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CONTROL OF CELL pH IN IMMATURE PRIMITIVE RED CELLS FROM CHICK EMBRYO

ULRICH SIEGER, CHRISTOPH REINHARDT and ROSEMARIE BAUMANN*
Physiologisches Institut, Universität Regensburg, Postfach 397, 84 Regensburg, Germany
(Tel. 0941-943-2956; Fax 0941-943-4315)

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Abstract—1. The intracellular pH in primitive red cells from 4 day chick embryos was measured with the digitonin null-point method and the fluorescent indicator SNARF-1. At physiological pH_e of 8.0 red cell pH is 7.39 at day 4.

2. The calculated proton equilibrium potential of −38 mV is in good agreement with previous measurements of E_m (Engelke *et al.*, 1988) and supports the conclusion that the E_m is dominated by a proton conductance.

3. The sodium–proton exchanger is present in primitive red cells but quiescent under physiological conditions.

4. The results indicate that the bicarbonate–chloride exchange via Band 3 protein is impaired.

INTRODUCTION

Primitive erythrocytes which originate in the yolk sac of higher vertebrates constitute the first red cell population of the embryo. In the chick embryo a closed circulatory system is established by the end of the second day of incubation. The primitive red cells enter the vascular system as a cohort of immature erythroblasts which carry out their terminal differentiation (including several mitoses) inside the vascular system. While the oxygen binding properties of the primitive red cells have been assessed from day 3 onwards (Baumann *et al.*, 1982; Lapennas and Reeves, 1983) the mechanisms that control red cell pH—a major determinant of hemoglobin oxygen affinity—during early development have not yet been studied in greater detail.

In adult red cells of birds and mammals the distribution of chloride, bicarbonate and protons follows the Donnan equilibrium (Hladky and Rink, 1977; Kregenow, 1977), and rapid equilibrium between chloride and bicarbonate is achieved through the presence of the Band 3 anion transporter. During embryonic development Band 3 first appears in the red cell membranes of 3-day-old chick embryos (Chan, 1977).

Direct measurements of red cell pH (in the absence of carbon dioxide) have been obtained only for mature primitive red cells from 6 day chick embryos. They demonstrated a large pH difference of about 0.6 units (Baumann *et al.*, 1982, Baumann and Haller, 1983).

Measurements of the membrane potential of primitive red cells have demonstrated the presence of a proton conductance and the measured potential is considerably more negative than the chloride equilibrium potential (Engelke *et al.*, 1988). These data suggest that Band 3 function is altered in primitive red cells. In the present investigation we have measured directly the pH of red cells from early chick embryos in the absence and presence of carbon dioxide and assessed the presence of the sodium–proton antiport.

The results show that the sodium–proton exchanger is present in these cells but is quiescent under physiological conditions. Furthermore we have obtained additional experimental evidence to support the hypothesis that in immature primitive erythrocytes the hetero-exchange of bicarbonate and chloride across the Band 3 protein is impaired, causing a disequilibrium of bicarbonate and chloride distribution across the red cell membrane.

MATERIALS AND METHODS

Fertilized eggs were incubated for 4 days at 37.5°C and 60% r.h. in an incubator with automatic rotation. At day 4 the eggs were opened and red cells aspirated from extracellular blood vessels and transferred into cold buffer (pH 7.4, 20 mmol/l Tris, 146 mmol/l NaCl, 4 mmol/l KCl, 1.5 mmol/l $CaCl_2$ and 5 mmol/l glucose). The cells were washed twice in the buffer and then prepared for the cell pH measurements.

Measurement of cell pH

The cell pH of the red cells was determined by two methods:

The digitonin null-point method (*Rink* et al., 1982). This method has been shown to give reliable estimates of mean cytoplasmic pH (Rink *et al.*, 1982). The main disadvantage of the method is that it needs

*To whom all correspondence should be addressed.

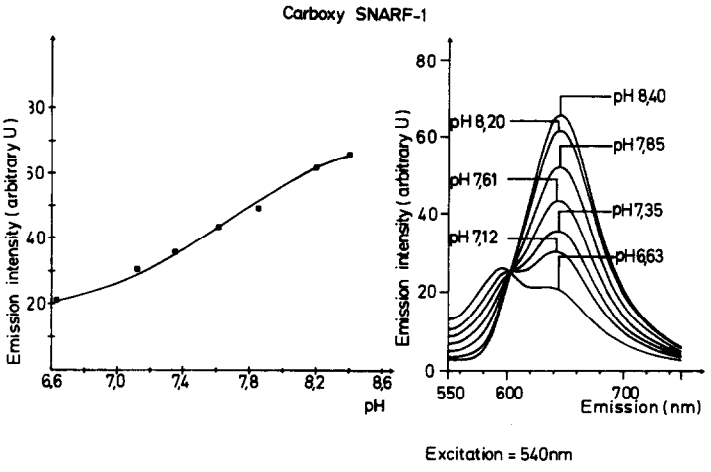

Fig. 1. Left panel: pH dependent emission of SNARF-1 at 640 nm, excitation at 540 nm and 37°C. Right panel: pH dependent emission spectrum of SNARF-1 after digitonin lysis of red cell sample.

fairly large amounts of material and cannot be used to determine cell pH in the presence of CO_2, since measurements are made in unbuffered solutions. For the cell pH determinations the cells were first incubated for 30 min in the respective buffer solution (140 mmol/l NaCl, 5 mmol/l KCl, 2 mmol/l $CaCl_2$, 1 mmol/$MgCl_2$, 5 mmol/glucose, 20 mmol/l Tris or Hepes). The pH of the incubation buffer varied from pH 7.0 to 8.5. At the end of the incubation period the solution was rapidly centrifuged and 5 μl of the packed red cells transferred to 1.5 ml unbuffered solution with the same composition as above except for Tris/Hepes. The pH of this solution was adjusted to the value of the respective incubation buffer by addition of NaOH or HCl.

Immediately after resuspension the cells were permeabilized by addition of 20 μl of a 20 mmol digitonin stock solution. The change of the extracellular pH (pH_e) was registered on an x–t recorder.

The cell pH is taken to correspond to the external pH where no change in pH_e is recorded after addition of digitonin.

Measurement of the cell pH with the fluorescent indicator SNARF/AM-1 (Molecular Probes Inc.). The commonly used indicator BCECF was found unsuitable for cell pH measurements due to large quenching artifacts and rapid leakage of BCECF from the embryonic erythrocytes.

We have now established a protocol for use of the recently introduced fluorescent dye Carboxy-SNARF-1 (Molecular Probes. Inc) which allows reliable measurements in the physiological pH range. Figure 1 shows the emission spectrum of SNARF/-1 as a function of pH. The excitation wavelength was 540 nm.

For our measurements the fluorescence signal was taken in the ratio mode ($Emission_{640}/Emission_{603}$) with excitation at 540 nm. All measurements were carried out on a Shimazdu RF-540 fluorescence-spectrophotometer.

Calibration

The presence of an independent electrogenic proton conductivity in the membrane of the embryonic red cell (Engelke *et al.*, 1988) does not allow the usual calibration of the fluorescence signal with nigericin (Thomas, 1986), which requires that protons follow the distribution of the potassium ions. This condition is only met if other pathways for proton transport are negligible, since otherwise their presence acts as a proton shunt. In keeping with this we observed only minimal changes of the fluorescence signal in the presence of nigericin.

We therefore used the following calibration procedure which initially relied on the cell pH values obtained with the digitonin-null point method.

Red cells were incubated with SNARF/AM-1 (final concentration 1.5 μmol) at pH 8.0 for 30 min at 37°C. Under these conditions the intracellular pH as determined by the digitonin-nullpoint method is 7.4 (Fig. 2).

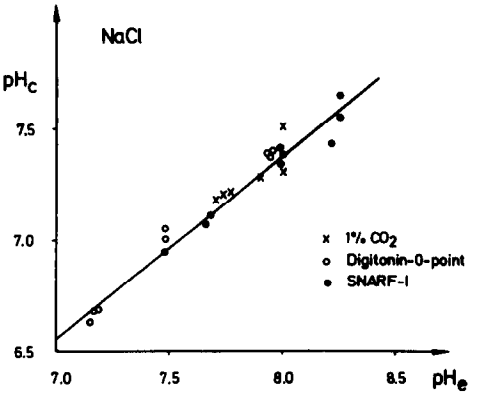

Fig. 2. Dependence of cell pH on external pH as measured with the digitonin-null point method (open circles) or SNARF-1 (filled circles). Cell pH measurements in the presence of carbon dioxide were obtained with SNARF-1. All measurements were performed at 37°C.

At the end of the incubation period cells were washed twice for 20 sec with incubation buffer of increased density (50 g Ficoll 400/l added). The increased density facilitates removal of extracellular dye compared to the results obtained when washing with normal buffer.

The pellet (2 μl packed cells) was transferred into the cuvette containing 2 ml buffer (pH 8.0). The emission ratio was recorded, the suspension rapidly centrifuged and the cell pellet quickly transferred into buffer of pH 7.4 (so that initially $pH_e = pH_i$). The ratio was immediately recorded before and after addition of digitonin.

Under the assumption that $pH_i = pH_e$ and provided that little or no intracellular quenching occurs, the fluorescence signal obtained before and after addition of digitonin should be identical.

We found in a typical set of experiments that there was only a small, undirected change of the ratio after addition of digitonin, amounting at most to an error of cell pH determination of about 0.05 to 0.1 pH units. Since these results suggest no specific quenching artifacts, one can use the simple digitonin lysis method for standardization of the fluorescence measurements; i.e. cells were lysed with digitonin after the measurement and the emission ratio recorded for stepwise changes of pH using 0.5 M HCL as a titrant. The main set-back of the method is that although dye leakage is much smaller than for BCECF is obtained only for the first few min. Therefore the dye cannot be used to monitor cell-pH continuously in embryonic red cells.

All readings were performed within 3 min after transfer of the cells to the fluorescence spectrophotometer.

For measurements of cell pH in the presence of CO_2 the following procedure was adopted. Cells were incubated with dye for 30 min in bicarbonate buffer (composition as above except that Tris was replaced by a mixture of Hepes and bicarbonate to yield the desired pH) in a gas mixture of 1% CO_2 in air. The gas mixture was saturated with water vapor and was provided by a Wösthoff gas mixing pump Type SA 27 F.

After incubation the sample was transferred anaerobically with a blood–gas syringe into Eppendorf tubes (2.2 ml) filled with 200 μl incubation buffer which had been equilibrated with the same gas mixture. The sample was layered under the buffer and centrifuged for 20 sec. The cell pellet was then washed rapidly twice with CO_2 equilibrated incubation buffer of higher density and after the final wash the pellet was resuspended in 40 μl fresh medium and immediately transferred with a gas-tight Hamilton syringe into anaerobic cuvettes (Fa. Hellma) sealed with teflon caps. The cuvettes contained 1960 μl incubation buffer equilibrated with the CO_2/air gas mixture. The pH of the buffer was measured anaerobically using the AVL Gas Check 939 blood gas analyser. All other procedures were as described above.

To test the dependence of cell pH on the presence of external sodium or chloride the ions were substituted by choline and gluconate respectively.

Red cell concentrations of sodium and potassium were determined by flame photometry, chloride by coulometric titration (Aminco Cotlove) as described previously (Engelke *et al.*, 1988). All concentrations are given as mmol/kg cell water. The wet and dry weights were determined as described elsewhere (Engelke *et al.*, 1988).

SNARF/AM-1 was obtained from Molecular Probes and a stock solution of 1 mmol/l dissolved in DMSO was prepared. Amiloride was a kind gift of Merck, Darmstadt, Germany. All other reagents were obtained from Sigma chemicals St Louis, MO. A stock solution of DIDS (4,4′diisothiocyano-2,2′-disulfonic acid stilbene) was prepared daily at 1×10^{-2} M in aqueous solution.

RESULTS

Red cell pH in norminally CO_2 free solutions

Figure 2 shows the combined results of the cell pH measurements obtained with either the digitonin-null point method or the fluorescent indicator SNARF/1 under standard conditions. Both sets of data are in good agreement. With an estimated pK of around 7.5 SNARF/1 is a sensitive indicator in the physiological pH range.

Previous measurements of extracellular pH in various parts of the embryonic circulation at day 4 (Meuer *et al.*, 1989) have shown that the pH in the vitelline vein, which carries the oxygenated blood to the embryonic heart is 8.0, whereas the pH in the intraembryonic circulation drops to 7.64 (jugular vein). Thus the physiological extracellular pH range for erythrocytes of the 4 day embryo is 8.0–7.6 and the corresponding values for pH_i are 7.39 and 7.09 as calculated from the common regression equation (equation 1).

$$pH_i = 0.73085 + 0.833\, pH_e; \quad r = 0.98469 \qquad (1)$$

When the embryonic red cells were treated with DIDS (30 min incubation in the dark with a final concentration of 5×10^{-4} mol) in order to inhibit anion-transport via the Band 3 protein, cell pH did not change (at $pH_e = 8.0$ $pH_i = 7.37$, mean value from six experiments). On the other hand substitution of external chloride by gluconate (in the absence of DIDS) caused a moderate alkalinization (Fig. 3). After 30 min incubation in gluconate the intracellular pH increased by about 0.15 pH units. At pH_e 8.0 the pH_i is 7.56.

When external sodium was replaced by choline during the incubation period (Fig. 4) we observed no significant decrease of the intracellular pH compared to the controls. This indicates that sodium–proton exchange is quiescent under these conditions (i.e. in the physiological pH range).

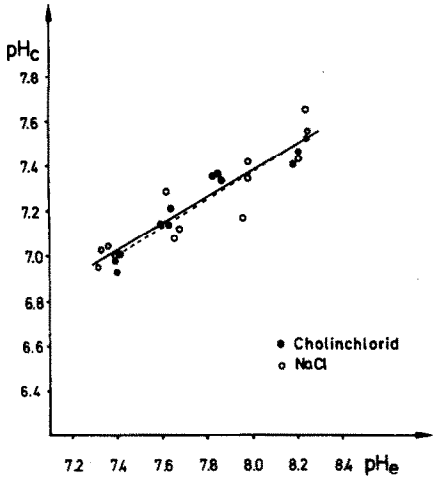

Fig. 3. Effect of substitution of external sodium by choline on cell pH.

Note also that the possible gradient for the Na^+–H^+ exchange is much smaller than for adult red cells, since the intracellular sodium concentration is 97 and 83 mmol/kg H_2O at pH_e 7.0–8.0 (mean values from 4 experiments).

Since these results gave no indication for the presence of a sodium proton exchange we conducted a second series of experiments. We incubated the red cells for 20 min in buffer with high extracellular sodium (140 mmol/l) at a low extracellular pH of 6.6 (cell pH is 6.15 as measured by digitonin-nullpoint). Under these conditions the intracellular sodium concentration is about 100 mmol/kg H_2O. The cell suspension was rapidly centrifuged and the pellet (20 μl) transferred into an unbuffered (1.5 ml) solution of 140 mmol choline chloride pH 6.6.

This causes a steep inward gradient for the uptake of protons. The initial rate of proton influx was recorded in the absence and presence of 0.5 mmol/l amiloride by following the change in pH_e. We found that in the absence of amiloride initial proton uptake was 4.69 picomole/10^6 cells per min (SD 0.03, $N = 12$) and 1.27 picomole (SD 0.75, $N = 10$) in the presence of amiloride (the residual influx is probably caused by incomplete inhibition of the exchanger). This result suggests that the exchanger is present, but apparently inactive under physiological conditions.

Chloride distribution

In the range of pH_e 7.0–8.0 the chloride concentration decreases from 95.9 mmol/kg cell water to 73.8 mmol/kg cell water (mean values from 5 experiments). With total extracellular chloride of 148 mmol/kg water the ratio Cl_i/Cl_e falls from 0.65 to 0.5. These values are much higher than those for the proton distribution ratio, which are 0.24 at pH 8.0 and 0.36 at pH 7.0. Since the calculated proton equilibrium potential of -38 mV is close to the previously measured membrane potential of -44 mV at day 4 (Engelke *et al.*, 1988), the intracellular chloride concentration is kept above the electrochemical equilibrium.

Cell pH measurements in the presence of CO_2

Figures 2 and 4 also contain the results of the cell pH measurements carried out in the presence of 1% CO_2 compared with data obtained at the same external pH but in the absence of CO_2. Although all experiments were carried out in the absence of the Band 3 inhibitor DIDS the cell pH values are the same in the absence and presence of CO_2. This result supports our previous suggestion that Band 3 protein in primitive red cells is not involved in substantial bicarbonate–chloride heteroexchange (Engelke *et al.*, 1988) sufficient to establish equal distribution ratios for both ions.

DISCUSSION

The immature primitive red cells from the chick embryo that we investigated in the present paper are still dividing at day 4 and will undergo an estimated two additional divisions before final maturation (Campbell *et al.*, 1971).

The neccessity to carry out oxygen transport without compromising the last proliferative cycles may require a pH regulation different from that of mature red cells, where the presence of Band 3 protein causes equal distribution ratios for protons, chloride and bicarbonate, so that cell pH can be calculated from the chloride distribution ratio (Van Slyke *et al.*, 1923; Funder and Wieth, 1966; Hladky and Rink, 1977). Indeed the results of the present study show a pattern of pH control in the early embryonic red cell that differs substantially from that of the adult red cell.

Steady state pH in embryonic red cells

Measurements of cell pH in non-transformed proliferating erythroid cells have not been carried out before. We found a good agreement between the two methods used in our study to assess cell pH, in particular the fluorescent label SNARF/1 seems well

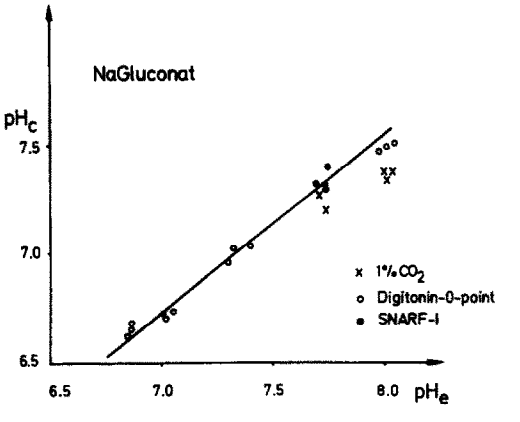

Fig. 4. Effect of substitution of external chloride by gluconate on cell pH. Measurements in the presence of carbon dioxide were obtained with SNARF-1.

suited to monitor cell pH in the range above pH 7.0. Nevertheless leakage still prevents continuous registration of cell pH and we are currently trying to reduce it with aid of organic anion transport inhibitors.

The pH difference of 0.61 pH units between red cell and extracellular medium at pH_e 8.0 is of the same size as at day 6 (Baumann *et al.*, 1982). The principal mechanism controlling cell pH of embryonic red cells during early development seems to be the proton conductance present in the membrane, whose molecular mechanism is not established (Engelke *et al.*, 1988).

The steady state pH is unchanged when the Band 3 protein is blocked with DIDS. Furthermore red cell pH is unaltered when measured in the presence of carbon dioxide and hardly affected by substitution of gluconate for chloride. Replacement of sodium by choline has no measurable influence on steady state pH in the range pH_e 7.4 to about 8.3 (corresponding to an intracellular pH of around 6.9 to 7.55).

While the experiments carried out at pH_e 6.6 in the absence of external sodium demonstrate the presence of a sodium dependent proton flux one must conclude that even at acid pH_e the physiological importance of the exchanger is reduced. Under normal conditions the driving force for sodium–proton exchange is less than in adult red cells, due to the high intracellular sodium concentrations which under our experimental condition e.g. with an external sodium of 140 mmol/l amounts to about 80 mmol/kg cell water. Further experiments have to clarify the mechanisms that cause the high intracellular sodium concentration and keep the intracellular chloride concentration well above electrochemical equilibrium, a possible candidate is the Na–K–Cl cotransport system.

Role of Band 3 protein in embryonic red cells

The presence of Band 3 protein in the membrane of primitive red cells is well documented. Chan (1977) as well as Cox *et al.* (1987) found Band 3 protein as early as day 3.

Our results demonstrate that there is no substantial heteroexchange of bicarbonate for chloride in early embryonic red cells. The presence of significant heteroexchange should result in an increase of red cell pH in the presence of bicarbonate since the Band 3 protein tends to equilibrate the bicarbonate and chloride distribution and r_{Cl} is much higher than r_{H+}.

However our cell pH data are the same in the absence or presence of CO_2, this implies that chloride and bicarbonate are not in equilibrium with each other. It follows that the hetero-exchange function of the Band 3 protein is altered in embryonic red cells. We do not know the cause for the altered functional behaviour. The presence of covalent modifications affecting the cytoplasmic domain is one possibility since a tyrosine kinase which *in vitro* phosphorylates Band 3 has been demonstrated (Hillsgrove *et al.*, 1987) in avian erythrocytes. Further experiments have given evidence that two Band 3 protein sequences exist in avian red cells, which apparently differ at the cytoplasmic domain (Kim *et al.*, 1988). There is as yet no information available as to whether the two proteins differ in their functional properties and if their insertion into the plasma membrane is developmentally regulated.

In the adult chick red cell Band 3 protein has the same functional characteristics as Band 3 of human red cells (Jay, 1983). The divergent results obtained for embryonic red cells demonstrate that the regulation of cell pH and of Band 3 protein function during development is more complicated than allowed for by the present models describing these functions in adult red cells. Further experiments are necessary to extend the model and identify the developmentally regulated control mechanisms.

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