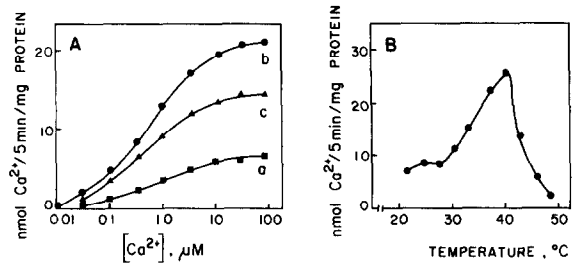

Fig.2. Dependence of Ca^{2+} binding to PM on $[Ca_f^{2+}]$ (A) and temperature (B). Incubation medium contained 0.1 M KCl, 0.5 μM EGTA, 5 mM $MgCl_2$, 40 mM imidazole (pH 7.4, 37°C; in A), 0.464 mM (in B) or different concentrations (in A) of $^{45}CaCl_2$ and 2 mM ATP (all experiments except a). Passive Ca^{2+} binding measured by $Ca^{2+}_{cold}/^{45}Ca^{2+}$ exchange was registered after 1 min incubation of 0.06 mg PM protein in $^{45}Ca^{2+}$ medium. ATP-dependent Ca^{2+} binding was stopped 5 min after ATP addition. a, passive Ca binding; b, Ca^{2+} binding after ATP addition; c, net ATP-dependent binding.

capacity of PM are significantly lower than in cardiac SR fragments.

A steady-state level of phosphorylation of PM in the presence of Ca^{2+} (fig.3B) is reached at shorter intervals than found in [5]. The velocities of phosphoprotein formation in PM and cardiac SR are similar. Assuming the degree of phosphorylation

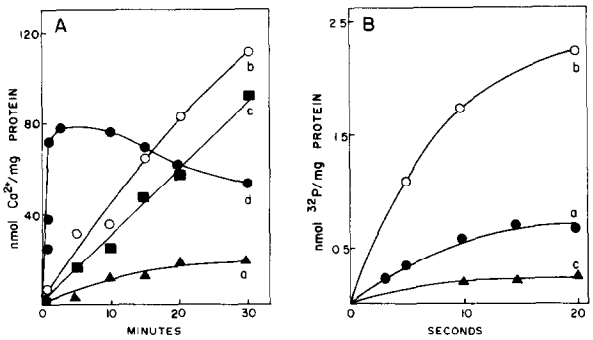

Fig.3. Time course of Ca^{2+} uptake (A) and phosphorylation (B) in PM and SR. (A) Ca^{2+} uptake with PM (a–c) and guinea pig heart SR (d) was measured at 37°C in a medium containing 0.1 M KCl, 0.1 mM EGTA, 0.1 mM $^{45}CaCl_2$, 5 mM $MgCl_2$, 2 mM ATP, 20 mM Tris–HCl (pH 7.3) without (a,d) and in the presence (b,c) of 10 mM potassium oxalate. (B) Phosphorylation of PM (a,c) and cardiac SR (b) was measured in a medium containing 0.1 M KCl, 10 mM $CaCl_2$ (or 1 mM EGTA, curve c), 30 μM [γ-^{32}P]ATP, 10 mM Tris–HCl (pH 7.3), 0.2 mg protein/ml.

of each Ca^{2+}-ATPase molecule to be the same for both preparations, one may conclude that the amount of the enzymes in PM is much lower than in the cardiac SR fraction (fig.3B). Autoradiography of gels performed after electrophoresis of phosphorylated PM at pH 2.4 demonstrated ^{32}P incorporation into a band of M_r very close to that of SR Ca^{2+}-ATPase (not shown).

3.2. Charge redistribution in PM and SR

Experiments were performed to follow charge changes in the microsomes upon energization of the Ca^{2+}-pump systems. It has been shown that the lipophilic anions tetraphenylboron and PCB^- move electrophoretically across membranes of energized submitochondrial particles [13]. Besides, PCB^- binds to hydrophobic regions of proteins and polysaccharides [15]. Fig.4 shows that albumin, Ca^{2+}-binding protein calsequestrin, fragments of PM and SR adsorb PCB^- effectively. MgATP addition increases PCB^- binding to the microsomes. Identical results were obtained with tetraphenylboron anion (not shown). In the presence of EDTA, EGTA and the Ca^{2+} ionophore A23187, the effects of microsome energization on PCB^- binding are negligible. The lipophilic anion which is adsorbed by membranes in the presence of MgATP is released into the medium after additions of EGTA and A23187.

It is evident that the additional PCB^- adsorption accompanies the process of Ca^{2+} uptake by microsomal fragments. This adsorption can be conditioned either by generation of the transmembrane potential (positive inside the vesicles) as a result of uncompensated Ca^{2+} uptake or by an increase of positive charge in membrane sites after their saturation by Ca^{2+}. The first possibility is very unlikely as is demonstrated in fig.4(f). In the presence of Cl^-, which penetrates readily across SR membrane and shunts the membrane potential [16], the ATP-dependent PCB^- binding does not change.

The interrelation between the saturation of Ca^{2+}-binding sites and PCB^- adsorption is confirmed in fig.4(i). Addition of A23187 to EGTA-containing medium causes a partial release of passively bound PCB^- from SR fragments. Increase in free $[Ca^{2+}]$ to 1 mM results in rebinding of PCB^- to the membranes. PCB^- release and its readsorption are evidently provided by an increase in accessibility of low-affinity Ca^{2+}-binding sites in

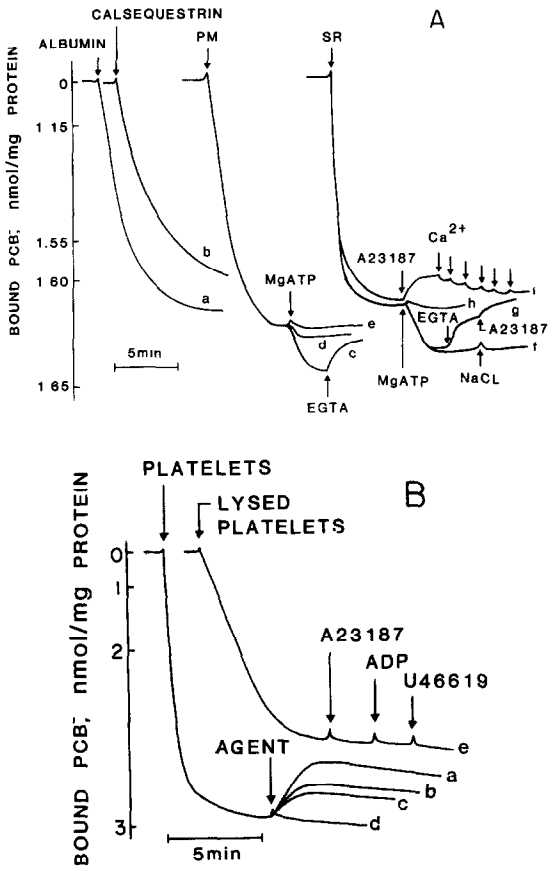

Fig.4. PCB^- binding to proteins, PM, SR fragments (A) and platelets (B). (A) The following additions were made: 0.1 mg BSA, 0.3 mg calsequestrin, 0.3 mg protein of PM and skeletal muscle SR fragments, 1 mM MgATP, 1 μM A23187, 15 mM NaCl. Incubation media (10% sucrose, 40 mM imidazole, pH 7.0) were also supplied with 1 μM A23187, 0.1 mM EGTA (e,h) and 1 mM EDTA (d). The consequent increase in $CaCl_2$ concentration (arrows in i) was 0.1, 0.2, 0.4, 0.8, 1 and 2 mM. (B) Incubation medium contained 10% sucrose, 40 μM EGTA, 40 mM histidine (pH 7.0). Additions: 0.6 mg protein of platelets, 0.1 mM ADP (a), 10 μM U46619 (b), 1 μM A23187 (c) and 0.1 mM adenylyl imidodiphosphate (d).

the presence of the Ca^{2+} ionophore.

It has been clearly shown that Ca^{2+} concentration in the cytoplasm of activated platelets increases due to mobilization of intracellular pool(s) of the cation [1]. We suggested that Ca^{2+} dissociation from binding sites in platelet membranes could result in a decrease of their positive charge. As seen from fig.4B, the agents which induce platelet activation (ADP, A23187, U46619), but not adenylyl imidodiphosphate, cause partial release of PCB^- passively bound to intact platelets. In a control experiment performed on lysed platelets these compounds did not decrease PCB^- binding. These results indicate that platelet activation is accompanied by a decrease in positive charge in hydrophobic regions of the cells.

In summary, the data obtained demonstrate that the mechanisms of operation of Ca^{2+}-pump systems localized in microsomal membranes of platelets and muscle cell are, in essence, quite similar. It remains to be determined whether lower Ca^{2+}-pumping activity and phosphoenzyme level in PM, as compared to those in cardiac SR fragments, and differences in the protein composition of PM and SR fragments, and differences in the protein composition of PM and SR arise from a unique structure or from an impurity of the first pump system. In microsomes from both types of cells, ATP-dependent Ca^{2+} binding correlates with the increase in positive charge in hydrophobic regions. PCB^- release from platelets which is induced by A23187, ADP and U46619 confirms the conclusion [17] about redistribution of Ca^{2+} in the activated cells from a nonpolar phase to a polar one.

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