Calcium transport by red blood cell membranes from young and adult cattle

A. Zimmermann and H. J. Schatzmann

Department of Veterinary Pharmacology, University of Bern, Länggassstrasse 124, CH-3012 Bern (Switzerland), 24 August 1984

Summary. It is shown using inside-out membrane vesicles that cattle red cells extrude calcium by means of a calmodulin sensitive Ca-pump whose activity is high in calves and extremely low in adult cows. The decline is not due to loss of calmodulin susceptibility nor to a drop in Ca-affinity.

Key words. Red blood cells, bovine; calcium transport; membrane vesicles; calmodulin; calcium concentration, intracellular.

Cattle red cells (like those of other ruminants) are peculiar in that certain membrane functions differ in fetal and adult life. Thus a majority of animals in each breed carry in the adult state red cells with a high intracellular Na (and low K) concentration, whereas they are born with high-K (low-Na) cells[1-6]. The alteration is due to a change in the kinetic behavior of the Na-K-pump during the first months of life[4-6]. A similar metamorphosis after birth occurs with regard to the membrane (Ca^{2+}+Mg^{2+})-ATPase; its activity is high in newborn calves and declines with a half-time of 17 days to about $^1/_{100}$ of the original value in adults cows[7]. As we have reason to believe that the (Ca^{2+}+Mg^{2+})-ATPase reflects the presence of a Ca-pump it seemed interesting, therefore, to investigate Ca^{2+} pumping in cattle red cells and to see whether Ca^{2+} transport undergoes a similar reduction in rate after birth. If indeed there is a decline in the rate of Ca^{2+} pumping with age the question may be asked what properties of the Ca-pump bring about the change.

Cattle red blood cells were treated according to the original method of Steck et al.[8] to produce inside-out vesicles (IOVs). A difficulty encountered was that regularly a much smaller percentage of IOVs was obtained from bovine red cells (5–40%) than from human cells (40–70%) The reason for this in unknown.

^{45}Ca uptake into IOVs from a medium with 50 μM $^{45}Ca^{2+}$ was studied as described before[9] (see legend to fig.1). As will be demonstrated, 50 μM Ca^{2+} is a concentration saturating the high affinity Ca^{2+} site of the Ca-pump in cattle red cells also.

Figure 1 shows the time course of net Ca^{2+} uptake by originally Ca^{2+}-free red cell inside-out vesicles (IOVs) from one cow and two calves. In all three, Ca^{2+} uptake was strictly ATP-dependent and the Ca^{2+} accumulated was promptly released when 10 μM of the ionophore for divalent cations A 23187 was added to the medium, showing that the transport occurred in an uphill fashion and that Ca^{2+} was indeed concentrated in the free water space of the vesicles. Thus there is an ATP driven Ca-pump even in cattle red cells. However, figure 1 indicates that the calf IOVs took up Ca^{2+} at a much faster rate than the cow IOVs.

The average initial rate of Ca^{2+} transport in the presence of (added or endogenous) calmodulin was determined in a series of five adult cows, varying the method of membrane preparation. Either a method that conserves endogenous calmodulin[10,11] or one that deliberately removes calmodulin[11] were used. Contrary to figure 1 the transport rate was referred to total protein rather than to IOV protein, because in some preparations the percentage of IOVs was too low to be determined accurately (< 10%).

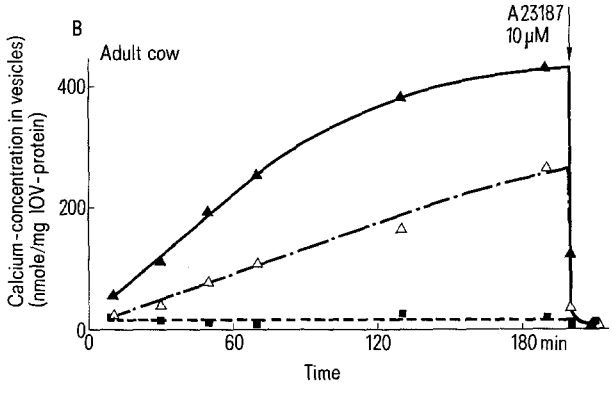
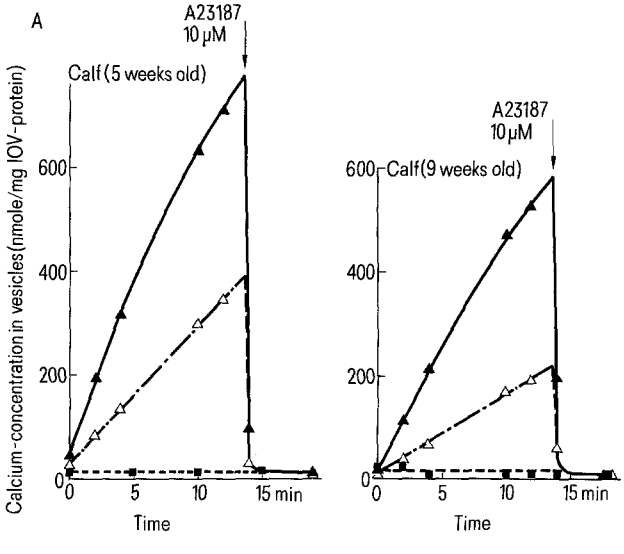


Figure 1. Ca^{2+}-uptake into bovine red cell inside-out vesicles prepared from blood of two calves (A) (initial rate 4.1 and 2.9 μmoles/h·mg IOV-protein) and one cow (B) (initial rate 0.21 μmoles/ h · mg IOV-protein). Notice the difference in the time scale in A and B. For measuring the Ca^{2+} uptake the vesicles were incubated at 37 °C in tris-Cl 20 mM (pH 7.4 at 37 °C), KCl 130 mM, $MgCl_2$ 2 mM, $CaCl_2$ 0.05 mM (^{45}Ca 0.4 μCi·ml^{-1}), with ▲ or without △ added calmodulin 1 μg·ml^{-1}. The Ca^{2+} uptake was started by adding Na_2ATP 1.2 mM (■, no ATP). At the arrow Ca-Mg-ionophore A 23187 was added to give 10 μM. 100–500 μl samples (adjusted to contain approximately 50 μg protein) were passed through Millipore filters (pore diameter 0.45 μm) which retained all vesicles. The filters were immediately washed by filtration with about 6 ml ice-cold KCl 140 mM, tris-Cl 20 mM (pH 7.4 at 2 °C), dissolved in 0.7 ml ethoxyethanol and supplemented with 4 ml scintillator (299 TM, Packard). The ^{45}Ca activity was measured in a Packard Tri-Carb 460 C scintillation counter. Vesicles were prepared acording to Steck et al.[8]: Cells from freshly drawn blood (62.5 U/ml heparin added to prevent clotting) were washed four times with NaCl 155 mM, the white cells being discarded. 1 vol. of packed red cells was lysed in 40 vol. of ice-cold Na-phosphate 5 mM (pH 8 at 2 °C), the suspension centrifuged and the membranes washed twice in the same solution. The white membranes were vesiculated at room temparature in a 40-fold vol. of Na-phosphate 0.5 mM (pH 8 at 22 °C) and incubated at 0 °C for at least 1 h. After centrifugation the vesicles were homogenized by passing the suspension four times through a 26 G needle. They were resuspended in a 4-fold volume of KCl 140 mM, tris-Cl 20 mM (pH 7.4 at 2 °C), concentrated by centrifugation to about 2 mg protein · ml^{-1} and equilibrated at 4 °C during at least 12 h. The percentage of sealed inside-out vesicles was measured according to Steck and Kant[14]. IOV-protein was calculated from total protein and the fraction of acetylcholine esterase activity revealed by triton X-100 treatment. Protein was measured according to Lowry et al.[15] against a standardized human serum (Lab-Trol) (the amount of calmodulin remaining bound to the vesicles prepared by this method seems to vary from preparation to preparation).



This seems justified since there is no indication that the IOV content of the preparation depends on the animal's age. The initial rate was taken as the difference in slope of a plot of IOV Ca^{2+} content versus time obtained with and without ATP and was found to be 2.98 ± 0.92 nmoles/(mg protein)·h. Although exceedingly small, it differed significantly from zero ($p = 0.003$). This may be compared to 140 nmoles/(mg protein)·h in vesicles from a calf of 19 days of age studied under similar conditions. From figure 1 it may further be seen that calmodulin, added to the medium at 1 µg/ml, markedly increased the rate of ATP dependent Ca^{2+} uptake both in the adult and the young animals' cell membranes prepared in phosphate buffer. This is similar to what is known from human red cells and a host of other cells in various animal species: Unlike the sarcoplasmic reticulum Ca-pump the plasma membrane Ca-pump is well-known to be directly stimulated by calmodulin[12].

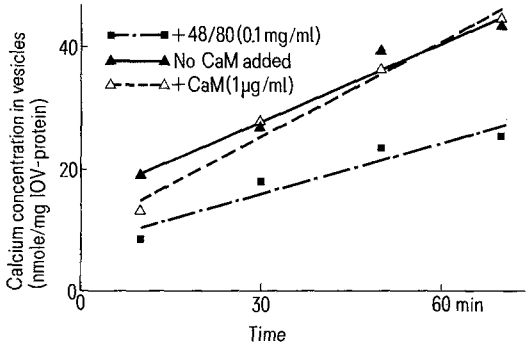


Figure 2. Ca^{2+} uptake by red cell inside-out vesicles from a cow, showing that Ca^{2+} transport is calmodulin sensitive and that the vesicles of this preparation contained endogenous calmodulin. Vesicle preparation and Ca^{2+} uptake measurement were the same as in figure 1. Additions: ▲, *none (control)*; △, *calmodulin (CaM) from beef brain*, 1 µg·ml^{-1}; ■, compound 48/80, 0.1 mg·ml^{-1}. The straight regression line calculated for ▲ and △ lumped together (not shown) has a significantly steeper slope than the regression line calculated for ■ ($p \sim 0.01$). The fact that added calmodulin did not appreciably stimulate transport shows that endogenous calmodulin was saturating the pump protein. This was not true for other preparations (see figs 1 and 3).

Figure 2 shows that compound 48/80 (Sigma C-4257), the most selective inhibitor of calmodulin activity known at present[13], was able to reduce the pump rate, which confirms the calmodulin dependence and suggests that calmodulin is present in cattle red cells.

Figure 3 demonstrates directly that cell-free hemolysate from cattle red cells contains an agent that stimulates the pump activity. This substance was enriched by passing the hemolysate over an Affi-Gel® phenothiazine column (BIO-RAD) and it was found that the major protein in the eluate had the same migration velocity as calmodulin in SDS gel electrophoresis.

In figure 4 the initial rate of Ca^{2+} transport into IOVs from two cows and two calves is plotted as a function of the Ca^{2+} concentration in the medium. It is obvious that the Ca^{2+} affinity is as high as in human red cell membranes ($K_{Ca} \sim 1$ µM at pH 7.4) and does not differ between young and adult animals.

From the present experiments it is clear that cattle red cell membranes are equipped with an ATP driven Ca-pump which can be stimulated by added calmodulin. The efficacy of the pump declines in adult animals to approximately $1/50$ of that observed in calves of 3–9 weeks of age, and the decline is not due to a loss of calmodulin sensitivity nor to a decrease in affinity for Ca^{2+} of the transport site at the internal face (outside of IOVs) of the membrane.

Interestingly, the transport rate in vesicles from one cow (shown in fig. 1) was intermediate between that of the vesicles from calves and the grand average of those from adult animals. Since we observed the presence of such outliers also with respect to $(Ca^{2+}+Mg^{2+})$-ATPase in cattle red cell membranes[3] the possibility exists that this is not simply due to chance variation but may indicate that there is a population of animals within a given breed that preserves a fairly high rate of Ca-pumping in adult life. The most common event, however, is a drop in activity of the Ca^{2+}-pump in adult animals to something like 1–5% of the activity observed in calves a few weeks after birth. This decline is

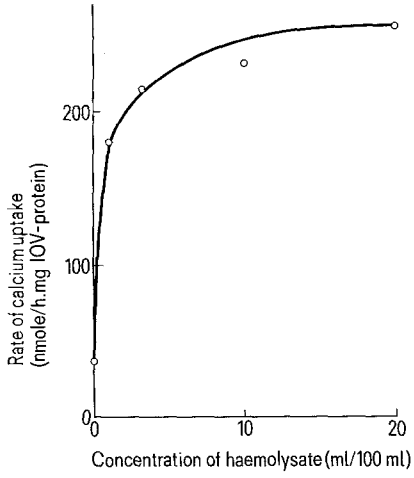


Figure 3. Stimulation of Ca^{2+} uptake into bovine red cell inside-out vesicles from a cow (same preparation as cow in fig. 1B) by cell-free hemolysate of cattle red cells. Single experiment. Hemolysate was prepared as follows: Red cells from freshly drawn blood were washed in NaCl 155 mM and lysed in a 9-fold volume of water. The ghosts were removed by centrifugation. The vesicle preparation and the Ca^{2+} uptake measurement were done as described in figure 1. The rate of Ca^{2+} uptake at any hemolysate concentration was obtained by linear regression through values at 0, 20, 40 and 60 min after initiating transport by addition of ATP.

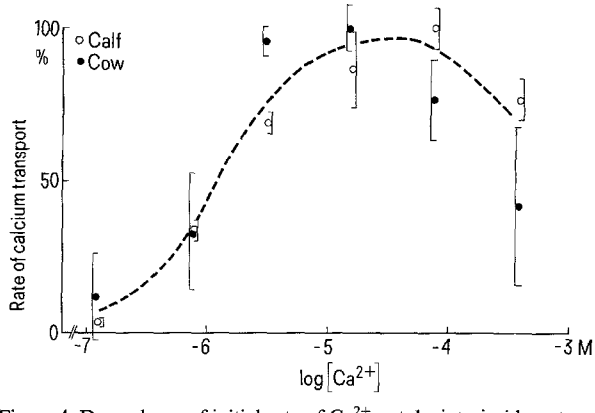


Figure 4. Dependence of initial rate of Ca^{2+} uptake into inside-out vesicles from cows and calves on the Ca^{2+} concentration of the medium. Each point shows the mean rate from two animals. The rate for one animal at a given Ca^{2+} concentration was determined in at least two experiments. The two calves were 5 and 9 weeks of age. The vesicles were incubated at 37 °C in tris-Cl 40 mM (pH 7.4 at 37 °C), KCl 260 mM, $MgCl_2$ 4 mM, calmodulin from beef brain 1 µg·ml^{-1}, ^{45}Ca 0.4 µCi·ml^{-1}. The required concentrations of free Ca^{2+} from 0.14 to 380 µM were obtained with tris-EGTA 0.2 mM and $CaCl_2$ concentrations varying from 0.12 to 0.7 mM. The constants[16] used for calculation were for EGTA: $K_1 = 3.47 \cdot 10^{-10}$, $K_2 = 1.41 \cdot 10^{-9}$, $K_3 = 2.09 \cdot 10^{-3}$, $K_4 = 1.0 \cdot 10^{-2}$, $K_{CA} = 2.24 \cdot 10^{-11}$, $K_{Mg} = 4.0 \cdot 10^{-6}$ and for ATP: $K_1 = 3.16 \cdot 10^{-7}$, $K_2 = 8.9 \cdot 10^{-5}$, $K_{Ca} = 4.79 \cdot 10^{-5}$, $K_{Mg} = 3.98 \cdot 10^{-5}$. The Ca^{2+} uptake was started by adding Na_2ATP 1.2 mM. All other conditions and the method of vesicle preparation were as described in the legend to figure 1.

not accounted for by a loss of calmodulin sensitivity nor to a drop in Ca^{2+} affinity and thus must be due to a lowering of the turnover rate of the pump, or more likely to a reduction in the number of pump sites per cell. A drastic increase of the Ca^{2+} affinity at the external membrane surface (inside IOVs) can be ruled out as a cause from the high steady state IOV Ca^{2+} concentration reached in cows' vesicles (fig. 1B).

The leak flux for Ca^{2+} in cattle cells has not been measured very accurately, but it seems to be extremely low[7], so that it is not surprising that a rather ineffective Ca-pump suffices to keep the intracellular Ca^{2+} concentration at a level which is not hazardous to cellular function.

1 Brown, A.M., Ellory, J.C., Young, J.D., and Lew, V.L., Biochim. biophys. Acta *511* (1978) 163.
2 Christinaz, P., and Schatzmann, H.J., J. Physiol. *224* (1972) 391.
3 Schatzmann, H.J., Nature *248* (1974) 58.
4 Ellory, J.C., and Tucker, E.M., J. Physiol. *204* (1969) 101P.
5 Israel, Y., Macdonald, A., Bernstein, J., and Rosenmann, E., J. gen. Physiol. *59* (1972) 270.
6 Tucker, E.M., Smalley, C.E., Ellory, J.C., and Dunham, P.B., J. gen. Physiol. *79* (1982) 893.
7 Schatzmann, H.J., and Scheidegger, H.R., Experientia *31* (1975) 1260.
8 Steck, T.L., Weinstein, R.S., Straus, J.H., and Wallach, D.F.H., Science *168* (1970) 255.
9 Wüthrich, A., and Schatzmann, H.J., Cell Calcium *1* (1980) 21.
10 Schatzmann, H.J., J. Membr. Biol. *35* (1977) 149.
11 Gietzen, K., Wüthrich, A., and Bader, H., Molec. Pharmac. *22* (1982) 413.
12 Martonosi, A.N., in: Muscle and Nonmuscle Motility, vol. 1, p. 233. Academic Press, New York and London 1983.
13 Gietzen, K., Adamczyk-Engelmann, P., Wüthrich, A., Konstantinova, A., and Bader, H., Biochim. biophys. Acta *736* (1983) 109.
14 Steck, T.L., and Kant, J.A., Meth. Enzym. *31* (1974) 172.
15 Lowry, D.H., Rosebrough, J.N., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
16 Sillen, L.G., and Martell, A.E., Stability constants of metal ion complexes, 2nd edn, Special Publication No. 17. The Chemical Society, Burlington House, London 1964.

0014-4754/85/060743-03$1.50 + 0.20/0

The effect of DDT on DNA replication in the larval salivary gland cells of *Drosophila melanogaster*

Z. Bahçeci

Atatürk University, Science Faculty, Department of Biology, Erzurum (Turkey), 12 August 1983

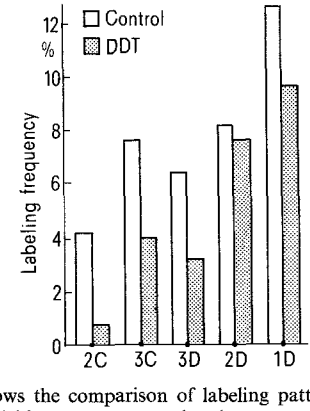
Summary. The inhibitory effect of DDT on the initial stage of the DNA replication process in polytene chromosomes of larval salivary gland cells of *Drosophila melanogaster* was investigated and possible mechanisms for the inhibition are discussed.
Key words. Drosophila melanogaster; salivary gland, larval; DNA replication; DDT; chromosomes, polytene.

Earlier reports on the Dipteran polytene chromosomes confirmed the replication process to be a temporally ordered sequence[1-4]. It has also been reported that some antibiotics[4,5] and toxic substances affect the sequential order of DNA replication. Here an attempt is made to determine how DDT (a pesticide) affects the DNA repliction in polytene chromsomes of third instar larval salivary gland cells of *Drosophila melanogaster*.

Materials and methods. In the experiments, the Oregon strain of *Drosophila melanogaster* was used. The stocks were obtained from the Biology Institute of Hacettepe University, Ankara. The salivary glands of the larvae were excised under Drosophila Ringer solution (pH = 7.2). One of the glands dissected from each larva was incubated in a drop of Drosophila Ringer containing ^{3}H-thymidine (1.0 mCi/ml, specific activity 42 Ci/mMol., Radiochemical Centre, Amersham, England) for 10 min as a control, and the other gland was first treated with 0.25 ppm DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane][6] solution for 5 min then incubated in a drop of Drosophila Ringer containing ^{3}H-thymidine, for 10 min (experimental group). DDT was dissolved in acetone, and control experiments did not show any effect of acetone on DNA replication. Later, each of the batches was fixed, stained with lactic-acetic orcein[7] and squashed on slides. These slides were carried through the routine autoradiographic procedures[8].

The differences in labeling frequency of the nuclei between the control and experimental groups was tested with Student's t-test[9].

Results. In both experimental and control slides, those polytenic cells in S-phase of DNA replication were labeled with ^{3}H-thymidine. The polytene chromosomes in the third instar larval salivary gland cells showed five types of labeling pattern with ^{3}H-thymidine. These are, in sequence; 2C (light continuous), 3C (heavy continuous), 3D (heavy discontinuous), 2D (middle discontinuous) and 1D (lightly discontinuous) labeling patterns. It has generally been accepted[10,2,5,11] that these patterns represent the initial stage (2C), middle stage (3C-3D-2D) and the terminal (1D) stage of the DNA replication cycle. In the control group, a total of 492 nuclei have been examined and 195 out of these were seen to be labeled (table). Therefore, the labeling frequency of the controls with ^{3}H-thymidine was calculated to be 39.6%. A detailed analysis of the frequencies of various labeling patterns was as follows: 4.3% 2C, 7.7% 3C, 6.5% 3D, 8.5% 2D and 12.6% 1D. On the other hand, 494 nuclei were examined in the experimental group and only 123 (24.8%) of these were labeled with ^{3}H-thymidine. In the



The figure shows the comparison of labeling patterns with ^{3}H-thymidine of the nuclei in DDT-pretreated and nontreated salivary gland cells of *Drosophila melanogaster*.