Analysis of Calcium Handling in Erythrocyte Membranes of Genetically Hypertensive Rats

MARIE-AUDE DEVYNCK, PH.D., MARIE-GABRIELLE PERNOLLET, ANNE-MARIE NUNEZ, M.D., AND PHILIPPE MEYER, M.D.

SUMMARY Calcium handling by erythrocyte membranes was compared in genetically hypertensive (SHR) and normotensive (WKR) rats by direct measurement of calcium binding, passive influx, and adenosine triphosphate (ATP)-dependent extrusion. The SHR erythrocyte membranes exhibited the following abnormalities: 1) the binding capacity of the high affinity Ca\textsuperscript{2+}-binding sites located on the inner side of the membrane was 0.84 ± 0.07 nmole/mg protein compared with 1.17 ± 0.08 nmole/mg protein in WKR, 2) ATP-dependent Ca\textsuperscript{2+} extrusion, measured as the Ca\textsuperscript{2+} influx into inside-out vesicles, was also lower than the WKR, as was the La\textsuperscript{3+}-sensitive, Ca\textsuperscript{2+}-dependent ATP hydrolysis, indicating reduced activity of the calcium pump; 3) the passive calcium influx into ATP-depleted red blood cells was slightly accelerated. These abnormalities in Ca\textsuperscript{2+} binding and transport probably enhanced intracellular Ca\textsuperscript{2+} concentration, and were observed under both prehypertensive and hypertensive conditions, in 3-week-old and adult SHR respectively. Similar membrane defects in excitable cells may help to explain the pathogenesis of hypertension, since they may increase vascular tone and/or catecholamine release. (Hypertension 3: 397-403, 1981)

KEY WORDS • spontaneous hypertension • calcium binding • calcium transport • Ca,Mg-ATPase • erythrocytes • inside-out vesicles

Several abnormalities have been described in red blood cells of spontaneously hypertensive rats (SHR), including changes in the Na\textsuperscript{+} and K\textsuperscript{+} transmembrane fluxes\textsuperscript{1-3} and in membrane polyphosphoinositide distribution,\textsuperscript{4} and reduction in calcium binding.\textsuperscript{4} These alterations could stem from an intrinsic membrane defect inherited with hypertension.

An extensive biochemical study is necessary to understand how the various erythrocyte alterations are linked together, and this requires careful analysis of each one. In the present study we aimed at describing erythrocyte calcium handling by means of direct calcium binding and transport evaluation procedures.

The defect in calcium regulation by the erythrocyte membrane demonstrated here shows interesting similarities with the changes observed in membranes from vascular smooth muscle and which appear to be of great pathogenic importance.\textsuperscript{6-12} It is therefore possible that a genetically transmitted and diffuse membrane defect is involved in the development of hypertension, and that erythrocytes constitute a convenient tool for extending the investigation to the molecular level.

Methods

Male Okamoto spontaneously hypertensive rats (SHR) and the corresponding Wistar-Kyoto (WKR) normotensive controls derived from the National Institutes of Health (NIH) stock were supplied by Iffa-Credo (France). They were studied at 3 and 15-24 weeks of age. Systolic arterial blood pressure (BP) was recorded by tail plethysmography. Values (in mm Hg, mean ± SEM) were as follows: at 3 weeks, SHR = 115 ± 3, controls = 105 ± 3; at 22 weeks, SHR = 195 ± 7, controls = 129 ± 5. Both SHR and WKR were given a standard diet with free access to tap water. Blood was sampled by cardiac puncture performed on stunned animals, and collected in heparinized tubes (10 IU/ml). It was centrifuged at
800 g for 10 minutes at 4°C, and the red blood cells were washed three times in 150 mM NaCl. The white buffy layer was eliminated as completely as possible. Experiments were performed either on intact red blood cells or on isolated erythrocyte membranes.

Calcium Binding

Experiments were performed on sealed inside-out vesicles, prepared using the method of Steck and Kant modified by Blöstein & Chu, and on crude membrane vesicles (randomly oriented) obtained prior to the dextran density gradient step described in these procedures. Sideness and sealing quality of vesicles were measured by the activity of acetylcholine esterase, a marker enzyme of the exterior surface of the membrane; the enzyme was measured according to Steck and Kant in the absence and presence of 0.2% Triton X 100, which makes the internal enzyme sites accessible to the substrate and allows the determination of the maximal enzyme activity. Sealed inside-out vesicles percentages, assessed by the inaccessibility of acetylcholine esterase activity, were 59.1% ± 3.9% and 55.2% ± 4.1% (mean ± SEM, n = 7) for WKY and SHR erythrocytes respectively. Membrane fractions (10 to 60 µg protein) were incubated at 37°C in 10 mM histidine buffer, pH 7.4, containing 140 mM NaCl and 4CaCl2 (10 mCi/m mole), free calcium concentrations ranged from 10-8 to 10-4 M. Ca-EGTA buffers were used for free calcium concentrations (10 to 60 µg protein), prepared as for calcium binding, were incubated for 30 minutes in 10 mM histidine buffer, pH 7.4, containing 140 mM NaCl, and 10 mM histidine buffer, pH 7.4, containing 10-4 M 4CaCl2 (10 mCi/m mole). Incubation was stopped after 15 minutes and 1, 2, 3, and 4 hours, and erythrocytes were separated from the medium by centrifugation on a dibutyl phthalate cushion. The pellets were resuspended in water and treated with 5% TCA, and the supernatant was counted by liquid scintillation. The red blood cell volume was estimated by the hematocrit.

Trapping of Membranes

Inside-out vesicles were incubated for 5 to 60 minutes at 37°C in the presence of 200 to 1000 µg/ml trypsin (Boehringer). Trypsin inhibitor from soybean (Boehringer) at concentrations 10 times higher than trypsin was added before binding and transport studies.

Protein Determination

Membrane protein content was determined according to Lowry et al.

**Results**

**Calcium Binding**

**Inside-out Vesicles**

Experiments were performed on 3-week-old and 22-week-old rats, with similar results. Even with the lowest free calcium concentration, binding reached equilibrium within 30 minutes at 37°C and was reversible by the addition of an excess of unlabelled calcium (fig. 1). No significant difference was measurable between the rate constants in SHR and WKY membranes, but the amount of specifically bound calcium was lower in SHR than WKY membranes. Scatchard analysis of binding at free calcium concentrations ranging from 1 x 10-8 M to 3 x 10-4 M...
**Figure 1.** Time-course of "Ca" binding to inside-out vesicles of SHR erythrocytes. Binding was measured at 37°C with 10^8 M free "Ca in the absence (●) and the presence (○) of an excess unlabelled Ca" (10^3 M). Reversibility of the binding is shown by the dissociation of bound "Ca on addition of 10^-3 M unlabelled CaCl₂ as indicated by the arrow (one out of three similar experiments, each measurement was performed in duplicate).

**Figure 2.** Scatchard plots of "Ca" binding to inside-out vesicles from SHR (●) and WK (○) erythrocytes. Free Ca concentration ranged from 10^-1 M to 3 x 10^-1 M. Binding at higher Ca concentrations corresponding to low affinity and high-capacity binding sites is not illustrated (one of 10 independent experiments).

M is shown in figure 2. Within this concentration range, only one class of sites was detectable. Although for Ca concentrations above 3 x 10^-5 M, we observed low affinity-high affinity capacity binding sites, we considered only high affinity binding sites in our comparison of SHR and WK erythrocytes.

The apparent dissociation constants (K_D) of calcium binding were similar in SHR and WK erythrocytes (table 1). Conversely, the binding capacity was lower in genetically hypertensive rats. In erythrocytes from 3-week-old rats, then number of binding sites (Nₘ) was 0.84 ± 0.07 and 1.17 ± 0.08 nmole/mg protein on SHR and WK membranes respectively (mean ± SEM, n = 8, p < 0.01). This abnormality in calcium binding was not restricted to young rats since Scatchard analysis of Ca²⁺ binding on inside-out vesicles from adult rats indicated similarly a lowered binding capacity without any significant change in the affinity (table 1).

**Randomly Oriented Membranes**

In both SHR and WK erythrocytes, K_D values were the same and were also identical to those measured in inside-out vesicles. The binding capacity was reduced compared to that of inside-out vesicles, but no significant difference in this respect was observed between SHR and WK membranes (0.71 ± 0.08 and 0.73 ± 0.09 nmole/mg protein respectively).

### Table 1. Characteristics of Calcium Binding on Inside-Out Vesicles from Rat Erythrocytes

<table>
<thead>
<tr>
<th>Rat age group</th>
<th>Binding capacity (nmole/mg protein)</th>
<th>Apparent affinity constant (K_D) (10^-8 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>SHR</td>
</tr>
<tr>
<td>Young (3 week-old)</td>
<td>1.17 ± 0.08 (n = 8)</td>
<td>0.84* ± 0.07 (n = 7)</td>
</tr>
<tr>
<td>Adult (15-24 week-old)</td>
<td>1.28 ± 0.15 (n = 5)</td>
<td>0.95* ± 0.15 (n = 5)</td>
</tr>
</tbody>
</table>

*Significantly different from WKY values (by paired Student's t test (p < 0.01).
Ca**.Mg**-ATPase

We observed no significant difference between the rates for ATP hydrolysis by SHR and WKR membranes respectively, either with regard to basal Mg**-dependent hydrolysis, or Ca**-dependent extra splitting (fig. 3). However, when we measured the inhibition of Ca**-dependent ATPase by lanthanum, we found that the amounts of both La**+-sensitive and La**+-resistant activities differed (fig. 4). In the presence of 0.25 mM La**, which maximally inhibits ATP hydrolysis,18-20 the La**-resistant ATPase activities in SHR and WKR erythrocyte membranes respectively were 0.296 ± 0.024 and 0.230 ± 0.010 μmoles Pi released per milligram (mg) of protein per hour (p < 0.02). La**-sensitive ATPase activities in SHR and WKR membranes were respectively 63.5% ± 2.9% and 70.5% ± 2.6% of the Ca**-dependent ATP hydrolysis (p < 0.02 by paired Student t test, n = 8). For both substrains, we observed 50% inhibition for 2.5 × 10^-4 M La**.
ATP-Dependent Ca\(^{2+}\) Transport

Inside-out vesicles from SHR and WKR rats accumulated calcium in the presence of ATP, and this process has been shown to reflect ATP-dependent Ca\(^{2+}\) extrusion from intact cells.\(^{21,24}\) Maximal intravesicular concentrations were reached in less than 40 minutes. When 1.5 \times 10^{-4} M A23187 ionophore was added, more than 99% of the intravesicular \(^{44}\)Ca was lost in less than 2 minutes, and a return to the same binding level was observed as in the absence of ATP.

Studies were performed on young and adult rats (3–4 and 15–22 week-old animals), with similar results. The Ca\(^{2+}\) uptake by SHR erythrocyte vesicles was lower than the Ca\(^{2+}\) uptake by WKR erythrocyte vesicles, whatever the ATP or Ca concentrations (fig. 5). This seems to be due to a lower apparent V\(_{\text{max}}\) (fig. 6) without any change in the apparent affinities for calcium (1.2 \times 10^{-7} M) and ATP (1.2 \times 10^{-4} M) of either SHR or WKR membranes.

Passive Calcium Influx

Passive membrane permeability to calcium was tested on ATP-depleted erythrocytes. The rate of calcium influx in the presence of 10^{-4} M extracellular \(^{44}\)Ca\(^{2+}\) was 0.61 ± 0.11 and 0.44 ± 0.09 nmoles/liter of cells per hour for SHR and WKR erythrocytes respectively (mean ± SEM, n = 6). Thus, SHR erythrocytes appear to show an increased passive calcium influx compared to control WKR erythrocytes, although this difference is not statistically significant.

Nature of the Sites Involved in Membrane Changes in SHR Erythrocytes

Trypsin digestion of membranes increased calcium binding (fig. 7) and totally suppressed ATP-dependent transport in both SHR and WKR erythrocytes. The difference between their respective calcium-binding capacities was gradually reduced by trypsin treatment, without altering their apparent affinities.

Discussion

A previous investigation has shown that erythrocyte membranes have a lower calcium-binding ability in spontaneously hypertensive rats than in normotensive controls.\(^{4}\) The present experiments on genetically hypertensive Okamoto rats and normotensive Wistar-Kyoto rats, usually considered to be appropriate controls, confirm the existence of this biochemical abnormality. Additional insights were gained into calcium handling in red blood cell membranes.

Kinetic analysis showed that calcium binding to erythrocyte membranes is a reversible phenomenon that concerns various classes of sites with limited capacities and different affinities. Comparison of calcium-binding capacities in sealed inside-out vesicles and randomly oriented membranes showed that the high affinity Ca\(^{2+}\)-binding sites were mainly located on the internal side of the membrane. The Ca-binding site concentration measured in this study on the internal side of the membrane corresponds to that reported by Postnov et al.\(^{5}\) The apparent affinity of SHR and WKR membranes for Ca\(^{2+}\) binding was found to be 7.5 \times 10^{-7} M. Given the reversibility of the binding measured here and the intracellular Ca\(^{2+}\) concentration, this observation suggests that these Ca\(^{2+}\)-binding sites might be involved in regulating the cytosolic calcium concentration, as well as helping to determine membrane structure and function.

We confirmed that Ca\(^{2+}\) binding was lower in SHR than WKR erythrocytes, and demonstrated that this
difference was connected to a reduction in the binding capacity of the high affinity class of sites located, as suggested by Postnov et al., on the inner side of the membrane, with no change in the apparent affinity constant. The reduction of the membrane calcium-binding capacity could thus constitute a mechanism capable of enhancing free intraerythrocytic calcium content in genetically hypertensive rats.

From the present data, however, other mechanisms might also be involved. One could be the increased passive calcium influx indicated in our experiments. This higher membrane permeability to calcium may result from the reduction in calcium binding to SHR erythrocyte membranes, since bound calcium has a stabilizing effect that may inhibit the passive downhill calcium fluxes from the extra- to intracellular compartments.

The other mechanism capable of increasing intraerythrocyte calcium content appears to be a lowering of ATP-dependent calcium extrusion. This is suggested by the present measurements of calcium transport in the presence of ATP, made on inside-out membrane vesicles. Direct biochemical Ca-ATPase measurements indicated that the reduction of the active calcium efflux is probably secondary to the slight slowing down of the calcium pump activity, which up until now has been considered the only process capable of active calcium extrusion from the cells.

The reduction in active calcium transport observed in hypertensive rat erythrocytes might, according to the Lineweaver-Burk representation, reflect a reduction in the Vmax of enzyme activity without any concomitant change in affinity constants. The drop in Vmax might in its turn indicate a diminution in the number of enzyme molecules or of the enzyme turnover.

Another interesting result is that trypsin treatment of the membranes does not abolish calcium binding but suppresses the difference between normotensive and hypertensive animals. This suggests that the calcium-binding sites are either not proteic in nature or inaccessible to trypsin, as reported recently for the high affinity calcium-binding protein from sarcoplasmic reticulum membranes. The trypsin-induced suppression of the difference between calcium-binding capacities in normotensive and hypertensive rat erythrocytes may also indicate that the difference could stem from a proteic membrane component regulating calcium binding, which in genetic hypertension, would constitute an abnormality.

Further studies are obviously necessary to understand the molecular mechanisms that reduce the calcium-binding capacity in SHR erythrocytes. In particular, it seems important to investigate the cAMP-dependent protein kinase and membrane polyphosphoinositides. This is suggested by the reduction observed in cAMP-dependent kinase activity, which in turn enhances the energy-dependent calcium sequestration in aortic microsomes from spontaneously hypertensive rats. Diphosphoinositides and triphosphoinositides were also shown to be involved in calcium binding by human erythrocytes.

There is considerable evidence that calcium controls the properties of the red cell membranes by regulating membrane conformation. Abnormal cationic fluxes have been observed in red blood cells from genetically hypertensive rats. They included in particular, enhanced unidirectional Na efflux, increased Na and K net fluxes after ouabain treatment, enhanced Na and Li unidirectional fluxes, and reduced net Na extrusion, which was observed in both young and adult hypertensive rats. The relationship between these flux abnormalities is still obscure, as is their relationship with the calcium-handling abnormalities reported in the present investigation. However, they could conceivably all result from a common membrane abnormality. It is worth mentioning that the membrane-bound calcium appears to be directly involved in both the Na and K permeability and in the regulation of ATP-dependent transport across the membrane.

One could question whether the above abnormalities and the changes in the surface area previously reported to characterize this strain are linked. However, no causal relationship exists between these two characteristics since this last abnormality appears only when the rats reach a weight of approximately 150 g, whereas the changes in calcium handling already exist in very young rats. Furthermore, the calcium membrane interaction has been described to be abnormal in other SHR tissues. Recent experiments from our laboratory have shown reduced calcium binding in plasma membranes from the hepatocytes of spontaneously hypertensive rats (unpublished observations). Reduced calcium binding has also been observed in the microsomal fraction of arterial smooth muscle cells from spontaneously hypertensive rats, compared with controls. This reduction seems to be combined with decreased ATP-dependent calcium transport. As suggested by Jones, the abnormality leading to the reduction of membrane calcium binding might be responsible for the changes in cation membrane permeability described in smooth muscle cells from genetically hypertensive rats.

One may suppose that defective calcium handling by membranes is of great pathogenic importance. If it exists in excitable cells, the resultant intracellular cationic changes, particularly the enrichment of calcium and sodium would be able to initiate the series of molecular events capable of inducing hypertension such as increased catecholamine release in catecholaminergic nerve endings. In this hypothesis, membrane changes such as those detected in red blood cells of SHR, reflected by alterations in calcium handling, belong to the inherited abnormalities leading to hypertension.

Extensive investigation of excitable cells are of course necessary before the link between the membrane defects and hypertension can be firmly established. However, two observations have already suggested that erythrocyte membrane abnormalities are related to the development of hypertension: 1) they are observed in young rats before any detectable rise...
in blood pressure, and in various strains genetically prone to develop hypertension," 2) they are quite similar to those observed in human essential hypertension also linking the reduction in calcium binding, similar to those observed in human essential hypertension. "

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