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THE EFFECT OF IN VITRO AND IN VIVO CELLULAR AGING ON THE ACTIVE CALCIUM TRANSPORT IN HUMAN INSIDE-OUT RED CELL MEMBRANE VESICLES

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Modelling of the in vivo and in vitro aging processes in the human red cell has stressed the following features of the active calcium uptake by inside-out vesicles: 1) it is higher in the outdated, in vitro aged, than in the fresh red cell ($p<0.0005$), and in the densest, in vivo aged fraction than in the lightest, young fraction ($p=0.08$); 2) it increases following stimulation by excess calmodulin to values that are not significantly different; 3) it decreases to the same value in the absence of endogenous calmodulin and inhibitor, with and without exogenous calmodulin; 4) it is the target of a non-competitive inhibition, that is stronger in the fresh than in the outdated red cell. We conclude that the aging process does not involve neither membrane Ca-ATPase nor calmodulin, but rather the interaction of the calcium pump with the inhibitor of Ca-ATPase.

The choice of a suitable cell model to study the mechanism underlying cellular aging is driven by the relative importance of the cell and by its simple anatomy and physiology. Thus, the human RBC has been widely regarded as optimal model, and its aging process has been characterized by metabolic depletion (1), deformability and shape changes (2), altered enzyme activity (3), and oxygen transport (4). These modifications seem to share a common pathway with the change of intracellular free calcium, that is currently recognized as regulator of several cell functions (5, 6). It was indeed observed that high cytosolic free calcium potentiates the oxidative injury leading to cell death (7-9), and follows the cell metabolic depletion (10), but the mechanism underlying these phenomena is still to be established. The cal-

The abbreviations used are: IORCMV: inside-out red cell membrane vesicles; MCHC: mean cellular hemoglobin concentration; RBC: red blood cell.

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cium pump is likely to be involved in the alteration of the calcium homeostasis because it is the main controller of cytosolic free calcium in the RBC (11), and was chosen in this study as the probe to characterize the RBC aging, with the aim to define its role and which of its components, i.e., Ca-ATPase, calmodulin, and the inhibitor of Ca-ATPase, is primarily involved during RBC aging. Inside-Out Red Cell Membrane Vesicles (IORCMV) provide an unique opportunity to characterize the calcium transport across the plasma membrane, because, unlike intact cells, they do not require unphysiological calcium loads, and the reversed orientation of the membrane exposes the enzyme to the medium, allowing the control of the cofactors of the calcium pump.

MATERIALS AND METHODS

Red cells. Fresh heparinized blood was obtained from normal healthy volunteers of both sexes recruited in the laboratory. In vitro aging was modeled comparing fresh vs outdated RBC, obtained from the blood bank. In vivo aging was modeled separating the densest and lightest fractions from fresh blood, following a described method (12). Briefly, two solutions of Percoll (Sigma Chemicals) were prepared, one of low (1.009 g/ml) and one of high (1.020 g/ml) density. The two solutions were adjusted with an hyperosmotic solution (2.66 M NaCl, 0.09 M KCl) to 300 mosmol/l. The pH was 7.4 at room temperature. By mixing the two solutions in appropriate ratios, we obtained the desired gradient. Centrifuge tubes (95 mm x 10 mm i.d.) were then loaded with 1.5 ml of the densest Percoll solution, and 3 ml of well stirred blood was carefully layered over the Percoll. The tubes were centrifuged 1 000 g x 15 min at 4° C with no brake. Plasma and the buffy coat was removed, and the dense and light layers were recovered by gentle aspiration with a Pasteur pipette. Dense cells were resuspended in isotonic saline and washed throughout three times. Light cells were centrifuged again in a less dense Percoll. Usually, the cycle was repeated 2 to 3 times until 10-20% of the lightest cell fraction was obtained. The efficiency of the separation was checked measuring the MCHC of the separated fractions. Preparations with light MCHC/dense MCHC ratio less than 1.2 were discarded.

Inside-out RBC membrane vesicles. Washed RBC were lysed with 40 vols of 5 mM sodium phosphate, pH 8 at room temperature, and the ghosts washed three times with the same buffer (22 000 g x 10 min). Alternatively, to remove calmodulin and inhibitor of the calcium pump, this step was replaced by 3 washings with 1 mM EDTA in isotonic phosphate-buffered saline (5 mM sodium phosphate and 150 mM NaCl, pH 8) or in 5 mM phosphate alone, followed by three further washings with 5 mM phosphate to remove EDTA (13). The resulting ghosts were resuspended in 0.5 mM sodium phosphate and incubated overnight at 4° C. Finally, the suspension was centrifuged (28 000 g x 30 min), the surnatant was removed, and the ghosts, resuspended in an equal volume of the same buffer, were passed three times through a G26 needle.

Active calcium uptake. The incubation medium was 30 mM imidazole (pH 6.8 at 37° C), 1 mM $MgCl_2$, 5 mM NaN_3, 100 mM KCl, and 0.1 mM Arsenazo III (Sigma Chemicals). Arsenazo III was previously freed of calcium ions by passage through Chelex-100 resin (Bio-Rad Laboratories). The calmodulin-activated rate was determined in the presence of excess (350 I.U.) calmodulin (Sigma Chemicals). The reaction was recorded in a dual wavelength spectrophotometer DW2A (American Instrument Company) operating at 685-675 nm, in the presence of 0.02 mM $CaCl_2$ and 0.5 mM ATP (Sigma). The amount of mem-

brane with correct sidedness was determined by the acetylcholinesterase accessibility test (14), and total protein was measured with the BCA assay (Pierce Chemical). Data are expressed as uM of calcium uptaken per min per mg protein per percentage of vesicles with correct sidedness. No correlation was found between the percentage of inside-out vesicles and RBC age, nor any effect of different calcium loads in the 2 μM to 40 μM range on the active calcium uptake rate.

RESULTS AND DISCUSSION

The active calcium uptake in the outdated, in vitro aged RBC is higher than in the fresh RBC (3.50 +/- 1.08 μM/min/mg protein, mean +/- S.D., n=7, vs 1.43 +/- 0.57, n=9). The difference is highly significant ($p<0.0005$). Excess calmodulin increases the uptake of fresh and outdated RBC by different factors (2.4 and 1.4, respectively), and the difference becomes less significant (3.48 +/- 1.94 vs 4.95 +/- 1.96, $p=0.11$, fig. 1). The in vivo aging induces changes in the calcium uptake that have the same trend as those observed for the in vitro aging, but to a lower extent (Table 1). Likely, the in vitro aged RBC is more damaged than the in vivo aged RBC for the lack of nutrients that do never limit the existence of the RBC aged in vivo in the bloodstream.

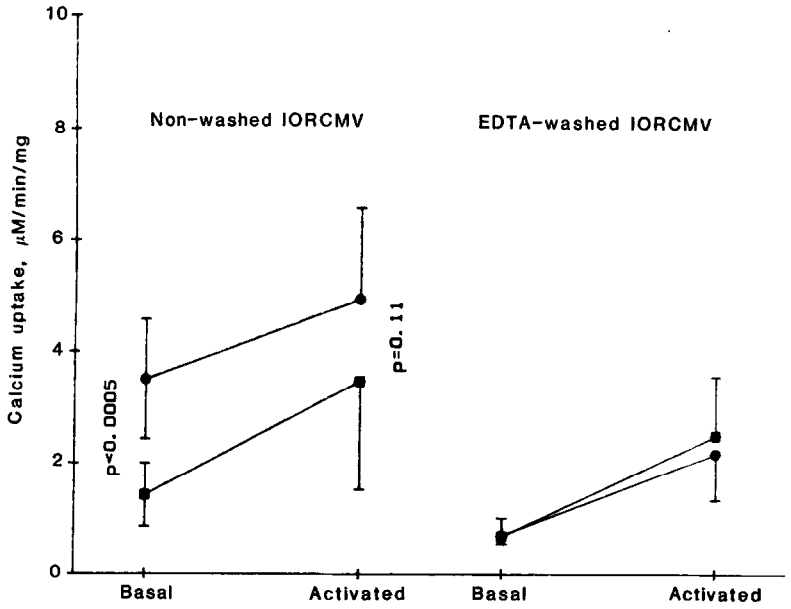

Figure 1: Calcium uptake (μM/min/mg protein, corrected for the actual percentage of IORCMV) in fresh (squares) and outdated (circles) RBC. The vertical bars represent the S.D., the significativity level (unpaired Student's t-test) is reported where appropriate.

TABLE 1

Calcium uptake (μM/min/mg protein) in IORCMV from the light and dense RBC fractions

	Light RBC		Dense RBC	
Exp.	Basal	Activated	Basal	Activated
1	1.0	3.7	1.8	4.9
2	1.2	3.2	1.6	5.4
3	0.9	2.7	1.0	3.0
mean	1.03	3.20	1.47	4.43
S.D.	0.15	0.50	0.42	1.27

The paired Student's t-test indicates that the difference light vs dense is significant at the p=0.08 level for both the basal and the activated conditions.

These data indicate that the calcium pump system is affected by the cellular aging. The pertinent literature is rather controversial because of inhomogeneity in methods and materials. Some investigators have found that Ca-ATPase is inactivated by aging (15) as most of RBC enzymes (16), and as suggested by the high sensitivity of Ca-ATPase to the oxidative damage led by activated oxygen (17) and glutathione depletion (18). Other investigators have reported that the Ca-ATPase activity is similar in the fresh and the outdated RBC, and that Ca-ATPase in the outdated RBC is activated by calmodulin more than in the fresh RBC (19). It was also reported that the calmodulin-stimulated Ca-ATPase activity of the densest RBC is non-significantly lower than that of the lightest RBC (20). Still other investigators have found that the Ca-ATPase activity is higher in the densest RBC, but have also cautioned against extreme data variability (21).

The here used IORCMV model allows a deeper investigation into the RBC aging process than hitherto reported, because the IORCMV is an integrated system composed by three components, i.e., Ca-ATPase, the activator (calmodulin) and the inhibitor, that was recently reported to be a membrane protein (22). To define the role of these components, RBC ghosts were washed with 1 mM EDTA in 5 mM phosphate to remove endogenous calmodulin and the inhibitor. The active calcium uptake of such IORCMV decreases to the same value for the fresh and the outdated RBC (0.70 +/- 0.14, n=4, vs 0.72 +/- 0.31 μM/min/mg protein,

n=5, fig. 1), regardless of the ionic strength of the washing buffer. In duplicated experiments, the active calcium uptake in IORCMV washed in isotonic phosphate-buffered saline was 0.60 and 0.69 μM/min/mg protein, for the fresh and the outdated RBC, respectively. The uptake in the presence of excess calmodulin is the same (2.52 +/- 1.06, vs 2.20 +/- 0.85 μM/min/mg protein) for both the fresh and the outdated RBC, suggesting that the aging process in RBC involves the inhibitor protein rather than the enzyme itself or calmodulin, that is always in a very large excess in human RBC (11).

The occurrence of inhibitory phenomena was confirmed by measuring the active calcium uptake of the fresh and the outdated RBC in the presence of subsaturating calmodulin, and reporting the obtained kinetic data in a double reciprocal plot (fig. 2). The inhibition is stronger for the fresh than for the outdated RBC, and is similar in the non-competitive character to that observed using purified Ca-ATPase (22). The approximate K_M value is 12 I.U./mg IORCMV.

The proteolytic phenomena led by calpain both in vivo and during the assay can not be ruled out, but the following considerations indicate that

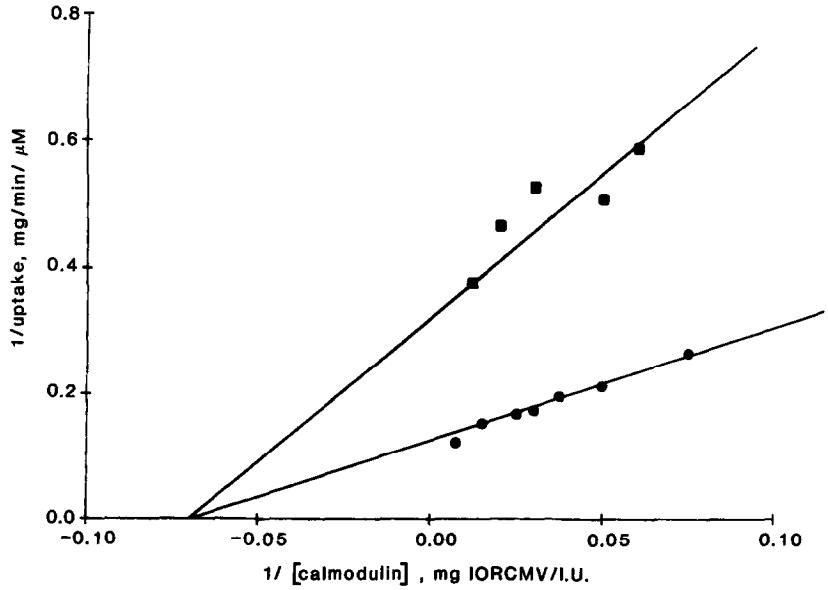

Figure 2: Double reciprocal plot for the activation by calmodulin of the fresh (squares) and the outdated RBC (circles). The lines intersect on the X-axis, indicating a non-competitive inhibition, that is stronger for the fresh than for the outdated RBC.

they are unlikely. First, calpain is activated at calcium concentrations of about 10 uM (23), and the usual intracellular free calcium is submicromolar (24). Then, the primary target of the action of calpain is hemoglobin (23), that is in a very large excess over the components of the calcium pump. In addition, calpain being a cytoplasmatic factor, it is completely removed during washings before the experiments. Finally, should some proteolytic activity be still present in the assay, then it is inconsistent with the observed straightforward linearity of the uptake of calcium for at least 10 min. Thus, we believe that the effect of calpain in this sytem is a minor one, although this possibility should be carefully evaluated.

In conclusion, it appears that the active calcium transport plays a key role during the cellular aging processes. It remains to be demonstrated whether the alteration of the calcium transport is primary or secondary to the accumulation of calcium in the cytosol. If primary, it is likely that the inhibitor of the calcium pump is deactivated by the stressing factors in the environment of the aging RBC as most of cellular enzymes. If secondary, the accumulation of calcium occurs first, and then the inhibitor is deactivated by some intracellular agent, possibly calcium itself. In both cases, it appears that the age-dependent accumulation of calcium in the cytosol depends on the cell metabolic energy depletion rather than on the calcium pump activity.

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