Erythrocyte membrane Ca\textsuperscript{2+}-pumping ATPase of hypertensive humans: Reduced stimulation by calmodulin

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The Ca\textsuperscript{2+}-pumping ATPase of erythrocyte plasma membranes of hypertensive humans (HTN) show, in the absence of calmodulin, a low \(V_{\text{max}}\) comparable to that of the enzyme of the erythrocyte membranes of normotensive humans (NTN). Although the addition of calmodulin (1.5 \(\mu\)g per ml) increased the maximum activity of the calcium pump of membranes of HTN and NTN individuals by at least 2-fold and 4-fold, respectively, the activator protein partially purified from the erythrocytes of HTN individuals enhanced the activity of the enzyme in a fashion similar to that of the protein obtained from the haemolysate of NTN individuals. A determination of the dependence of the activity of the pump on concentration of ATP revealed that the \(K_m\) (ATP) of the enzyme of membranes of HTN individuals is 52% higher than that of the enzyme of membranes of NTN individuals, while the \(V_{\text{max}}\) (1.75 ± 0.28 \(\mu\)mol ATP mg protein\(^{-1}\) h\(^{-1}\)) of the pump is 46% lower in the membranes of HTN humans than that of the enzyme of membranes of normal individuals (3.25 ± 0.42 \(\mu\)mol ATP mg protein\(^{-1}\) h\(^{-1}\)). It seems likely from these results that elevated erythrocyte Ca\textsuperscript{2+} concentration associated with essential hypertension may be due to a defective interaction between the Ca\textsuperscript{2+}-pumping ATPase and the calmodulin Ca\textsuperscript{2+} complex.

Although Ca\textsuperscript{2+} has to fulfil several messenger functions within the cell, elevated intracellular Ca\textsuperscript{2+} concentration has been shown to cause an increase of K\textsuperscript{+} leak transport, an inhibition of Na\textsuperscript{+}-K\textsuperscript{+} ATPase, and a destruction of the plasticity and normal shape of red blood cells (1). There is evidence that Ca\textsuperscript{2+} plays a role in the pathogenesis of essential hypertension, a disease which is characterized by a high blood pressure (2). The abnormalities of Ca\textsuperscript{2+} metabolism in hypertensive humans (HTN) and in spontaneously hypertensive rats (SHR) include a chronic depression of serum ionized Ca\textsuperscript{2+}, a lowered membrane binding of Ca\textsuperscript{2+} and an excessive accumulation of Ca\textsuperscript{2+} by erythrocytes (3-5). It is now well established that the Ca\textsuperscript{2+}-pumping ATPase is responsible for the active extrusion of Ca\textsuperscript{2+} from erythrocytes, thus maintaining the erythrocyte intracellular concentration below 1 \(\mu\)M while the Ca\textsuperscript{2+} level in the surrounding plasma is about 1-2 mM (6-7). The kinetics and physiological properties of the enzyme obtained from human

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erythrocyte ghost membranes have been extensively studied (8-10). Both the membrane-bound and purified enzymes are stimulable by calmodulin, a multifunctional calcium-dependent modulator protein which mediates the effects of Ca\(^{2+}\) on many enzymes and cellular processes (11). In view of the observation that there is no appreciable difference in the rate of Ca\(^{2+}\) influx into erythrocytes of individuals with essential hypertension when compared with normotensive control humans (3), it seems pertinent to assess the ability of the Ca\(^{2+}\)-pumping enzyme to regulate erythrocyte intracellular Ca\(^{2+}\) concentration in hypertensive humans. In this paper, we report that the basal activity of the Ca\(^{2+}\)-pumping ATPase of the erythrocyte ghost membranes of hypertensive humans is identical to that of normotensive individuals, although the calmodulin-responsiveness of the enzyme is lower in the former than in the latter, irrespective of the source of calmodulin.

Materials and Methods

Reagents

All reagents were of the highest purity available. ATP (vanadium-free), 4-(2 hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and fatty acid-free bovine serum albumin were purchased from Sigma Chemical Co., Missouri, U.S.A. DEAE-cellulose (DE-52), sodium dodecylsulphate (SDS) and ethyleneglycol-bis-(2 aminoethyl ether)\(\text{N}_2\text{N}_4\text{N}_4\text{N}_4\)-tetra-acetic acid (EGTA) were obtained from Fluka AG, Zurich, Switzerland. Sephadex G-100 (Superfine) was purchased from Pharmacia, Uppsala, Sweden. All other reagents were of analytical grade.

Preparation of calmodulin-deficient erythrocyte ghost membranes

Blood samples were obtained from individuals who were newly identified as having essential hypertension by the Cardiac Clinic at the University College Hospital (U.C.H.), Ibadan. The subjects were not receiving any medication or dietary therapy at the time of collection of blood. A set of normotensive individuals was drawn from healthy volunteers at the same hospital. Calmodulin-deficient erythrocyte ghost membranes were prepared by haemolysis in 1 mM EDTA (ethylene-diamine-tetra-acetic acid) by the procedure of Niggli et al. (9). Packed erythrocytes were obtained from whole blood samples by centrifuging at 5800 g for 10 min. The plasma and buffy layers were removed by aspiration. The erythrocytes were washed twice in 5 vol. of 130 \(\mu\)M KCl, 20 \(\mu\)M Tris (pH 7.4) by centrifuging the cell suspension at 5800 g. The cells were now haemolysed in 5 vol. of 1 mM EDTA (Na salt), 10 mM Tris-HCl (pH 7.4) and centrifuged at 18 000 g for 30 min. This step was repeated four times to ensure complete haemolysis. The membranes were then washed eight times in 10 mM HEPES (pH 7.4). Calmodulin-deficient erythrocyte ghost membranes were finally resuspended in 130 mM KCl, 20 mM HEPES pH 7.4, 500 \(\mu\)M MgCl\(_2\), 50 \(\mu\)M CaCl\(_2\) and stored at -4°C.

Partial purification of calmodulin

Calmodulin was partially purified from the lysate of the erythrocytes of normotensive and hypertensive individuals according to the
procedure of Jarret and Penniston (17). 5 g of purified DEAE-cellulose previously washed and equilibrated in 10 mM Na-PIPES (pH 6.5) was carefully stirred into one litre of membrane-free haemolysate. The suspension was stirred vigorously for 1 h and allowed to settle for at least 45 min. The supernatant was carefully aspirated while the mixture that remained was filtered using a Buchner funnel. The cellulose was washed extensively with 50 mM NaCl in 10 mM Na-PIPES pH 6.5 until free from haemoglobin. The DEAE-cellulose was washed twice with 150 mM NaCl in 10 mM Na-PIPES, pH 6.5 and thrice with 300 mM NaCl in 10 mM Na-PIPES, pH 6.5. Calmodulin was eluted from the DEAE-cellulose using 600 mM NaCl in 10 mM Na-PIPES pH 6.5. This step was repeated twice. The eluate was dialysed extensively against distilled water using Spectropor 3 (cut-off mol.wt. 3500) and concentrated in vacuo. The pH of the eluate was adjusted to 7.0 using NH₄OH in 50% saturated (NH₄)₂SO₄. Solid (NH₄)₂(SO₄) was added in small portions to the eluate to achieve 50% saturation. The solution was stirred for an hour and centrifuged at 18 000 g for 30 min at 4°C. The pH of the supernatant therefrom was adjusted to 4.0 using 0.5 M H₂SO₄ in 50% saturated (NH₄)₂SO₄, stirred for an hour and centrifuged at 18 000 g for 20 min at 4°C. The supernatant was discarded. The final pellet was dissolved in 100 mM NH₄HCO₃ and the centrifugation repeated in order to remove particulate matter. The solubilized material was loaded onto a 2.5 x 80 cm column of Sephadex G-100 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated with 100 mM NH₄HCO₃. The column was eluted at 20 ml per hour using two bed volumes of 100 mM NH₄HCO₃ while monitoring the absorbance at 235 nm. The fractions containing calmodulin as detected by ability to activate Ca²⁺Mg²⁺-ATPase were pooled and concentrated.

Assay of Ca²⁺-ATPase and Mg²⁺-ATPase

Ca²⁺Mg²⁺-ATPase activity was assayed in the presence or absence of calmodulin (10 μg/ml) by measuring the rate of release of inorganic phosphate from ATP by a slight modification of the procedure of Ronner et al. (13). The assay medium contained 120 mM KCl, 300 mM HEPES, pH 7.2, 2 mM MgCl₂, 0.2 mM CaCl₂ (or 0.5 mM EGTA) and 100-200 μg of erythrocyte membrane protein in a total of 0.8 ml. The mixture was pre-incubated for 3 min at 37°C prior to initiation of reaction by addition of 1 mM ATP (final concentration). The reaction was allowed to proceed for 30 min and terminated by addition of 0.2 ml of 5% sodium dodecylsulphate (SDS). All assays were run in triplicate. Individual experiments were repeated at least twice. Blanks were run to correct for non-enzymic hydrolysis of ATP. Mg²⁺-ATPase was assayed by the same protocol except that 0.5 mM EGTA was added to the reaction medium in place of 0.2 mM CaCl₂. To obtain the basal Ca²⁺-ATPase activity, Mg²⁺-ATPase activity was subtracted from the Ca²⁺Mg²⁺-ATPase.

Determination of protein

Membrane protein was determined by the method of Lowry et al. (14) using deoxycholate and trichloroacetic acid to prevent interference with HEPES etc.
Results and Discussion

The molecular properties of the Ca²⁺-pumping ATPase of human erythrocyte plasma membrane are now well established (10). The results obtained from several laboratories indicate that the purified Ca²⁺-pumping ATPase has, in general, the properties expected of the functional Ca²⁺-pump of unfractionated erythrocyte membranes (15). This enzyme shows a low \( V_{\text{max}} \) and a relatively low affinity for Ca²⁺ in the absence of calmodulin (16). Acid phospholipids have been shown to mimic the action of calmodulin (13). These findings indicate that the enzyme has two distinct molecular configurations depending on the presence or absence of calmodulin. There is evidence that calmodulin interacts with a high affinity mainly with the Ca²⁺-ATPase and not with any other ghost membrane proteins (17). It has been demonstrated that when the switch from low \( V_{\text{max}} \) is evoked calcium ions are bound to one calmodulin molecule which in turn forms a one-to-one complex with the pump protein (18-21). Results of experiments from a number of laboratories indicate that the higher \( V_{\text{max}} \) and the higher calcium affinity found in the presence of calmodulin is an intrinsic property of the activated pump (22-24). The specific activities reported for purified Ca²⁺-ATPase preparations in the absence of calmodulin range from 9.0-186.0 \( \mu \text{mol mg protein}^{-1} \text{ h}^{-1} \) as compared with the usual 0.3-3.0 \( \mu \text{mol mg protein}^{-1} \text{ h}^{-1} \) for whole membranes (16). There is no report on the properties of the Ca²⁺-ATPase of erythrocyte membranes of HTN individuals. In this study, calmodulin-deficient erythrocyte plasma membranes were isolated from blood samples of ten hypertensive (HTN) and ten normotensive (NTN) individuals as described under 'Materials and Methods'. The basal Ca²⁺-ATPase of these membranes are shown in Table 1. The data show that the basal activities of the calcium pumping enzyme in these membranes are comparable. This finding suggests that the degree of interactions of the lipids of the erythrocyte membranes of HTN individuals with the Ca²⁺-pumping enzyme is identical to that of the enzyme of the erythrocyte membranes of NTN individuals since these interactions play a role in the activity of the enzyme (21).

Calmodulin was isolated and partially purified from the haemolysates of the erythrocytes of HTN and NTN individuals as described.

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<tr>
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<th>Membranes from normotensive individuals</th>
<th>Membranes from hypertensive individuals</th>
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<tbody>
<tr>
<td>Mg²⁺-ATPase</td>
<td>0.36 ± 0.04</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-calmodulin</td>
<td>0.69 ± 0.06</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>+calmodulin</td>
<td>2.86 ± 0.21</td>
<td>1.78 ± 0.10</td>
</tr>
<tr>
<td>+calmodulin²</td>
<td>2.74 ± 0.26</td>
<td>1.83 ± 0.20</td>
</tr>
<tr>
<td>Stimulation factor</td>
<td>4.1¹</td>
<td>2.6²</td>
</tr>
<tr>
<td>by calmodulin</td>
<td>4.2²</td>
<td>2.5²</td>
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Fig. 1. Stimulation of the basal rate of erythrocyte plasma membrane Ca\(^{2+}\)-pumping ATPase of normotensive (NTN) humans (○) and hypertensive (HTN) humans (●) by different concentrations of calmodulin. Stimulation factor corresponds to the ratio of Ca\(^{2+}\)-ATPase activity in the presence and absence of calmodulin. The assay medium contained 120 mM KCl, 300 mM HEPES, pH 7.2, 2 mM MgCl\(_2\), 0.2 mM CaCl\(_2\) (or 5 mM EGTA) and 100-200 µg membrane protein in a total of 0.8 ml. Ca\(^{2+}\)-ATPase was assayed as described under Materials and Methods.

under 'Materials and Methods'. Significant stimulation (4-fold) of the basal Ca\(^{2+}\)-ATPase by calmodulin was observed in the erythrocyte membranes of NTN individuals irrespective of source of calmodulin (Table 1). In contrast, the calmodulin-responsiveness of the enzyme in the erythrocyte membranes of HTN individuals was only 50% that of normal membranes. This degree of stimulation was also observed in the presence of calmodulin obtained from haemolysates of HTN erythrocytes. These results show clearly that HTN calmodulin is quite normal. A profile of the dependence of stimulation of basal Ca\(^{2+}\)-ATPase on calmodulin is presented in Fig. 1. It appears from this figure that the enzyme of the membranes of NTN individuals shows a higher affinity for calmodulin than the enzyme of HTN individuals. For instance, the extent of stimulation of the enzyme in the membranes of HTN individuals by 2 ng of calmodulin per mg of membrane protein was only 66% that of the enzyme of the membranes of NTN individuals. It seems probable from these results that the

<table>
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<tr>
<th>Membrane</th>
<th>K(_m) (µmol ATP)</th>
<th>V(_\max) (µmol ATP/mg protein/h)</th>
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<tr>
<td>Normotensive</td>
<td>20.6 ± 5.1</td>
<td>3.25 ± 0.42</td>
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<tr>
<td>Hypertensive</td>
<td>31.3 ± 4.6</td>
<td>1.75 ± 0.28</td>
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Table 2. K\(_m\) and V\(_\max\) values for the Ca\(^{2+}\)-ATPase of human erythrocyte ghosts during essential hypertension. Each value is a mean of several determinations (at least 10) ± S.D.
binding between the calmodulin-Ca\textsuperscript{2+} complex and the enzyme of HTN membranes is defective since the machinery of the red cell Ca\textsuperscript{2+}-pump is normally turned off until it encounters the right calmodulin-Ca\textsuperscript{2+} complex. The kinetic properties of the Ca\textsuperscript{2+}-ATPase of unfractionated erythrocyte membranes have been well documented. Table 2 summarizes the data obtained from determinations of the $K_m$ (ATP) and $V_{\text{max}}$ of the Ca\textsuperscript{2+}-ATPase of the erythrocyte membranes of NTN and HTN individuals in the absence or presence of calmodulin (Fig. 2). The affinity of the membrane-bound enzyme for ATP was determined in five NTN and five HTN individuals in the presence or absence of calmodulin. The results show that in the presence of the calmodulin, the $K_m$ (ATP) of the enzyme of the erythrocyte plasma membranes of HTN individuals is significantly higher than that of the enzyme of the membranes of NTN individuals. $V_{\text{max}}$ values were lower in the membranes of HTN individuals ($1.75 \pm 0.28$ µmol ATP mg protein\textsuperscript{-1} h\textsuperscript{-1}) than in NTN individuals ($3.25 \pm 0.42$ µmol ATP mg protein\textsuperscript{-1} h\textsuperscript{-1}). These results demonstrate that the Ca\textsuperscript{2+}-pump of the erythrocyte membranes of HTN individuals is less active than that of normal membranes even in the presence of calmodulin. It appears from these findings that the defect in the affinity of the HTN enzyme for calmodulin could be responsible for the reduced $V_{\text{max}}$ and increased $K_m$ values. The consequence of this is a reduction of the rate of calcium pumping and an increase in intracellular Ca\textsuperscript{2+} ions in the erythrocytes of HTN individuals. It seems likely therefore that the observed elevated intracellular Ca\textsuperscript{2+} ion concentration in the erythrocytes of HTN individuals may be due to a defective Ca\textsuperscript{2+}-ATPase/calmodulin-Ca\textsuperscript{2+} interaction. Although the erythrocyte membranes of patients with essential hypertension are now known to be more diffuse (25) than those of normal individuals, the exact nature of the defect in the Ca\textsuperscript{2+}-ATPase/calmodulin-Ca\textsuperscript{2+} interaction remains unknown.

![Fig. 2. ATP-dependence of the Ca\textsuperscript{2+}-pumping ATPase of normotensive (NTN) and hypertensive (HTN) humans in the presence (NTN, •; HTN, △) and absence (NTN, ○; HTN, ▲) of calmodulin (1.5 µg/ml).](image-url)
References