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VOLTAGE PULSATION OF SICKLE ERYTHROCYTES ENHANCES MEMBRANE PERMEABILITY TO OXYGEN

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Summary

Treatment of sickle red cells (SS homozygous) with a voltage pulse of less than 0.8 kV/cm and duration of 20 μs caused a change in the cell membrane, so as to facilitate the permeation of oxygen. The unsickling of the treated cells after a re-introduction of oxygen took place at a much faster rate. Neither leakages of Na^+ and K^+, nor a change in the cell volume occurred as the result of the low voltage pulsation. The effect of the voltage treatment persisted for hours at 25°C but disappeared rapidly at 37°C. The result suggests that a selective modification of membrane permeability may be achieved by the voltage pulsation technique.

Previous studies have shown that the exposure of a red cell suspension to an electric pulse of intensity of a few kV/cm and duration of microseconds lead to a total hemolysis of the red cells [1—6]. The hemolysis is the result of the colloid osmotic swelling, which in turn is caused by the electric perforation of the cell membranes [5—8]. Several effects of the voltage pulsation have been discussed [4]. Among these are dielectric breakdown, electromechanical compression, electrophoresis of membrane proteins, and thermal osmosis effect. Results obtained in our laboratory indicate that the voltage pulse can generate a transmembrane potential, and at a critical potential (approx. 0.9 V), it opens up pores of limited size in the red cell membrane. The size of pores can be controlled, and resealing of these pores has been accomplished without hemolysis of the treated cells [7,8]. In an attempt to develop a drug entrapping technique for the treatment of sickle red cells we have found that sickle cell mem-

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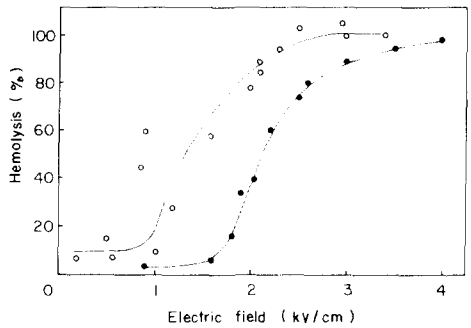
branes are more susceptible to voltage perforation, and that sickle cells treated with a pulse at a subthreshold field intensity [6] become resistant to sickling at low oxygen tensions. It is known that hemoglobin S gellates only in the deoxygenated state [9]. The result presented here would indicate that the inhibition of sickling by the voltage pulsation was probably due to an increased oxygen permeability of the treated cells.

Blood was obtained either from healthy adult donors or from patients with sickle cell anemia (SS homozygous), using heparin as an anticoagulant. Red cells were washed four times with 150 mM NaCl/7 mM phosphate buffer mixture at pH 7.4, and buffy coats were removed. The oxygen tension of red cell suspensions was measured using BMS 3MK2 Blood System (The London Company). A solution of low oxygen tension was prepared by bubbling nitrogen gas for at least 30 min. The oxygen tension was reduced to less than 35 mmHg, and the cells sickled in this solution as they did in the presence of 10 mM dithio nite.

The high voltage pulse was applied to the red cell suspensions by a method described in detail elsewhere [5]. 40 μl of cell suspension was placed between a parallel pair of stainless steel electrodes. The pulse applied to the sample was rectangular single wave of 20 μs duration (See Fig. 1 of Ref. 5 for the waveform). Unless otherwise stated the pulsation was carried out at room temperature of 23—25°C. In all experiments, the suspensions contained 7 mM phosphate; thus, no variation in the effect of phosphate concentration [10] would be expected. Na^+ penetration and K^+ leakage were checked by a Corning Model 450 Flame Photometer.

A nuclepore filtration technique was used to assay the degree of sickling. Light microscopy was also used occasionally to check the filtration result. The filtration method worked as follows. The time required for a fixed volume, 1.2 ml, and a fixed concentration, 0.3 % (v/v), of red cell suspension to pass through a polycarbonate nuclepore filter (25 mm, Nuclepore Corp.) was measured. We used a membrane of 3 μm mean pore diameter. The membrane was placed in a filter holder which was connected to a glass tube with diameter of 0.6 cm and a length of 12.5 cm. An initial pressure of 100 mmH_2O was added to the other end of the glass tube. The flow time for the normal red cell suspensions was 10 ± 3 s and was independent of the oxygen tension. The flow time depended dramatically on the oxygen tension for the sickle cell suspensions. At a high oxygen tension (greater than 70 mmHg) the flow time of sickle cell suspensions was comparable to that of the normal red cell suspension. However, under a low oxygen tension solution, sickle cells sickled and the flow time could be as high as 500 s. In our analysis of data we used a non-dimensional unit, i.e., the ratio of flow times under deoxygenated (t_d) and oxygenated (t_o) suspensions, to express the degree of sickling.

Fig. 1 shows the relation between the extent of hemolysis and the pulsated voltage for typical sickle and normal red cell samples at equilibrium. The hemolysis of the sickle cells occurred at lower voltage (with a mid-point at 1.5 ± 0.3 kV/cm) as compared to that of the normal cells (2.2 ± 0.1 kV/cm), under the present conditions. The hemolysis curve of the sickle cells was found to be rather insensitive to changes in the oxygen tension of the suspension in the range 30 to 75 mmHg. This is surprising in view of the fact that



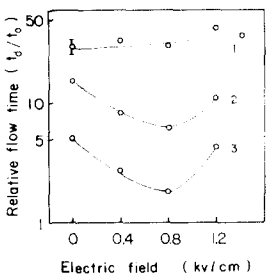


Fig. 1. The extent of hemolysis for sickle and normal cells. Red cells suspended in 10% sodium-phosphate buffer/90% sucrose isotonic mixture (cell concn. 20% v/v) were subjected to a single electric pulse of various intensities with pulse duration of 20 μs. After pulsation the sample of 60 μl was mixed with 4.0 ml of the same buffer solution. After about 20 h, the suspension was centrifuged and the extent of hemolysis was determined from the absorbance of hemoglobin in the supernatant at 410 nm. The value for 100% hemolysis was determined by hypotonic hemolysis. The oxygen pressure was not controlled in the experiment. ○, sickle cells; ●, normal cells. Results obtained for the sickle cells at 35 mmHg and 70 mmHg oxygen tensions are similar.

Fig. 2. The ratio of flow time for the deoxygenated sickle cell suspension (t_d) to that of the oxygenated (t_o) plotted against the electric field. The pulse was applied to the suspension with the hematocrit 30% without controlling the oxygen pressure (i.e. oxygen tension > 65 mmHg). The pulse duration was 20 μs. After pulsation the red cells were suspended in the deoxygenated buffer solution at the hematocrit of 0.3% and were kept for 15 min before measuring the flow time. In the curves 2 and 3, a small amount of air bubble was introduced into the deoxygenated suspension through a glass capillary, which was connected to a rubber ball. The measurement of flow time was started within 1 min after introducing air bubble. The oxygen tension, pO_2, of the samples was: 1, 34.6 mmHg (unpulsated, not air bubbled); 2, 55.0 mmHg (pulsated, air bubbled); and 3, 75.1 mmHg (pulsated, air bubbled).

voltage perforation is sensitive to cell shape, cell volume and medium composition [5,6,10]. However, consistently more susceptibility to the voltage perforation was found for sickle blood cells from several patients. Sickle cells from these patients assumed normal biconcave shape and cell volume at a oxygen tension of 70 mmHg. At a oxygen tension of less than 35 mmHg, these cells sickled within 10 min. It is known that repeated cycles of sickling and unsickling in vitro lead to permanent impairment of the cell membrane and the formation of irreversibly sickled cells [11]. Irreversibly sickled cells showed increased hemolysis coupled with loss of deformability [12]. The result in Fig. 1 indicates that damage to the cell membrane occurs before a permanent impairment of the cell membrane.

There are two steps in the formation of pores by the voltage pulsation. One is the initiation of pores and the other a subsequent growth of pore size [6]. The initiation of pores requires a transmembrane potential greater than a threshol whereas the latter process is influenced by some factors such as the ionic strength, the applied field strength and pulse duration [6]. The resistance of red cell membrane to the pulsation and the subsequent colloid osmotic hemolysis depend also on the membrane properties, as is demonstrated in Fig. 1.

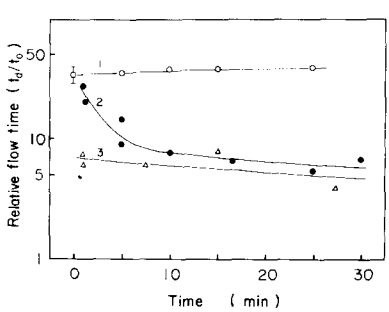
Fig. 2 shows the ratio of the flow times for the deoxygenated and the oxygenated sickle cell suspensions plotted against the field strength of the applied voltage pulse. The flow time was almost independent of the field intensity when the sickle cells were suspended in the low oxygen tension buffer (curve 1). Introduction of a small air bubble, or an increase in the oxygen tension, to the de

genated suspension caused a decrease in the flow time within 1 min (curves 2 and 3). Furthermore, the flow time decreased with the increase of field strength, as can be seen in curves 2 and 3. The increase in the flow time beyond the threshold voltage, in this case about 1 kV/cm as shown in Fig. 1, was due to the osmotic swelling of cells by the pulsation [5].

In fact, the introduction of air bubbles also reduced the flow time of untreated sickle cell suspension but only after a 10 min incubation period (25°C). No difference in the flow time between the unpulsated and pulsated samples was observed beyond this incubation period. This is shown in Fig. 3. At 37°C, the effect of the voltage pulsation was hardly detectable as the perforated membranes rapidly resealed and regained the normal permeation barrier to oxygen. At 25°C the resealing took place at a much slower rate. The pores were stable for at least 2—3 h. To demonstrate that this was indeed the case we undertook the experiment, the results from which are shown in Fig. 4.

Sickle cells voltage treated were depleted of oxygen for at least 15 min. Aliquots were then taken at different time intervals and bubbled with air, and the unsickling of the cells was determined within 1 min of air bubbling. If voltage-induced pores were still present, cells would unsickle rapidly and the flow time would be low. On the other hand if the perforated membranes resealed, unsickling could not occur within 1 min and the flow time should be close to that for the sickled cells. Fig. 4 indicates that voltage induced pores were indeed stable for at least 2—3 h at 25°C.

In all of our experiments, unless the field intensity exceeded the threshold voltage, 1kV/cm of applied field in this case, no leakage of K^+ and Na^+ was detectable by flame photometry. Cell volume was also constant to within ±3%



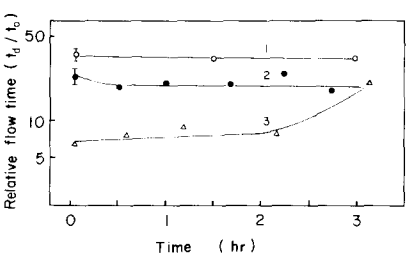


Fig. 3. The ratio of flow time for deoxygenated sickle cell suspension to that for oxygenated one plotted against the elapsed time after introducing the air bubble into the suspension. The pulsation was carried out at the field intensity 0.8 kV/cm and the duration of 20 μs. After pulsation the red cell was suspended in a deoxygenated buffer solution at the hematocrit of 0.3% and kept at 25°C for 15 min. Then the air bubble was introduced into the sample and the flow time was measured at each interval. The oxygen tension was: 1, 32.3 mmHg (unpulsated, not air bubbled); 2, 65.4 mmHg (unpulsated, air bubbled); and 3, 60.0 mmHg (pulsated, air bubbled).

Fig. 4. Recovery of oxygen barrier after the voltage treatment of sickle cells. The pulsation was carried out under the same conditions as in Fig. 3. After pulsation the sample was kept in the buffer without control of the oxygen pressure. At each interval, the red cells were spun down and deoxygenated solution was added into the packed cells. After 15 min the air bubble was introduced into the deoxygenated sample and the measurement of flow time was started within 1 min. The first point in each curve was obtained as following. After pulsation the sample was kept in the deoxygenated buffer solution for 15 min and then the measurement was begun within 1 min after bubble was introduced. The oxygen tension of the samples was: 1, 33.8 mmHg (unpulsated, not air bubbled); 2, 63.1 mmHg (unpulsated, air bubbled); and 3, 62.3 mmHg (pulsated, air bubbled).

by hematocrit measurements. The treated cells were stable and no hemolysis occurred beyond a controlled level. These results suggest strongly that even at a voltage far below the threshold voltage originally thought for pore initiation [5,6], the cell membranes were already modified to an extent where oxygen permeation was greatly facilitated. Since no leakage of K^+ and Na^+ occurred, as the result there was no colloid osmotic swelling, and hence no hemolysis of the treated cells. This indicates that a selective opening of membrane channels by the voltage pulsation technique can be achieved without doing severe damage to the cell membranes.

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