Irreversible Effects of Calcium Ions on the Plasma Membrane Calcium Pump

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Abstract. The calcium pump of human red cells can be irreversibly activated by preincubation of the membranes in the presence of calcium ions, with a pattern reminiscent of that produced by controlled trypsin attack. With 1 mM Ca$^{2+}$, the activity of the basal enzyme increases three to fourfold over 30 to 60 min, to levels about half those obtained in the presence of calmodulin. On the whole, the effect occurs slowly, with a very low Ca$^{2+}$ affinity at 37°C and is unaffected by serine-protease inhibitors. The activation caused by 1 mM Ca$^{2+}$ is little affected by leupeptin (a thiol-protease inhibitor) and that obtained at 10 μM Ca$^{2+}$ is not inhibited. Preincubations at 0°C also lead to activation, to a level up to half that seen at 37°C, and the effect is not affected by leupeptin or antipain. No activation is observed by preincubating soluble purified Ca,Mg-ATPase in Ca$^{2+}$-containing solutions at 37°C. Instead, calcium ions protect the detergent-solubilized enzyme from thermal inactivation, the effect being half-maximal between 10 and 20 μM Ca$^{2+}$. We conclude that the activation of the membrane-bound Ca,Mg-ATPase by Ca$^{2+}$ should result from an irreversible conformational change in the enzyme and not from attack by a membrane-bound protease, and that this change presumably arises from the release of inhibitory particles existing in the original membrane preparations.

Key words: Ca,Mg-ATPase — Erythrocytes — Ca$^{2+}$ activation — Detergents — Thermal inactivation

Introduction

The plasma-membrane Ca,Mg-ATPase or calcium pump transports calcium ions out of the cell against a steep electrochemical gradient and can maintain the cytoplasmic Ca$^{2+}$ concentrations at levels in the 10$^{-8}$ M range (Lew et al., 1982; Schatzmann, 1982, 1989). The enzyme is stimulated by calmodulin, which induces a conformation with high Ca$^{2+}$ affinity and turnover number (Bond & Clough, 1973; Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1978; Scharff & Foder, 1978). The enzyme can be purified close to homogeneity in detergent solution, by affinity chromatography on calmodulin-Sepharose 4 B columns (Niggli, Penniston & Carafoli, 1979; Gietzen & Kolandt, 1982), and several isoforms have been cloned and sequenced (Shull & Greep, 1988; Verma et al., 1988). In the red cell membrane, the enzyme is present as the dimer of the single polypeptide chain (Minocherhomjee et al., 1983; Cavieres, 1984).

The plasma-membrane Ca,Mg-ATPase can also be activated by proteolytic cuts with trypsin, chymotrypsin and calpain, an intracellular Ca$^{2+}$-activated protease (Enyedi et al., 1980; Taverna & Hanahan, 1980; Stieger & Schatzmann, 1981; Sarkadi et al., 1986; Papp et al., 1989; Wang, Roufogalis & Villalobo, 1989; Zvaritch et al., 1990). This type of activation appears as the calmodulin-binding peptide (found at the C-terminal extension of the primary structure) is removed and consists of broad kinetic changes that are apparently similar to those elicited by calmodulin binding. A third mode of activation of the pump, comparable in its effects, is obtained with fatty acids and acidic phospholipids, which are effective with both the cell membranes and the purified enzyme (Taverna & Hanahan, 1980; Niggli, Adunyah & Carafoli, 1981).

Yet a fourth form of activation arises from simply preincubating calmodulin-depleted red cell membranes in the presence of calcium ions (Klinger et al., 1981; Cavieres, 1987a; Au, Lee & Siu, 1989; Roufogalis et al., 1990). This modification is irreversible in the membrane-bound Ca,Mg-ATPase and also leads to a state of high Ca$^{2+}$ affinity. Because
of the possibility of a regulatory potential in this effect, the present study was carried out to decide whether or not a Ca\(^{2+}\)-activated protease could be responsible for the activation at near-physiological Ca\(^{2+}\) concentrations and to examine additional features of the activation process.

**Materials and Methods**

**Materials**

ATP was purchased from Boehringer Mannheim (Lewes, UK) and radioisotopes from New England Nuclear (Stevenage, UK). Bovine brain calmodulin (CaM), Sephadex G-50, trypsin, soybean trypsin inhibitor, benzamidine hydrochloride, leupeptin, antipain, bestatin, E-64 and other biochemicals were from Sigma (London) and \(p\)-toluenesulphonyl fluoride (pTSF) was from Aldrich (Gillingham, UK). Calmodulin-Sepharose 4 B was obtained from Pharmacia LKB (Milton Keynes, UK) and purified egg-yolk phosphatidylcholine from Lipid Products (Nutfeld, UK). All other chemicals were Analytical Reagent grade from Fisons (Loughborough, UK) or Analar from BDH Merck (Poole, UK). Double-distilled water was further purified with a Milli-Q Plus system (Millipore UK, Watford).

**Preparation of Calmodulin-depleted Red Cell Membranes**

Broken membranes from human erythrocytes were prepared from fresh heparinized blood from healthy donors or bank blood, as described (Cavieres, 1984). The membranes were resuspended in a Mg/HEPES solution, containing (mM): HEPES 15 (adjusted with NaOH to pH 7.4 at 20°C), MgCl\(_2\) 0.1, frozen in aliquots at 8–10 mg protein/ml (200% hematocrit on the original cells) and kept at -80°C.

**Preincubation of Red Cell Membranes**

The membranes were mixed in microcentrifuge tubes on an ice-bath with 11 or 12 volumes of a solution containing CaCl\(_2\). In early experiments, this was a phosphate-buffered saline (PBS), made up with (mM): NaCl 137, KCl 2.7, KH\(_2\)PO\(_4\) 1.5, Na\(_2\)HPO\(_4\) 8.1 (pH 7.45 at 20°C), MgCl\(_2\) 0.4 and CaCl\(_2\) 0.9, plus NaN\(_3\) 1 (which later proved to be an unnecessary precaution). Most experiments were in Mg/HEPES solution after the first spin.

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The controls were (i) preincubated membrane preparations mixed in Mg/HEPES solution, containing (mM): HEPES 15 (adjusted with NaOH to pH 7.4 at 20°C), MgCl\(_2\) 0.1, and (ii) for the NK plus Ca\(^{2+}\) preincubation: membranes kept at 0°C in NK solution plus 0.5 mM ethyleneglycol-bis(\(\beta\)-amino-ethyl ether) N,N\(^{-}\)-tetraacetic acid (EGTA). Preincubations at 37°C were stopped by cooling the tubes on an ice-bath for 5 min, followed by centrifugation of the membranes; 0°C preincubations were in fact terminated by the resuspension of the membranes in Mg/HEPES solution after the first spin. Preincubated and control membranes were centrifuged at 20,000 x g and 5°C in an Ole Dich refrigerated microcentrifuge (Camlab, Cambridge). The membranes were resuspended with chilled Mg/HEPES solution and washed with the same solution. The tube walls were wiped with a roll of Whatman 1 filter paper and the pellet was finally resuspended with Mg/HEPES, ready for the Ca,Mg-ATPase assay.

**Assay of the Ca,Mg-ATPase Activity of Red Cell Membranes**

Fresh, control or preincubated membranes were assayed for their ATPase activity as previously described (Cavieres, 1984). This was done in 40 ml of a medium containing 2 mM ATP (plus \(\text{[32P]}\)-ATP), 20 \(\mu\)M CaCl\(_2\) (3 \(\mu\)M Ca\(^{2+}\)), 1.4 mM MgCl\(_2\), 60 mM NaCl, 60 mM KCl, 15 mM HEPES (adjusted with NaOH to pH 7.2 at 37°C) and 0.1 mM ouabain (to inhibit Na,K-ATPase), with or without 0.5 mM EGTA, in triplicate initial and final tubes. The membrane suspension was equivalent to 12% hematocrit on the original cells (ca. 0.4 mg protein/ml). The final tubes were incubated at 37°C for 30 to 90 min, while the initials remained on the ice-bath. When calmodulin was used, this was at a concentration of 0.4 \(\mu\)M and the incubation was for 20 or 30 min. In these conditions, ATP hydrolysis does not exceed 25% and linear time courses are obtained (Cavieres, 1987b). The released \(32P\) i was extracted and counted together with acid-hydrolyzed \(\text{[32P]}\)-ATP standards (Brown, 1982; Cavieres, 1984). The Ca,Mg-ATPase activity was calculated as the difference between the enzymic activities measured in the absence and the presence of 0.5 mM EGTA.

**Purification of Ca,Mg-ATPase**

This was done by CaM-affinity chromatography in the presence of Triton X-100 and phosphatidylcholine, essentially as described by Niggli et al. (1979, 1981), except for the addition of thiol-protease inhibitors (500 \(\mu\)M leupeptin and bestatin). The red cell membranes were given a wash in a solution consisting of (mM) KCl 300, TES-triethanolamine 10 and dithiothreitol 2, before solubilizing with 5 mg Triton X-100 per ml and at 5 mg protein/ml. The solubilize was cleared by centrifugation at 100,000 x g in a Beckman L8-80 ultracentrifuge, before calmodulin affinity chromatography. The fractions eluted with 5 mM EDTA were supplemented with 5 mM CaCl\(_2\) and glycerol (to 20% v/v) before freezing and storage at -80°C. On average, the specific activity of the pooled fractions was 1 \(\mu\)mol · min\(^{-1} · mg\(^{-1}\) at 54 \(\mu\)M Ca\(^{2+}\) and 37°C.

**Preincubation and Assay of Purified Ca,Mg-ATPase**

Existing Ca\(^{2+}\) and EDTA were removed from soluble-purified Ca,Mg-ATPase by gel filtration (see below) through spun Sephadex G50 columns (Penevsky, 1977) which had been pre-equilibrated in medium S consisting of (mM): NaCl 100, KCl 100, HEPES 15 (adjusted with NaOH to pH 7.4 at 20°C), dithiothreitol 1, and also phosphatidylcholine 0.8 mg/ml, Triton X-100 4 mg/ml and 10% (v/v) glycerol. Aliquots of the gel-filtered enzyme were preincubated for 10 min at 37°C at varying Ca\(^{2+}\) concentrations (using buffers at 10 \(\mu\)M Ca\(^{2+}\) and below) in the medium above, supplemented with 500 \(\mu\)M leupeptin and 500 \(\mu\)M antipain.