RESTRICTION OF CALCIUM UPTAKE IN NORMAL AND SICKLE RED CELLS BY PROCAINE HYDROCHLORIDE AND P-AMINOBENZOIC ACID

Richard F. Baker, Darleen Powars and L. Julian Haywood

Department of Microbiology and Departments of Pediatrics and Medicine, University of Southern California School of Medicine, Los Angeles, California

Received January 26, 1977

SUMMARY: A reduced capacity for calcium uptake by human red cells after procaine hydrochloride and p-aminobenzoic acid treatment has been measured by atomic absorption spectrophotometry and X-ray microprobe techniques. Cell-associated calcium was lower by 45% and 38% in deoxygenated sickle cells pre-exposed to 10 mM procaine HCl and PABA and incubated 2 hr. at 37°C in plasma, as compared with untreated cells. Prior exposure of washed red cells to 10 mM PABA reduced the intracellular calcium level by 39% after incubation for 45 min. at 37°C with 40 μM calcium ionophore A23187. Procaine HCl was more efficient than PABA in inhibiting calcium uptake at equimolar concentrations.

INTRODUCTION

The normal human erythrocyte maintains a large calcium concentration gradient across its membrane due to a membrane-located diffusion barrier to inward transport, and an active outward-directed calcium pump (1). Elevated levels of cell-associated calcium have been implicated in red cell shape changes (2), a decrease in membrane deformability (3,4) and changes in sodium and potassium flux (5). In vivo, high calcium levels have been observed in hereditary spherocytosis (6) and in sickle cell anemia (7).

We report here a reduced calcium accumulation in vitro following prior treatment of human red cells with either procaine or p-aminobenzoic acid. Intracellular calcium was elevated in normal AA cells by metabolic depletion and by ionophore treatment; in sickle cells, by incubation while deoxygenated. Calcium levels were measured by atomic absorption, and alternatively, in single red cells by X-ray microprobe analysis.

MATERIALS AND METHODS

Blood

Normal blood was collected by venipuncture from human volunteers in heparin anticoagulant and used within 4 hours. After removing the buffy coat, washing was done 3 times in 15 volumes of phosphate buffer (11 mM KH_2PO_4; 60

ISSN 0006-291X

mM Na_2HPO_4; 147 mM NaCl; pH 7.4). SS blood was drawn from patients with homozygous sickle cell anemia. Diagnosis of SS anemia was based on the hematological parameters of hemoglobin electrophoresis, anemia, and demonstration of sickling. Hemoglobin F was less than 10% in all patients.

Calcium Measurements

Calcium was measured on a Perkin-Elmer atomic absorption spectrophotometer Model 360 or 290 using an air-acetylene flame or an HGA graphite furnace. Cells were prepared for analysis by digestion with 5 volumes of 10% nitric acid (Ultrex, J.T. Baker Co.) or 3% trichloracetic acid. Lanthanum oxide (0.5%) was added to all specimens and standards to inhibit phosphate interference. Alternatively, calcium levels in single red cells were measured with an X-ray microprobe (Applied Research Labs) after fixation with 2% buffered glutaraldehyde, critical point drying and mounting on a beryllium substrate. The cells were carbon coated by vacuum evaporation to ensure conductivity. The Ca Kα emission from the cells was excited with a 1.5 μm diameter electron beam at 10 kv. X-ray counts for single cells were totaled over a counting period of 120 sec. Background counts were obtained by defocussing the X-ray spectrometer off the Ca Kα wavelength without stage movement. Counts were made on 10 cells for each specimen and averaged after background subtraction.

Sickle Cell Incubation

Deoxygenation of freshly drawn SS blood was done by mixing one volume of whole blood with two volumes of 2% sodium metabisulfite (in distilled water). Milliosmolarity of this mixture was 252 mOsm, measured on a freezing point osmometer (Precision Systems, Newton, Mass.). Final extracellular pH was 6.0. Incubation of the blood-bisulfite mixture was at 37°C for 2-4 hr. Thirty minutes before deoxygenation and incubation, one aliquot of blood had added to it p-aminobenzoic acid (PABA, Matheson, Coleman and Bell) or procaine hydrochloride to a concentration of 10 mM. Echothiophate (0.2 μg/ml) was added to both aliquots to inhibit cholinesterase. After incubation, the cells were washed 3 times in isotonic saline before TCA or HNO_3 digestion.

Metabolic Depletion

Normal AA cells were incubated 48 hr. at 37°C in plasma, with or without 6 mM procaine hydrochloride at a 10% hematocrit. Six mM procaine (pH 7.2) was in contact with the cells for 30 min. at room temperature before incubation.

Calcium Ionophore

Calcium ionophore A23187 (Eli Lilly Co.) was dissolved in absolute alcohol (1 mg/ml; 1.9 mM). Two ml of 3x washed red cells in isotonic saline containing 2 mM $CaCl_2$ were incubated for 45 min. at 37°C with 40 μM ionophore.

A second 2 ml aliquot had 10 mM PABA added 30 min. before incubation. The pH of both aliquots was adjusted to pH 7.3 with NaOH. After incubation, the cells were washed 3x in cold isotonic saline before digestion with TCA or HNO_3. Control experiments established that calcium was not lost to the washing medium.

RESULTS

Sickle Cells:

Microprobe Analysis

The results of the microprobe analysis of single deoxygenated SS cells incubated at 37°C are summarized in Fig. 1, 2 and 3. Three concentrations of procaine, 0.1, 1 and 10 mM, were added to SS cells in the three experiments de-

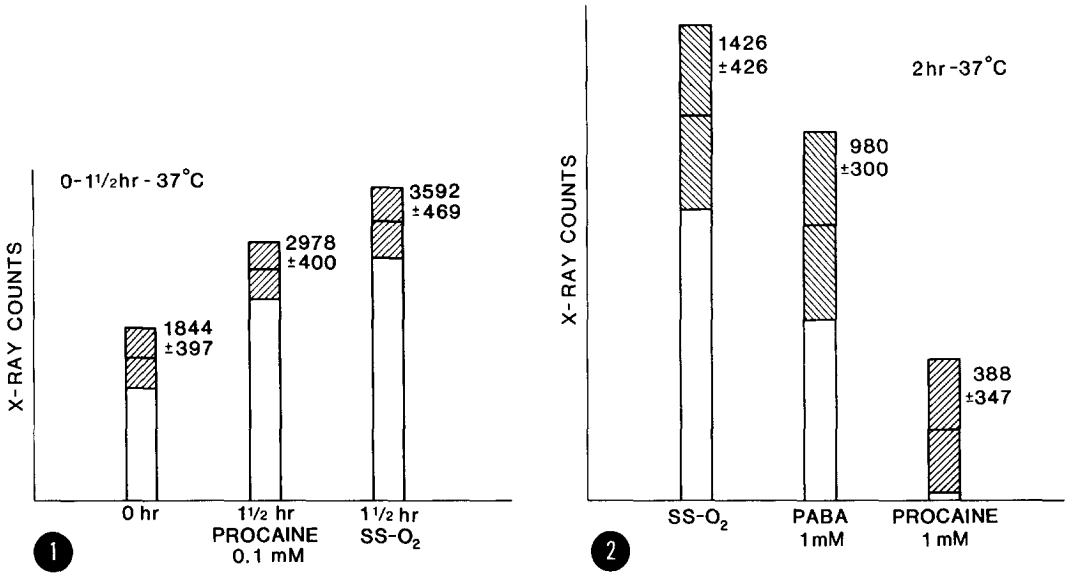

Figure 1: Relative calcium levels in individual SS cells, measured by an X-ray microprobe at zero time and after 1½ hr. at 37°C while deoxygenated in plasma with 2% sodium metabisulfite. Procaine HCl (0.1 mM) was added to one aliquot 30 min. before deoxygenation. Cells were prepared for analysis by glutaraldehyde fixation followed by critical point drying, beryllium substrate mounting and carbon coating. Each bar in Fig. 1, 2 and 3 represents the average count/cell for 10 cells. Calcium in untreated cells increased by a factor of 1.95 compared with 1.61 for treated cells.

Figure 2: SS cells in plasma, with and without 1 mM PABA and procaine HCl, were incubated 2 hr. at 37°C while deoxygenated. Cell-associated calcium for the procaine treated cells was 27% of that of the control cells, while for PABA-treated cells, the level was 69% of the control cells.

picted in Fig. 1, 2 and 3, while PABA at 1 mM is represented only in Fig. 2.

Atomic Absorption

Calcium levels (μg/ml cells) in deoxygenated control SS cells after 2 hr. at 37°C rose by 83%, from 2.57 μg/ml to 4.71 μg/ml (Table 1). Following 10 mM PABA treatment and incubation, cell associated calcium had increased by 14% while calcium in the procaine-treated cells had increased only slightly.

Normal AA Cells:

Ionophore Treatment

Incubation of normal red cells at 37°C with a calcium ionophore and extracellular calcium results in a rapid calcium influx and the attainment of high intracellular calcium levels. Forty-five min. incubation at 37°C in buffered

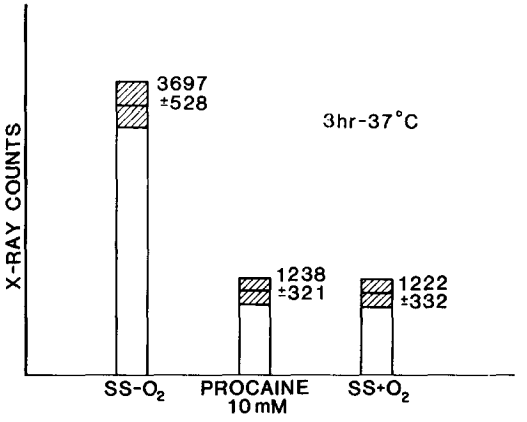

Figure 3: Following 3 hr. incubation at 37°C in plasma, relative calcium levels in deoxygenated SS cells with and without 10 mM procaine HCl were measured and compared with intracellular calcium in non-incubated oxygenated SS cells. The treated cells accumulated 33% as much calcium as the control cells.

saline and 40 μM ionophore A23187 resulted in an intracellular calcium level of 208 μg/ml cells, or 1.81x the extracellular calcium level of 115 μg/ml at the start of incubation. Cells exposed to 10 mM PABA for 30 min. at room temperature before addition showed a final calcium level of 127 μg/ml cells after 45 min. at 37°C, or 39% less calcium than the control cells.

Metabolic Depletion

Table 2 tabulates calcium levels after 48 hr. incubation in plasma at 37°C with and without 6 mM procaine hydrochloride. One aliquot of control cells was hemolyzed in 15 volumes of 20 mOsm phosphate buffer and calcium levels were measured in ghost pellet and hemolyzate to determine the ratio of membrane-bound to total cell-associated calcium. Cells pretreated with 6 mM procaine showed a calcium level of 0.90 μg/ml cells, slightly above the level of 0.6 μg/ml cells associated with normal fresh red cells (8).

DISCUSSION

The experiments reported here demonstrate that when normal human red cells are calcium loaded by metabolic depletion or ionophore action, or when sickle cells are calcium loaded by deoxygenation at 37°C, the calcium uptake may be re-

Table 1

Calcium Levels in Incubated Sickle Cells			
Control 0 hr.	Control 2 hr.	PABA 2 hr.	Procaine 2 hr.
2.57 ± .13	4.71 ± .20	2.93 ± .12	2.60 ± .13

Freshly drawn SS blood, after removal of buffy coat, was deoxygenated by addition of 2 volumes of 2% sodium metabisulfite and incubated at 37°C for 2 hr. Calcium was measured at zero time and after 2 hr. in untreated cells and cells pre-exposed to 10 mM procaine HCl and PABA, and is expressed as μg/ml packed cells.

Table 2

48 Hour Metabolic Depletion		
Sample	Ca μg/ml	%
Intact Cells	3.85 ± .19	100
Intact Cells + 6 mM procaine	0.90 ± .05	23.4
Membranes	2.40 ± .10	62.4
Hemolysate	1.35 ± .07	34.9

Calcium levels are listed for normal human red cells, with and without 6 mM procaine HCl, after 48 hr. in plasma at 37°C. Cell membranes and hemolysate of control cells after 48 hr. in plasma by a one step hemolysis and centrifugation, and measured separately to determine the membrane-bound fraction of intracellular calcium.

stricted by prior exposure of the cells to procaine HCl or p-aminobenzoic acid. The mechanism underlying this drug action is unknown, but it is reasonable to believe that the site of action is associated with the membrane.

It is known that the local positively charged anesthetics absorb to membranes and may displace membrane-bound calcium from binding sites on or near the interior membrane surface (9). The locus of such binding sites have not

been definitely established. Forstner and Manery (10) determined that in human red cell ghosts, the calcium was 79% protein bound and 16% lipid bound with 5% left unbound. The positively charged anesthetics also increase membrane area presumably by intercalating a lipophilic region into the inner half of the bilayer (9) and such intercalation has been suggested as a cause of red cell cupping or invagination (11). Also, the positively charged anesthetics inhibit the flux of cations including calcium (12) possibly by an inhibition of Na^+K^+ ATPase (13) and change in membrane potential.

At physiological values of pH, PABA will be negatively charged since the pKs are 4.80 and 4.65, and hence a mechanism of calcium displacement based on electrostatic binding to negative charge sites is untenable. PABA had no effect on red cell shape except below pH 4.6, when it acts as an invaginator (unpublished observation). Since PABA does not crenate red cells at pHs greater than 4.8, it probably is not expanding the exterior half of the bilayer in the same sense that the positively charged ampiphilic agents expand the interior half.

A number of reports on the loading of erythrocytes with calcium have appeared since the introduction of the calcium ionophore A23187 in 1972. These reports have dealt with shape changes (14), deformability changes, K^+ efflux and ATP decline (15), and production of 1,2 diacylglycerol (16). It was noted (17) that the level of cell-associated calcium after ionophore treatment may be greater than the external calcium level--a fact attributable to intracellular calcium binding. In our experiments, the total cell-associated calcium was 1.81x the exterior calcium level in control cells, while for PABA-treated cells, the increase was only 1.12x. It is not known whether the PABA acted to inhibit internal calcium binding, or influenced calcium influx by interacting with ionophore, or perhaps both. Quite unexpected was the finding that thorough washing of calcium-loaded cells in calcium-free buffer did not result in calcium extrusion from the cells, even though ionophore was presumably still present. Such a vectorial aspect to ionophore action in red cells has not been reported and remains to be investigated.

Metabolic depletion of red cells causes an increase in intracellular and membrane-bound calcium, due to a decline in calcium pump activity and the freeing of ATP-bound calcium (3). It has been reported (18) that calcium flux into the ATP-depleted cell is pH dependent, with very slow rates of entry for pHs below 7. To ensure that the lower calcium level in procaine-treated cells was due to the drug rather than to a lowered pH, care was taken that the pH remained above 7 during incubation.

Cation analysis of cells by the X-ray microprobe offers both advantages and pitfalls. The unique advantage is the possibility of correlating cation content with cellular morphology. Also, the microprobe sensitivity is extremely high (10^{-15} gm in a 1 μm^3 volume). An absolute calibration of the microprobe was not attempted in this study and hence elaborate precautions to avoid cation losses were not taken. Such loss, if it occurs, would not alter the conclusion of the experiment--that the drug-treated cells have accumulated less calcium than the untreated cells.

Membrane-bound calcium is one of the determinants of cell deformability (3). The reduction in cell-associated calcium demonstrated in this report is compatible with the previously reported reduction in the rate of formation of irreversibly sickled cells *in vitro* produced by procaine hydrochloride and p-aminobenzoic acid (19,20). Red cell deformability loss and shortened life span *in vivo* are markedly dependent on cellular calcium levels, and thus these results confirm and emphasize the need for further studies of drug-induced membrane changes which may have clinical significance.

Acknowledgements:

This work was supported by NIH Grant HL 15162 and the Robert E. and Mary R. Wright Foundation. We thank Jack Worrall for technical assistance with the X-ray microprobe.

REFERENCES

1. Schatzmann, H.J., and Vincenzi, F.F. (1969) J. Physiol. 201, 369-395.
2. Weed, R.I., and Chailley, B. (1973) Red Cell Shape, pp. 55-68, Springer Verlag, New York.
3. Weed, R.I., LaCelle, P., and Merrill, E.W. (1969) J. Clin. Invest. 48, 795-809.

4. Weed, R.I., and LaCelle, P. (1969) Red Cell Membrane, Structure and Function, pp. 318-338, J.B. Lippincott Co., Philadelphia.
5. Schatzmann, H.J. (1973) J. Physiol. 235, 551-569.
6. Feig, S.A., and Bassilian, S. (1975) Ped. Res. 9, 928-931.
7. Eaton, J.W., Skelton, T.D., Swofford, H.S., Kolpin, C.E., and Jacob, H.S. (1973) Nature 246, 105-106.
8. Harrison, D.G., and Long, C. (1968) J. Physiol. 199, 367-381.
9. Seeman, P. (1972) Pharmacological Rev. 24, 583-655.
10. Forstner, J., and Manery, J.F. (1971) Biochem. J. 124, 563-571.
11. Sheetz, M.P., and Singer, S.J. (1974) Proc. Nat. Acad. Sci. 71, 4457-4461.
12. Bianchi, C.P. (1968) Fed. Proc. 27, 126-131.
13. Anderson, N.B. (1968) Pharmacol. Expt. Ther. 163, 393-406.
14. White, J.G. (1974) Amer. J. Path. 77, 507-514.
15. Kirkpatrick, F.J., Hillman, D.G., and LaCelle, P. (1975) Experientia 15, 653-654.
16. Allan, D., Billah, M., Finean, J., and Mitchell, R.H. (1976) Nature 261, 58-60.
17. Edmondson, J.W., and Ting-Kai, L. (1976) Biochim. Biophys. Acta 443, 106-113.
18. Romero, P.J. and Wittam, R. (1971) J. Physiol. 214, 481-507.
19. Baker, R.F., Powars, D., and Haywood, J. (1974) Biochem. Biophys. Res. Comm. 59, 548-556.
20. Baker, R.F., Powars, D., and Haywood, J. (1975) Erythrocyte Structure and Function, pp. 437-453, Alan Liss, Inc., New York.