Control of the Erythrocyte Free Ca\(^{2+}\) Concentration in Essential Hypertension

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Since Ca\(^{2+}\) ions seem to directly participate in the control of erythrocyte membrane structure and deformability and because cell Ca\(^{2+}\) metabolism has been repeatedly proposed to be modified in hypertension, the intracellular calcium ion concentration ([Ca\(^{2+}\)]) was investigated in red blood cells from hypertensive and normotensive subjects. [Ca\(^{2+}\)], was measured by using the fluorescent Ca\(^{2+}\) chelator fura-2. Red blood cell [Ca\(^{2+}\)] was increased in hypertensive compared with normotensive subjects in the whole population and further increased when hypertensive were compared with age-matched normotensive subjects. An inverse relation between age and [Ca\(^{2+}\)] was observed when calculated with blood pressure adjusted. In hypertensive patients, high [Ca\(^{2+}\)], values were associated with a reduced erythrocyte deformability. The initial rate of \(^{45}\)Ca\(^{2+}\) uptake did not differ between the two blood pressure groups. Similarly, when the extracellular Ca\(^{2+}\) concentration was elevated from 1 to 2 mmol/l, [Ca\(^{2+}\)], increased by 16±4% (p<0.03) in red blood cells from both groups, thus maintaining a significant difference between hypertensive and normotensive subjects. Under these conditions, the addition of 10~7 mol/1 nicardipine, a dihydropyridine Ca\(^{2+}\) antagonist, decreased [Ca\(^{2+}\)], by 15±4% (p<0.05) and 7±5% in erythrocytes from hypertensive and normotensive subjects, respectively, thereby reducing the difference in [Ca\(^{2+}\)], observed between these two groups. This nicardipine effect was positively correlated to the initial [Ca\(^{2+}\)],. In the presence of 5 μmol/l W7, a calmodulin antagonist, [Ca\(^{2+}\)], increased significantly only in erythrocytes from hypertensive patients (26±6%, p<0.01). These results demonstrate that essential hypertension is associated, in the erythrocyte, with an increased cytosolic Ca\(^{2+}\) concentration, higher reduction of [Ca\(^{2+}\)], by nicardipine, and an enhanced activity of the calmodulin-dependent regulatory mechanisms. (Hypertension 1992;19:167-174)

The intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) in erythrocytes is the direct balance of both Ca\(^{2+}\) influx and Ca\(^{2+}\) pump activity with the participation of membrane and cytosolic Ca\(^{2+}\) binding proteins as an intracellular Ca\(^{2+}\) buffer. The calmodulin-stimulated Ca\(^{2+}\), Mg\(^{2+}\)-ATPase, the membrane outwardly directed Ca\(^{2+}\) pump, is the only mechanism by which these cells maintain a low intracellular Ca\(^{2+}\) concentration.1 Ca\(^{2+}\) ions participate in the control of cell membrane structure and deformability.2-4 In particular, Ca\(^{2+}\) and calmodulin directly regulate skeletal protein interactions in the erythrocyte membrane and Ca\(^{2+}\) loading induces changes in the activities of calpain and acid endopeptidases, which are responsible for the selective degradation of cytoskeletal proteins.5

At the erythrocyte level, essential hypertension is associated with abnormalities such as increases in volume, number, and aggregability; a decrease in deformability6-11; and an altered Ca\(^{2+}\) handling.12-14 The activity of the Ca\(^{2+}\) pump has been investigated by several groups. Vincenzi et al13,15 reported that, according to the anti-calmodulin agent used, trifluoperazine or the 48/80 compound, its basal activity was decreased or unchanged in hypertensive subjects. The basal activity was also found unchanged in EGTA-treated membranes, but its stimulation by calmodulin was reduced when compared with that of normotensive subjects.12,16 Essential hypertension has been also reported to be associated with decreased \(^{45}\)Ca\(^{2+}\) uptake17 and membrane Ca\(^{2+}\) binding14,18,19 and increased or decreased total intracellular calcium content, measured by flame spectrometry or flameless atomic absorption.20,21 Recently, the erythrocyte [Ca\(^{2+}\)], evaluated by nuclear magnetic resonance of a fluorinated derivative of quin-2 (2-[(2-bis[carboxymethyl]-amino-5-methylphenoxo)-methyl]-6-methoxy-8-bis[carboxymethyl]aminoquin-
were subjected to constant pressure. The intra-assay variability was 5±0.32% (six measures of each sample, n=6). Intracellular viscosity was mimicked by adding ionic strength (in mmol/l: NaCl 10, KCl 120, free Mg 0.4, glucose 10, HEPES 25, [N'-2-hydroxyethyl]piperazine-N'-(2-ethanesulfonic acid), pH 7.4 at 37°C) immediately before incubation. Erythrocytes suspended at 1% hematocrit in buffer A were incubated for 45 minutes at 37°C with 0.5 μmol/l fura 2-AM. They were then diluted to 0.1% hematocrit in buffer A containing 1 mmol/l MnCl₂ to quench the extracellular fura-2 signal and excitation spectra immediately recorded on a Spex fluorolog CM111 equipped with a 450 W xenon lamp (Instrument SA; Jobin Yvon, Longjumeau, France). Excitation measurements corrected with rhodamine 6G quantum counter were performed with an excitation bandwidth of 1.8 nm at 510 nm emission wavelength and 9.0 nm bandwidth. All the experiments were performed at 37°C in quartz suprasil microcuvettes with optical pathways of 1 and 5 mm. Free Ca²⁺ concentrations were calculated according to the equation established by Poenie et al²⁸:

\[ [\text{Ca}^2+] = K \times \frac{(R - R_0)}{(R_s - R)} \]

with

\[ K = K_a \times (\text{Fo}/\text{Fs})^{385} \]

where R is the ratio of the fluorescence intensities measured at 335 and 385 nm, Ro is the same ratio in the absence of Ca²⁺, Rs is the same ratio when saturated with Ca²⁺, K is the dissociation constant of fura-2-calcium complex, and Fo and Fs are the fluorescence intensities with zero and saturating Ca²⁺, respectively, measured at a 385 nm excitation wavelength.

The parameters (Ro, Rs, K) were determined from fura-2-calcium fluorescence calibration experiments performed at 37°C with EGTA-calcium buffers, similar to the intracellular medium in terms of pH (7.2), ionic strength (in mmol/l: NaCl 10, KCl 120, free Mg²⁺ 0.4, glucose 10, HEPES 25, and variable Ca²⁺ concentrations ranging from 1 nmol/l to 20 μmol/l), and viscosity (14 mPa·s/sec). Under these conditions, Ro=0.88±0.04, Rs=13.2±3.1, and K=1.0±0.2 μM (n=6). Intracellular viscosity was mimicked by add-
ing 21 g/l polyvinylpyrrolidone to the incubation medium to produce a viscosity of 14 mPa s at 37°C, similar to that described in erythrocytes. The intracellular fura-2 concentration was similar in the two blood pressure groups (2.8±0.3 and 2.5±0.2 \cdot 10^{-5} \text{ mol/l} \text{ in the normotensive and hypertensive groups, respectively). As observed previously in normotensive subjects, [Ca^{2+}]_i values were observed to be stable between 15 and 90 minutes. The kinetics of fura 2-AM uptake and de-esterification were not markedly different in hypertensive patients since [Ca^{2+}]_i values were stable between 30 and 90 minutes (92.2±6.6 versus 86.2±6.7 nmol/l, respectively). An incubation time of 45 minutes was used for [Ca^{2+}]_i determinations. The de-esterification of the dye was completed under these conditions since the spectral characteristics of lysed cells (50 \mu l fura-2-loaded cells in 450 \mu l bidistilled water) were similar to those of fura-2 in the presence of saturating Ca^{2+} concentration (maximal intensity at 336 nm, Rs=34±8, n=5).

The interassay and intra-assay variabilities were 6.3±2.1% and 5.8±2.0% (n=6–8), respectively. All determinations were performed at least in duplicate.

**Determination of \text{^{45}Ca^{2+} Influx in Intact Red Blood Cells**

\text{Ca^{2+} influx experiments were performed according to a method derived from that described by Waller et al.}\text{\cite{30}. Packed erythrocytes isolated as described above were washed twice by centrifugation for 5 minutes at 1,500g and 4°C in nine volumes of physiological saline, twice in nine volumes of buffer A without Ca^{2+} (buffer B), and then suspended in the same buffer at 20–30% hematocrit. After incubation for 30 minutes at 37°C, the experiment was initiated by addition of CaCl2 and \text{^{45}Ca^{2+} (Amersham, Les Ulis, France) at final concentrations of 1 mmol/l and 5 \mu Ci/ml, respectively. Aliquots (0.1 ml) of cell suspension were withdrawn as a function of time, diluted with 0.9 ml buffer B at 0°C, and centrifuged for 5 seconds at 12,000 g in an Eppendorf microcentrifuge. The supernatants were discarded, and the pellets were suspended in 1 ml buffer B and centrifuged as above. This washing procedure was repeated twice. The whole procedure took less than 5 minutes. Erythrocyte pellets were then lysed by successive additions of 0.5 ml bidistilled water and 0.5 ml of 5% (wt/vol) trichloroacetic acid under vigorous agitation. After centrifugation for 2 minutes at 12,000g, 0.9 ml supernatant was diluted in 5 ml picofluor (Packard, Downers Grove, Ill.) and \text{^{45}Ca was counted.}

To check that the accumulated [Ca^{2+}]_i indeed represented an intracellular uptake and not a binding, erythrocytes were first Ca^{2+} loaded in the presence of 1 mmol/l CaCl2 and 25 \mu Ci/ml \text{^{45}Ca^{2+}, washed twice in medium B, and resuspended in this medium at the same hematocrit as initially. Then 2 \mu mol/l ionomycin (Calbiochem, San Diego, Calif.) was added, and 0.1-ml aliquots withdrawn as a function of time were treated as described above. For each donor, uptake experiments were performed at least in duplicate.

**Statistical Analysis**

Results are expressed as mean±SEM. The number of independent experiments is indicated by n. The significance of the differences between the groups was assessed by two-tailed unpaired or paired Student's t tests as indicated. Correlation coefficients between parameters were calculated by linear regression. \text{Ca^{2+} uptake initial rates were calculated by computerized polynomial analysis.}

**Results**

**Intracellular Free \text{Ca^{2+} Concentration in Red Blood Cells From Hypertensive Patients and Normotensive Subjects**

[Ca^{2+}], was determined in fura-2–loaded red blood cells from 50 untreated hypertensive patients and 37 normotensive subjects. The mean [Ca^{2+}] of hypertensive patients was significantly higher than that of the normotensive subjects (85±2 versus 77±2 nmol/l, \text{respectively). As observed previously in normotensive subjects, [Ca^{2+}] values were observed to be stable between 15 and 90 minutes. The kinetics of fura 2-AM uptake and de-esterification were not markedly different in hypertensive patients since [Ca^{2+}] values were stable between 30 and 90 minutes (92.2±6.6 versus 86.2±6.7 nmol/l, respectively) An incubation time of 45 minutes was used for [Ca^{2+}] determinations. The de-esterification of the dye was completed under these conditions since the spectral characteristics of lysed cells (50 \mu l fura-2-loaded cells in 450 \mu l bidistilled water) were similar to those of fura-2 in the presence of saturating Ca^{2+} concentration (maximal intensity at 336 nm, Rs=34±8, n=5).

The interassay and intra-assay variabilities were 6.3±2.1% and 5.8±2.0% (n=6–8), respectively. All determinations were performed at least in duplicate.
p<0.01). In the whole population, [Ca\textsuperscript{2+}] values did not correlate with age (r=-0.062), with blood pressure (r=0.124), or with BMI (r=0.015) (Figure 1). However, when the correlation between [Ca\textsuperscript{2+}], and age was calculated with blood pressure adjusted, it became significant (r=-0.202, p=0.05). Similarly, when the correlation between [Ca\textsuperscript{2+}], and blood pressure was calculated with age adjusted, it reached significance (r=0.239, p<0.05). In contrast, [Ca\textsuperscript{2+}] did not correlate with BMI when calculated with blood pressure or age adjusted (−0.035 or 0.035, respectively).

When data were analyzed separately for each sex, [Ca\textsuperscript{2+}], remained similarly correlated with age (n=59, r=0.300, p<0.05 with blood pressure adjusted) and mean blood pressure (r=0.441, p<0.01 with age adjusted) in men but not in women. When only male hypertensive patients were considered, a clear inverse relation between age and erythrocyte [Ca\textsuperscript{2+}] was observed (n=30, r=−0.584, p<0.001). It remained valid when calculated with blood pressure adjusted (r=0.634, p<0.001).

Since erythrocyte [Ca\textsuperscript{2+}], seemed to be influenced by two factors, age and blood pressure, it was also compared in age-matched normotensive and hypertensive subgroups. As indicated in Table 1, a clear difference in [Ca\textsuperscript{2+}], between hypertensive patients and normotensive subjects was obtained (p<0.001). It was more marked in men than in women. In male subjects only, erythrocyte [Ca\textsuperscript{2+}], was positively correlated to mean blood pressure (n=29, r=0.441, p<0.01) (Figure 2).

**Rheological Parameters**

Since Ca\textsuperscript{2+} ions participate in the control of membrane structure and deformability,\textsuperscript{2–5} the rigidity index of red blood cells was measured in 30 hypertensive patients (17 men and 13 women). Its values ranged between 4.7 and 12.8 with a mean value of 8.6±0.6 AU. The individual values were positively correlated with those of [Ca\textsuperscript{2+}], measured in parallel (r=0.373, n=30, p=0.042).

**Variations in Ca\textsuperscript{2+} Influx and Calmodulin Activity in Erythrocytes From Hypertensive and Normotensive Subjects**

To investigate the respective roles of Ca\textsuperscript{2+} influx and calmodulin in the high [Ca\textsuperscript{2+}], observed in erythrocytes from hypertensive and normotensive subjects.
RBCs from hypertensive patients, [Ca\(^{2+}\)], was measured under various experimental conditions. These experiments were performed with 12 hypertensive patients (eight men and four women; mean age, 50±3 years) and nine normotensive subjects (five men and four women; mean age, 46±2 years. These two age-matched subgroups were representative of the age-matched groups studied above in terms of mean blood pressure and [Ca\(^{2+}\)].

**Influence of Ca\(^{2+}\) influx.** To evaluate the participation of Ca\(^{2+}\) influx in the increase of [Ca\(^{2+}\)], observed in RBCs from hypertensive patients, the initial rate of 45Ca\(^{2+}\) uptake was measured in intact red blood cells (Figure 3). The addition of ionomycin, a Ca\(^{2+}\) ionophore, induced a rapid and total release of the accumulated 45Ca\(^{2+}\), thereby confirming that we were dealing with intracellular uptake and not with membrane binding. As illustrated in Figure 4, the initial rate of 45Ca\(^{2+}\) uptake did not differ between hypertensive and normotensive subjects (45±6 and 47±5 μmol/l cells/hr, respectively).

The influence of a rise in extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) on [Ca\(^{2+}\)] was also evaluated in hypertensive and normotensive subjects. When [Ca\(^{2+}\)]\(_o\) was increased from 1 to 2 mmol/l, [Ca\(^{2+}\)], increased significantly by 17±7% (from 84±4 to 97±4 mmol/l, p<0.023) and by 15±5% (from 71±2 to 81±4 mmol/l, p=0.028 by paired t tests) in RBCs from hypertensive and normotensive subjects, respectively (Figure 5). In the presence of 2 mmol/l extracellular Ca\(^{2+}\), the difference in [Ca\(^{2+}\)], values between hypertensive and normotensive subjects was maintained, (97±4 versus 81±4 mmol/l, respectively, p<0.01 by unpaired t test). The addition of 10\(^{-7}\) mol/l nicardipine, a dihydropyridine Ca\(^{2+}\) antagonist, decreased [Ca\(^{2+}\)], by 15±4% (from 97±4 to 83±3 mmol/l, p=0.013) in RBCs from hypertensive patients and by 7±5% (from 81±4 to 74±3 mmol/l) in those from normotensive subjects (Figure 5). The nicardipine-dependent decrease in [Ca\(^{2+}\)], was positively correlated to the initial [Ca\(^{2+}\)] value (r=0.65, p<0.01, n=21) (Figure 6) indicating that the higher the [Ca\(^{2+}\)], was, the larger the nicardipine effect. In spite of the low number of subjects, this correlation was also significant when results from each blood pressure group were analyzed separately (r=0.62 and 0.65, respectively, p<0.05 for both groups). This suggests that the nonsignificant effect obtained in normotensive subjects was related to their low initial [Ca\(^{2+}\)] values.

**Effect of the calmodulin antagonist W7 on [Ca\(^{2+}\)].** Since the activity of the Ca\(^{2+}\)-pump is regulated by the Ca\(^{2+}\)-calmodulin complex, the role of calmodulin in the high [Ca\(^{2+}\)], observed in RBCs from hypertensive subjects was evaluated by measuring [Ca\(^{2+}\)], in the presence of W7 [N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride], a calmodulin antagonist. W7 is reported to affect the Ca\(^{2+}\), induced conformational change of calmodulin, thus preventing its interaction with the target enzyme.\(^{31}\)

In the presence of 5 μmol/l W7, [Ca\(^{2+}\)], was enhanced significantly by 26±6% (from 84±4 to 101±5 nmol/l, p<0.01) in RBCs from hypertensive patients, whereas it increased slightly but not signif-
concentration in the erythrocyte varies
free Ca
neutral nuclear magnetic resonance. et al with chelator fura-2 or that were determined by Resnick trations that we measured with the fluorescent Ca
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selective electrode, reported an increase of [Ca
i in erythrocytes from hypertensive patients.
using a whereas Zidek et al, 21 20 total calcium content, determinations were performed as described in "Methods." Erythrocytes were first incubated 10 minutes at 37 °C with or without 10-7 mol/l nicardipine (Ni), then 10 minutes with 1 or 2 mM CaCl, *p<0.05 by two-tailed paired Student's t test for values measured in the presence of 2 mM extracellular Ca
compared with those obtained with 1 mmol/l extracellular Ca
*p<0.05 by two-tailed paired Student's t test for values measured in the presence of nicardipine compared with those obtained in its absence. **p<0.01 by two-tailed unpaired Student's t test for values from hypertensive patients compared with those from normotensive subjects.

Figure 6. Scatterplot shows influence of the initial intracellular Ca
concentration ([Ca
i] level on the nicardipine-dependent decrease of [Ca
i], in hypertensive (●) and normotensive (○) subjects. Nicardipine-dependent decreases are expressed as percent of the control [Ca
i], values measured in the absence of nicardipine.

Discussion
Numerous abnormalities of Ca
metabolism have been reported to be associated with essential hypertension. In particular, altered Ca
handling has been described in various blood cells.12-23,35

In erythrocytes, the presence of hemoglobin has hampered until recently the investigation of [Ca
i] by fluorescent indicators in hypertension.22 Engelmann and Duhm,26 using flameless atomic absorption, observed a slight but not significant reduction of total calcium content,21 whereas Zidek et al,29 using a Ca
selective electrode, reported an increase of [Ca
i] in erythrocytes from hypertensive patients. However, the Ca
concentrations were in the 1-10 mmol/l range, values similar to the total calcium contents determined by Engelmann and Duhm23 and much higher than the intracellular free Ca
concentrations that we measured with the fluorescent Ca
chelator fura-2 or that were determined by Resnick et al with 39F-nuclear magnetic resonance.23

The present study demonstrates that the cytosolic free Ca
concentration in the erythrocyte varies significantly (from 71±2 to 84±7 nmol/l) in normotensive subjects (Figure 7).

In hypertensive patients compared with those from normotensive subjects. Cell preparation and [Ca
], determinations were performed as described in "Methods." Erythrocytes were first incubated 10 minutes at 37 °C with or without 10-7 mol/l nicardipine (Ni), then 10 minutes with 1 or 2 mM CaCl, *p<0.05 by two-tailed paired Student's t test for values measured in the presence of 2 mM extracellular Ca
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The present study demonstrates that the cytosolic free Ca
concentration in the erythrocyte varies with two factors, age and blood pressure. It was increased in erythrocytes from hypertensive patients compared with normotensive subjects of the whole population. It further increased when hypertensive patients were compared with age-matched normotensive subjects. In this subpopulation, [Ca
i], correlated positively with mean blood pressure. For the whole population studied, erythrocyte [Ca
i], and mean blood pressure correlated positively only when calculated with age adjusted. The influence of age on [Ca
i], was indicated by the observation of a negative correlation between age and [Ca
i], when calculated with blood pressure adjusted. This was also observed when only data from male subjects were considered. The absence of correlation between [Ca
i], and blood pressure or age in women may be due to the small number of subjects studied or to the influence of additional factors such as hormonal variations. The effect of age seemed to be more marked in male hypertensive patients than in normotensive subjects. Previous results have demonstrated that, in spite of the decreased erythrocyte fluidity and the increased aggregation tendency observed in hypertensive patients, there was only in these patients an inverse relation between age and aggregation tendency. Tsuda and Masuyama,38 with use of the electron spin resonance method, have also observed a relation between age and Ca
-induced changes in erythrocyte membrane fluidity when only hypertensive patients were considered.

Erythrocyte Ca
content has been reported to participate in the control of cell deformability, which is reduced by calcium load23 and improved by Ca
antagonists.25 The erythrocyte rigidity indexes measured in this study in patients with moderate hypertension were similar to those previously determined using the same methodology.11 In the patients of the
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present study, the indexes were found to positively correlate with the cytosolic free Ca\(^{2+}\) concentration. This supports the role of Ca\(^{2+}\) ions in the control of erythrocyte deformability and indicates the physiological relevance of the [Ca\(^{2+}\)] measurements.

In age-matched populations, the increase of [Ca\(^{2+}\)], averaged 24%, which is in the same range as that described in blood platelets.** Its presence in erythrocytes argues for a major role of the plasma membrane in the altered Ca\(^{2+}\) handling observed in essential hypertension. In this cell, [Ca\(^{2+}\)], is only regulated by two plasma membrane mechanisms, Ca\(^{2+}\) influx and extruding pump.

The initial rates of Ca\(^{2+}\) influx measured by our method were in the same range as those determined previously in quin-2–loaded erythrocytes by others, thus indicating a real Ca\(^{2+}\) influx without significant participation of Ca\(^{2+}\) efflux. The initial rate of \(\text{Ca}^{2+}\) uptake did not differ between erythrocytes from hypertensive and normotensive subjects, in accordance with the previous study of Ronquist and Frithz, who observed that the fast component of \(\text{Ca}^{2+}\) uptake was similar in the two groups. A rise of the extracellular Ca\(^{2+}\) concentration from 1 to 2 mmol/l, which has previously been described to nearly double the initial rate of \(\text{Ca}^{2+}\) uptake and to increase [Ca\(^{2+}\)], observed measured in its absence. *p<0.05, **p<0.01 by two-tailed unpaired Student’s t test for values from hypertensive patients compared with those from normotensive subjects.

A modified Ca\(^{2+}\)-extruding pump activity, as assessed by a decreased degree of calmodulin stimulation, has been reported in both erythrocytes and platelets from essential hypertensive patients. In this study, we observed that the presence of a calmodulin antagonist increased further the level of [Ca\(^{2+}\)], in erythrocytes from hypertensive patients. This might reflect either a higher sensitivity of the calmodulin-calmodulin pump complex to this antagonist or a higher stimulation of the Ca\(^{2+}\) pump by the calcium-calmodulin complex in hypertensive patients. This last hypothesis is more likely since this may reflect a higher activity of the Ca\(^{2+}\) pump in hypertensive patients than in normotensive subjects in response to the increase in [Ca\(^{2+}\)]. This may suggest that the Ca\(^{2+}\) pump cannot reestablish normal [Ca\(^{2+}\)], levels in hypertensive patients.

In conclusion, we demonstrate with the fluorescent calcium chelator fura-2 that the cytosolic Ca\(^{2+}\) concentration is increased in erythrocytes from essential hypertensive patients. This increase in [Ca\(^{2+}\)], is associated with an increased nicardipine-sensitive effect and an altered function of calmodulin in the erythrocyte Ca\(^{2+}\) handling.

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References


**KEY WORDS** • calcium • red blood cells • erythrocyte deformability • calmodulin • fluoroscence dyes • essential hypertension
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