Clinical and Experimental Pharmacology and Physiology (1989) **16**, 387–394

CALCIUM CONTENT OF THE ERYTHROCYTES: A SENSITIVE AND EASY HANDLING METHOD FOR MEASURING FREE CALCIUM IONS, AND MODULATION OF THE Ca^{2+} ION CONCENTRATION BY THE CALCIUM ANTAGONISTS NIFEDIPINE AND PENTOXIFYLLINE

E. Friederichs,[1,2] T. Rädisch[1] and H. Winkler[2]

[1]*Department of Pediatrics, University of Göttingen, Federal Republic of Germany* [2]*Max-Planck-Institute for Biophysical Chemistry, Göttingen, Federal Republic of Germany*

(Received 3 November 1988; revision received 3 January 1989)

SUMMARY

1. A method for determining free Ca^{2+}-ions in the erythrocyte is described, using a commercially available ORION-Ca-electrode and calomel reference electrode assembly, where changes in free Ca^{2+}-ion concentration upon addition of 0.01% digitonin could be measured.

2. The average value found for fresh cells from 20 healthy donors at 37°C (pH = 7.4) was 0.20 ± 0.04 μmol/L referred to a haematocrit of 10%.

3. Decrease of the simultaneously determined adenosinetriphosphate (ATP) concentration indicates that ATP is presumably needed to activate the Ca-ATPase.

4. *In vitro* addition of the calcium antagonists pentoxifylline and nifidepine, respectively, induced a normalization of the intraerythrocytic Ca^{2+}-ion concentration after previous increase with the ion carrier ionophore A23187.

5. The advantages and possible clinical applications of this method are discussed.

Key words: calcium antagonist, erythrocytes, intracellular calcium, ionophore.

INTRODUCTION

Ca^{2+}-ions play an important role in several basic physiological functions, such as neuromuscular conditions, bone formation and preservation of cell membrane permeability. Its concentrations reflect the functional state of calcium and are a good indicator of the clinical condition. The relative distribution of ionized Ca^{2+}, complexed Ca^{2+} and protein-bound Ca^{2+} in plasma is altered by many factors, for example pH and changes in plasma protein concentrations. Increase of plasma proteins leads to an increase of the total calcium concentration, however, the ionized Ca^{2+} level may remain in the normal range. Decrease of plasma proteins consequently leads to a diminished total calcium level, but does not influence the ionized Ca^{2+} which still remains in the same order of magnitude.

Correspondence: Dr med. E. Friederichs, Department of Physiology and Biophysics, University of Southern California Medical School, 1333 San Pablo Street, Los Angeles, CA 90033, USA.

Much is already known about the total calcium level inside the red blood cell, but the regulation of intra-erythrocytic ionized Ca^{2+}-ion concentration is still an object of investigation. A variety of methods can measure the total calcium concentration (Bolibrohe *et al.* 1959; Valberg *et al.* 1964; Harrison & Long 1968). It would be more useful, however, to measure the free Ca^{2+}-ion concentration, particularly as inside the cells it might be the more important variable. The present work describes how Ca^{2+}-ion concentration was monitored with a commercially available CALCIUM electrode. The influence of temperature and the effects of the possible calcium antagonists nifedipine and pentoxifylline on the Ca^{2+}-ion concentration were investigated.

Already low concentrations of the free Ca^{2+}-ion inside the cell are recognized by a Ca-ATPase described by Schatzmann and Bürgin (1978), which in turn is regulated by the energy rich phosphate ATP. The concentration of this substance was determined and correlated with the Ca^{2+}-ion levels within the red blood cell.

METHODS

Blood was obtained from healthy blood donors and anticoagulated with heparine (8 iu/mL blood). Fresh blood cells (10 mL) were isolated by centrifugation in a refrigerated centrifuge (3000 r/min, 10 min, 4°C) and resuspension in a 310 mosm EDTA-phosphate buffer to obtain a reasonable low basal extracellular calcium level. To remove EDTA this solution was washed 3–5 times with normal 310 mosm phosphate buffer. Composition of the phosphate buffer: 822 mL 0.103 mol/L $Na_2HPO_4{}^*2H_2O$ and 178 mL 0.155 mol/L $NaH_2PO_4{}^*H_2O$ adjusted to pH 7.4.

Subsequently, the suspension of washed erythrocytes was normally suspended at 10% haematocrit in a medium containing 50 mmol/L HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethansulfonic acid), 100 mmol/L $MgCl_2$, 100 mmol/L sucrose and 50 mmol/L KCl where the pH 7.4 ± 0.1 at 37.0 ± 0.2°C was adjusted with 0.1 mol/L NaOH or HCl respectively. Ca^{2+}-ion measurements were taken by means of an ORION 93-20-01 CALCIUM electrode, and a reference electrode filled with 4 mmol/L KCl saturated with $AgCl_3$ both connected to an ORION 811 pH/mV-meter. Samples, usually 8 mL, were thermostated in a 37°C water bath and stirred with a magnetic stirrer. After temperature and pH equilibration the cell suspension was haemolyzed by addition of 0.01% digitonin. The potential before and after haemolysis was measured, the difference is directly proportional to the intracellular Ca^{2+}-ion concentration and gives an upper estimate of the intracellular Ca^{2+}-ion concentration.

Theory As has already been shown by Ferreira and Lew (1976) red cell cytoplasm buffers in a linear fashion which means that it behaves like a solution of a low affinity and high capacity buffer. With regard to the theoretical evaluations of Simons (1982) and Ferreira and Lew (1976) the change in external free calcium after haemolysis with digitonin is:

$$Ca'_{out} - Ca_{out} = h(Ca_{in} - Ca_{out})/(\alpha + h(1 - \alpha))$$

where Ca'_{out} = external free calcium after lysis, Ca_{out} = external free calcium before lysis, Ca_{in} = internal free calcium, h = haematocrit, and α = ionized fraction of calcium.

As already demonstrated by Simons (1982) intracellular calcium buffers become less effective in buffering calcium after haemolysis when the Mg^{2+} concentration is raised. In contrast to Simons we used a hypertonic medium with a $MgCl_2$ concentration of 100 mmol/L. It does not

significantly alter the calcium concentration (Simons 1982) and allows the simplification of the above equation to:

$$Ca'_{out} - Ca_{out} = h(Ca_{in} - Ca_{out}).$$

All of the cytoplasmic buffers are assumed to be bound by Mg^{2+} which results in a value of $\alpha \approx 1$.

The resolution of this equation to Ca_{in} gives:

$$hCa_{in} = Ca'_{out} - Ca_{out}\ (1 - h).$$

At low haematocrit as used in our measurements this gives a first approximation of:

$$Ca_{in} \approx (Ca'_{out} - Ca_{out})/h.$$

The difference in the electrode potential is, in first approximation, proportional to the difference in Ca^{2+}-ion concentration referred to the haematocrit.

Electrode calibration Three solutions with standardized Ca^{2+}-ion concentrations (0.1, 1.0, 10.0 mmol/L) were routinely used for calibrating the ionmeter. The fit to the equation $E\ (mV) = a + b \times p_{ca}$ ($p_{ca} = (-\log_{10}(Ca) = 4, 3, 2$ respectively) should result in a slope of 27 ± 2 mV indicating an optimal electrode function. According to the Nernst equation the activity of a cation transported is given by:

$$E = RT/ZF \ln C_1/C_2$$

where $RT/ZF = 29.58$ mV is the slope, C_1, C_2 are the activities of the transported cations in the two compartments separated by the erythrocyte membrane (R = gas constant, T = Temperature, Z = charge, F = Faraday constant = 96 500 C/mol). The slope found experimentally is in good agreement with the theoretical value of the Nernst equation (Simon & Carafoli 1979).

Calibration in the low concentration range In each experiment the electrode was first calibrated with the calibration standards to correct for the electrode drift. Calibration in the low concentration range was performed in a medium containing 50 mmol/L HEPES, 100 mmol/L $MgCl_2$, 100 mmol/L sucrose and 50 mmol/L KCl. To this medium, also used in the original Ca^{2+} measurements, subsequently were added 10 standardized Ca^{2+}-ion solutions (0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 µmol/L).

The determination of the difference in the electrode potential after successive addition of the Ca^{2+}-ion solutions results in a difference calibration curve ΔE (mV) as a function of ΔCa^{2+} (µmol/L). Contamination of the medium by calcium could thus be ignored.

Neutral ionophores The ionophore-cation complex allows the transfer of charge through the biological membrane (Pressman 1976; Simon & Carafoli 1979). The cell suspension was incubated with 25 µmol/L ionophore (0.5 h, 37°C, pH = 7.4) thus increasing the intracellular Ca^{2+}-ion concentration. Ionophore (A23187) could be removed by washing the blood solution with albumin (250 mg/dL)/phosphate buffer (composition described earlier). ATP concentration was determined according to Bücher (1947). Denaturation of the proteins was performed

immediately after obtaining the blood sample and determination of ATP had to be performed within the next hour in order to get reproducible results.

Erythrocyte suspensions were incubated by Nifedipine (Adalat, Bayer) and pentoxifylline (Trental, Hoechst), respectively, over a period of 30 min at 37°C.

RESULTS

Figure 1 shows the potential difference ΔE as a function of the Ca^{2+}-ion concentration difference ΔCa^{2+} in the low level range. This allows directly to read off the essential Ca^{2+}-ion concentration after haemolysis of the erythrocytes which results in a difference in the electrode potential. For our purpose a linear fit can be drawn between 0.1 μmol/L up to 1.0 μmol/L which is in the range of the expected intra-erythrocytic Ca^{2+}-ion concentration. At lower concentrations (< 0.1 μmol/L) this linear approximation cannot be used anymore. Figure 1 shows some linear fits with varying $MgCl_2$ concentrations in our standard solution. The optimal concentration seems to be 100 mmol/L as linearity could be observed down to 0.1 μmol/L Ca^{2+} and sufficient sensitivity could be achieved, as represented by the slope. The mean value of the Ca^{2+}-ion concentration in the erythrocyte from 20 healthy blood donors is given by 0.20 ± 0.04 μmol/L referred to a haematocrit of 10%.

The temperature dependence of the intra-erythrocytic free Ca^{2+}-ion concentration from different blood donors at pH = 7.4 is demonstrated in Fig. 2. In most of the cases the Ca^{2+}-ion concentration increases with higher temperatures. Figure 3 gives the result of an experiment where erythrocytes were loaded with Ca^{2+}-ions by means of the ion carrier ionophore A23187. The Ca^{2+}-ion concentration increases from 0.20 μmol/L at 0 μmol/L ionophore to 0.66 μmol/L when the ionophore concentration is 50 μmol/L following a 'classical' saturation function. There was no difference in this behaviour when blood was incubated with ionophore over a period of 0.5 h or 1 h (not shown). At the same time levels of the energy-rich phosphate ATP were measured. The increase in Ca^{2+}-ion concentration causes a decrease of the ATP concentration, indicating that ATP presumably is needed to activate the Ca-ATPase (Fig. 3).

Table 1 shows the calcium antagonizing effects of pentoxifylline and nifedipine. A decrease in Ca^{2+}-ion concentration could be achieved by incubation with increasing concentrations of

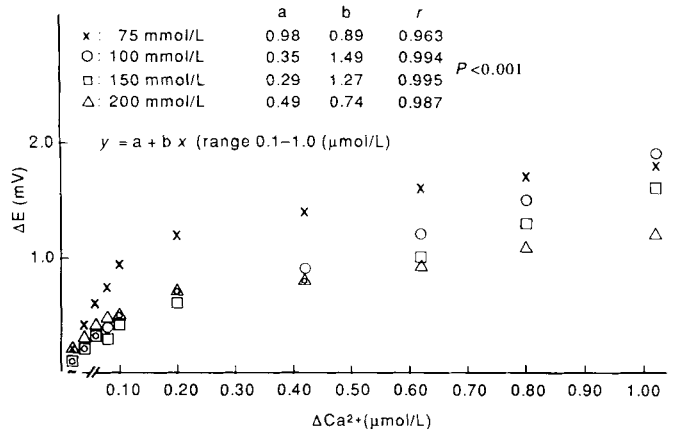

Fig. 1. Calibration of the Ca electrode at 37°C. Solution contained 50 mmol/L HEPES, 100 mmol/L sucrose, 50 mmol/L $MgCl_2$ and Ca^{2+}-ions in increasing concentrations. $MgCl_2$ was chosen in different concentrations and is represented by the different plots.

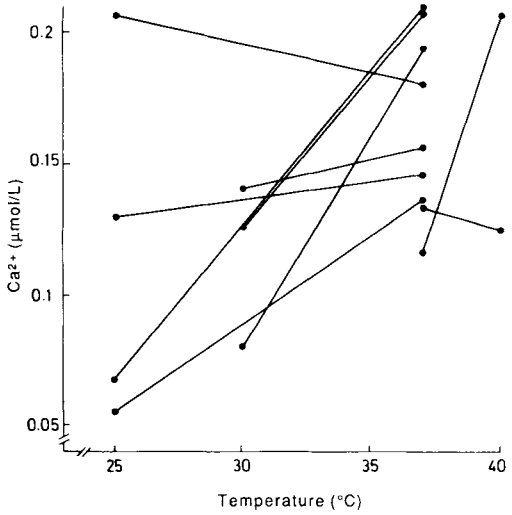

Fig. 2. Relationship between intra-erythrocytic free Ca^{2+}-ion concentration and varying temperature.

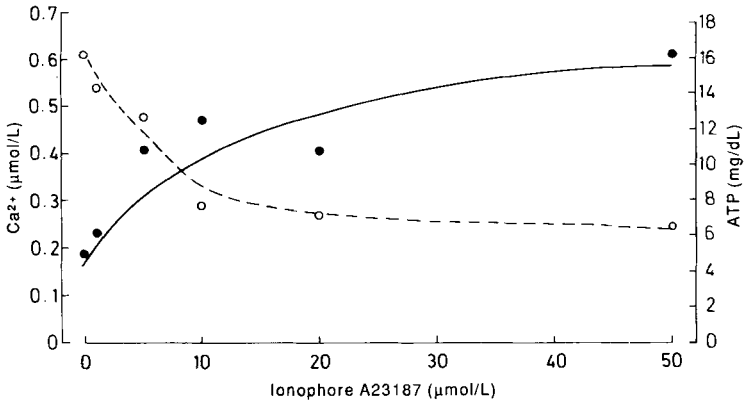

Fig. 3. Loading of the erythrocytes with Ca^{2+}-ions by means of the ion carrier ionophore A23187 (•) and influence on the ATP concentration (o).

these two substances. Previous addition of the ion carrier ionophore (50 µmol/L) increased the Ca^{2+} -ion concentration.

DISCUSSION

Red blood cells from adult humans are able to keep the calcium concentration in the cytosol several orders of magnitude below that in blood plasma. Most of the measurements of calcium concentrations reported so far are concerned with the total calcium content of the cell (Bolibrohe & Maizels 1959; Valberg *et al.* 1964; Harrison & Long 1968). Harrison and Long (1968) for example found an average of 16 µmol/L total calcium.

The use of Ca-chelators (Lew *et al.* 1982) is aggravated by the fact that due to the intense pigmentation of the red cells Ca^{2+} -ions could not be monitored spectrophotometrically from the spectral shifts as can be done in transparent cells. Recently David-Dufilho *et al.* (1988) described

Table 1. Ca^{2+}-ion concentration as a function of increasing concentrations of pentoxifylline and nifedipine, respectively (0.5 h, 37°C). Ca^{2+}-ion concentration inside the red blood cell could be increased by incubation with the ion carrier ionophore A23187 (0.5 h, 37°C)

	Concentration Ca^{2+} (μmol/L)
Nifedipine (μg/mL)	
0	0.20
0*	0.66
0.2*	0.36
0.5*	0.32
1.0*	0.22
2.0*	0.22
Pentoxifylline (μg/mL)	
0	0.20
0*	0.66
2.5*	0.30
5.0*	0.25
10.0*	0.24
20.0*	0.21

*Nifedipine plus 50 μmol/L ionophore A23187.

fluorescence measurements of free Ca^{2+} using the Ca^{2+} -indicator fura-2 which must still take into account the cytosol viscosity. Only a few authors used a calcium electrode to measure the ionized Ca^{2+} within the erythrocyte (Simons 1982; Kratje *et al.* 1983). In good agreement with the results of Simons we measured a Ca^{2+} -ion level of 0.20 ± 0.04 μmol/L (Fig. 2). This author used a null-point method for estimating intracellular Ca^{2+} -ions. By adjusting the external free Ca^{2+} -ion with low concentration of EDTA a null point could be obtained where there is no change in free Ca^{2+} -ions upon addition of digitonin. Simons (1982) got a Ca^{2+} -ion concentration value of 0.45 ± 0.13 μmol/L, where 0.40 μmol/L was regarded to be the upper limit due to experimental errors. These and our own measurements are in agreement with the simple assumption that digitonin haemolyses the cells thus causing a mixing of intracellular and extracellular Ca^{2+} -ion levels. Hence in our experiments we measured the difference between the Ca^{2+} -ion concentration before and after haemolysis using a hypertonic medium with 100 mmol/L $MgCl_2$. If one assumes that all cytoplasmatic buffering substances are bound by Mg^{2+}, the change in free calcium after haemolysis is due to the intracellular Ca^{2+} -ion concentration. The measured concentration of Ca^{2+}-ions might still be too high as it can include some tightly bound calcium from the membrane which possibly becomes released by digitonin. It should be noted that the calcium electrode does not always display a linear response down to 10^{-7} mol/L Ca^{2+} as it does in well buffered solution, leading to an overestimation of our results, too. This might partly explain the difference between our results and recently published lower calcium values (0.061 μmol/L) using magnetic resonance techniques (Murphy *et al.* 1986). Depending on cell and stimulus, the cytosolic Ca^{2+}-ion concentration may increase either as a consequence of an inflow of extracellular Ca^{2+}-ions or if Ca^{2+}-ions are released from membrane stores. Red cell calcium is known to be compartmentalized in endocytic inside-out vesicles of sickle cells (Lew *et al.* 1985; Rubin *et al.* 1985). If these vesicles are ruptured, at least a portion of this internally sequestered Ca^{2+} would be released into the cytoplasma of the cells. Already low concentrations of Ca^{2+}-ions are recognized by the membrane bound Ca-ATPase. The negative correlations between ATP concentration and Ca^{2+}-ion levels (Fig. 3) in our experiments could lead to the

hypothesis that ATP is used for the activation of the Ca-ATPase to maintain a normal Ca^{2+}-ion gradient (Schatzmann & Bürgin 1978).

From our results it is evident that nifedipine and pentoxifylline, respectively, lowered the intracellular Ca^{2+}-ion concentration in the erythrocytes. Three effects can be discussed: i) the calcium influx might be lowered through red blood cell calcium channels, ii) an activation of the calcium extruding pump might be induced by these two substances, iii) the drugs might affect inside-out vesicles. The sites of action of pentoxifylline and nifedipine are not known at this point and the results of the present study do not allow a discussion of the exact mechanism. Further investigations are required for example by using calcium influx–efflux techniques recently described (McNamara & Wiley 1986).

The understanding of the pathophysiology of many diseases, where increased intracellular calcium levels are known (Reig & Basrihan 1975; Wiley & Shaller 1977; Kretchman & Rogers 1981; Rachmilewitz *et al.* 1983; Turrini *et al.* 1985; Manrique 1988) might be widened as accurate methods for assaying the total calcium and Ca^{2+}-ions within the erythrocyte are established.

ACKNOWLEDGEMENTS

Pentoxifylline was donated by Hoechst, FRG, and nifedipine was obtained from Bayer, FRG.

REFERENCES

Bolibrohe, V. & Maizels, M. (1959) Calcium ions and the permeability of human erythrocytes. *Journal of Physiology*, **149**, 560–585.

Bücher, Th. (1947) Über ein phosphatübertragendes Gärungsferment. *Biochimica Biophysica Acta*, **1**, 292.

David-Dufilho, M., Montenay-Garestier, T. & Devynck, M. (1988) Fluorescence measurements of free Ca^{2+} concentration in human erythrocytes using the Ca^{2+}-indicator fura-2. *Cell Calcium*, **9**, 167–179.

Ferreira, H. G. & Lew, V. L. (1976) Use of ionophore A23187 to measure cytoplasmic Ca buffering and activation of the Ca pump by internal Ca. *Nature*, **259**, 47–49.

Harrison, D. G. & Long, C. (1968) The calcium content of human erythrocytes. *Journal of Physiology*, **199**, 367–381.

Kratje, R. B., Garrahan, P. J. & Rega, A. F. (1983) The effects of alkali neutral ions on active Ca^{2+} transport in reconstituted ghosts from human red cells. *Biochimica Biophysica Acta*, **731**, 40–46.

Kretchman, J. M. & Rogers, S. (1981) Sickle cell and Ca^{2+}. *American Journal of Medical Technology*, **47**, 561–566.

Lew, V. L., Tsien, R. Y., Miner, C. & Bookchin, R. M. (1982) Physiological $[Ca^{2+}]i$ level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. *Nature*, **298**, 478–481.

Lew, V. L., Hockaday, A., Sepulveda, M., Somlyo, A. P., Somlyo, A. V., Ortiz, O. & Bookchin, R. M. (1985) Compartmentalization of sickle-cell calcium in endocytic inside-out vesicles. *Nature*, **315**, 586–589.

Manrique, R. (1988) Sickle cell anemia. Pathophysiological role of increased intracorpuscular calcium and changes during treatment with pentoxifylline. *La Ricerca in Clinica e in Laboratorio*, **17**, 355–362.

McNamara, M. K. & Wiley, J. S. (1986) Passive permeability of human red blood cells to calcium. *American Journal of Physiology*, **250**, C26–C31.

Murphy, E., Levy, L., Berkowitz, L. R., Orringer, E. P., Abel, S. A. & London, R. E. (1986) Nuclear magnetic resonance measurements of cytosolic free calcium levels in human red blood cells. *American Journal of Physiology*, **251**, C496–C504.

Pressman, B. C. (1976). Biological applications of ionophores. *Annual Reviews of Biochemistry*, **45**, 501–530.

Rachmilewitz, E. A., Shinar, E., Shalev, Q., Milew, Y., Erusalimsky, J. & Schrier, S. L. (1983) Alterations in structure, function and Ca^{2+} content of thalassemic red blood cells. *Biomedica Biochimica Acta*, **42**, 27–31.

Reig, S. A. & Basrihan, S. (1975) Increased erythrocyte Ca^{2+} content in hereditary spherocytosis. *Pediatric Research*, **9**, 925–931.

Rubin, E., Schlegel, R. A., & Williamson, P. (1985) Endocytosis in sickle erythrocytes: A mechanism for elevated intracellular Ca^{2+} levels. *Journal of Cellular Physiology*, **126**, 53–59.

Schanne, F. A., Kane, A. B., Young, E. E. & Farber, J. L. (1979) Calcium dependence of toxic cell death: A final common pathway. *Science*, **206**, 700–702.

Schatzmann, H. J. & Bürgin, H. (1978) Calcium in human red blood cells. *Annals of The New York Academy of Sciences*, **307**, 125–147.

Simon, W. & Carafoli, E. (1979) Design, properties and applications of neutral ionophores. *Methods in Enzymology*, **516**, 439–449.

Simons, T. J. B. (1982) A method for estimating free Ca within human red blood cells with an application to this study of their Ca-dependent K^+ permeability. *Journal of Membrane Biology*, **66**, 235–247.

Turrini, F., Neitene, A., Mannuzzi, L., Pescarmone, G. & Arese, P. (1985). Increased red cell calcium, decreased calcium adenosine triphosphatase and altered membrane proteins during hemolysis in glucose-6-phosphate-dehydrogenase-deficient (mediterranean variant) individuals. *Blood*, **76**, 302–305.

Valberg, L. S., Holt, J. M., Paulson, E. & Sziock, J. (1964) Spectrochemical analysis of sodium, potassium, calcium, magnesium, copper and zinc in red blood cells by emission spectroscopy. *Analytical Chemistry*, **36**, 790–792.

Wiley, J. S. & Shaller, C. C. (1977) Selective loss of calcium permeability on maturation of reticulocytes. *Journal of Clinical Investigation*, **59**, 1113–1119.