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Evidence of Voltage-Induced Channel Opening in Na/K ATPase of Human Erythrocyte Membrane

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Summary. Previous studies have shown that human erythrocytes when subjected to a high voltage pulsation, in the microsecond time range, lysed in an isotonic medium. The hemolysis was the result of the colloid osmotic swelling, which, in turn, was caused by the voltage perforation of the red cell membranes. In this work we demonstrate that in a low ionic medium at least 35% of the pores was related to the opening of Na^+/K^+ ATPase channels. The membrane conductance generated by the externally applied electric field could be partially blocked by a specific inhibitor, ouabain, or by a specific cross-linking reagent, Cu^{++}-phenanthroline, of the ATPase. The effect of ouabain was saturable and had a mid-point of saturation at 0.15 μM. This value agrees with the physiological inhibition constant of the drug. K^+ ion in the external medium suppressed the effect of ouabain, as has also been demonstrated in physiological studies. Experiment presented in this communication also suggests that the Na^+/K^+ ATPase was not perforable in a high ionic medium, and that a large fraction of the voltage-induced pores occurred at as yet unidentified sites.

A cell can maintain a steady-state transmembrane potential by an asymmetric distribution of ionic species in both sides of the membrane. This transmembrane potential serves many different purposes, e.g., cell-cell communications, generation and transmission of neuro impulses, energy transductions, etc. When a cell is exposed to an externally applied electric field, the faculty of the cell electrogenecity is either perturbed or severally impaired. As a result, voltage pulse-treated cells usually lyse and release their cytoplasmic contents within a short period of time (Sale & Hamilton, 1968; Riemann, Zimmerman & Pilwat, 1975; Tsong & Kingsley, 1975; Tsong et al., 1976; Kinosita & Tsong, 1977*a*, *b*). This type of phenomenon has been extensively studied in the past, although, not until recently have mechanisms of the voltage-induced cell lyses been worked out to certain details for human erythrocyte (Riemann et al., 1975; Tsong et al., 1976; Kinosita & Tsong, 1977*a*, *b*, 1979).

It has now become clear that in human erythrocytes, pores of limited size are introduced in the cell membrane when the external field exceeds a threshold voltage of about 2 kV/cm for a 20 μsec pulse (Kinosita & Tsong, 1977*a*, *b*). The hemolysis following the voltage treatment is the consequence of the leakage of K^+ ion and the permeation of small molecules into the treated cells. This change in the membrane ionic permeability gives rise to a colloid osmotic swelling and eventually a puncture of the treated cells. The cause of the pore formation has also been demonstrated to be due to the field-induced transmembrane potential, but not to a sudden Joule heating of the cell suspension (Kinosita & Tsong, 1977*a*).

The maximum transmembrane potential generated by an external field can be estimated by the relation, $\Delta\Phi = 1.5\, a_o E$, where a_o is the outer radius of a cell and E is the field strength (Sale & Hamilton, 1968; Riemann et al., 1975; Tsong et al., 1976). By this relation it can be shown that a field strength of 2 kV/cm corresponds to a 0.9 V of transmembrane potential across the erythrocyte membrane. This unusually high breakdown potential casts a doubt on whether the phenomenon observed here has any relevance to the physiologically functional properties of the cell membrane. However, the erythrocyte membrane is a nonexcitable membrane, and the question of what is the physiological potential has little meaning. Instead, we have asked the question of whether there are specific sites of pore formation induced by the externally applied field.

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The mechanism of the voltage-induced pore formation has been shown to be a two-step process (Kinosita & Tsong, 1977*b*, 1979). A suprathreshold potential induces the initial perforation, in a few μsec, followed by the expansion of the pore size. This latter process is a function of pulse duration, field intensity and ionic strength, and it occurs in 100 μsec time range.

But if the kinetics is better known, the description of the events at a molecular level is still lacking. Previous studies (Kinosita & Tsong, 1977*b*) suggest that the perforation was due to a dielectric breakdown occurring in a highly localized region(s). These perforable areas may be either lipids or transmembrane proteins. However, because the voltage-induced pores have been found to be very stable even after days of incubation at low temperature (Kinosita & Tsong, 1977*a*, *b*), it is very unlikely that these pores occur at the lipid matrix[1]. Different transmembrane proteins involved in transport phenomena have been characterized in the red cell membrane. Each constitutes a potential target for the voltage-induced perforation.

In this paper we have tried to define the nature of these induced channels. This study was made possible because of the earlier development of a highly sensitive means for monitoring different steps of the perforation process, namely, by the conductivity measurement of red cell suspensions during and after the pulsation (Kinosita & Tsong, 1979). The use of the specific inhibitor, ouabain, for the protein responsible for the active Na^+/K^+ transport, and its consequences on the events linked to the permeability process (hemolysis, ion leakage, conductance changes, swelling rate) show clearly that this protein is one of the sites of the pore formation. Specific cross-linking experiment confirms this interpretation. Induced pores appear to be heterogeneous, and their sites may vary as the ionic strength of the suspending medium changes.

Materials and Methods

Fresh human blood was obtained from healthy young adults by venipuncture in the presence of heparin. Erythrocytes were washed three times by centrifugation at $1{,}000 \times g$ for 15 min with a solution containing 150 mM NaCl and 7 mM phosphate buffer, pH 7.2; buffy coats were carefully removed. Packed cells were suspended in a mixture of the 0.15 M NaCl phosphate buffer solution and a 0.272 M sucrose solution in the same buffer (both were assumed to be isotonic). Volume concentration (hematocrit index) was adjusted to 20% for conductance measurements and 2% for hemolysis experiments.

The voltage pulsation device was previously described (Kinosita & Tsong, 1977*a*, 1979). An erythrocyte suspension was placed between a pair of platinized platinum electrodes, and squarewave electric pulses, with a rise time of 30 nsec, were applied after thermal equilibration.

Conductance experiments were done as described (Kinosita & Tsong, 1979). A differential method was used. First, by use of subthreshold pulses (0.4 kV/cm) an adjustable voltage which was linearly related to the current passing through the red blood cell suspension was balanced by a voltage proportional to the applied tension. This gave the resting conductance of the sample. Both voltages were fed into the differential input amplifier of a storage oscilloscope. Then a pulse was applied and the change in conductance was recorded on the screen.

Hemolysis was measured as described (Kinosita & Tsong, 1977*a*). The pulsed samples (1% hematocrit index) were incubated in a lysing medium (0.15 M NaCl, 7 mM phosphate buffer) overnight, at 4°, in a dilution of 1:100. The sample was then spun down and the absorbance at 410 nm of the supernatant was recorded. One hundred percent hemolysis was obtained from a hypotonic shocked sample using pure water.

Ion leakage was measured by the flame photometry. After the pulse, the samples were kept standing for 1 hr then spun down, and the ionic content of the pellet was determined.

The rate of swelling was observed by light scattering measurements at 600 nm as described elsewhere (Kinosita & Tsong, 1977*c*). The pulsed sample (hematocrit index 1%) was diluted a hundredfold in the lysing solution and continuously stirred. The swelling of the cells was monitored by the decrease in the light scattering signal. The signal had been calibrated to cell volume as given by the hematocrit measurement.

Ouabain, 1–10 phenanthroline, and cupric sulfate were obtained from Sigma. Salts were of the analytical grade. Ouabain was dissolved in the 0.15 M NaCl, 7 mM phosphate buffer and kept in the dark at 4° until use. The stock concentration was 10 mM, and proper dilutions were done by using ethanol before applying to erythrocyte suspensions. The final concentration of ethanol in erythrocyte suspensions was less than 0.1%, and no effect of ethanol could be detected.

Inhibition by ouabain was performed as follows. The red blood cells were first incubated in the experimental mixture for 30 min at room temperature. Each sample was then separated into two batches, one for control and the other for experiment. A small aliquot of stock ouabain (less than 1% in volume) was added in the "experiment" sample and the same volume of buffer to the "control" sample. Both samples were incubated again for 30 min at room temperature. The conductance experiments were then carried out. Inhibition of active transport when it occurred (no K^+ ion in the external buffer) was detected either by a very small increase (about 5%) of the steady conductance of the suspension in low ionic content buffer (this was presumed to be due to a leakage of cytoplasmic K^+ ion) or measured by flame photometry in some experiments. If K^+ ion was present in the external buffer before addition of ouabain, no such changes were detected.

Cross-linking experiment was performed as described for ghosts (Steck, 1972) by using 0.2 mM 1–10 phenanthroline and 0.1 mM $CuSO_4$. The mixture was incubated at room temperature with open vials in order to obtain a good oxygenation of samples.

SDS electrophoresis was carried out as described for ghosts (Fairbanks, Steck & Wallach, 1971) using 5% acrylamide gels except that dithiothreitol was omitted. The staining procedure was described by Weber and Osborn (1975).

[1] Assuming that lipid molecule has a translational diffusion coefficient of $D \sim 1 \times 10^{-9}$ cm^2/sec (Edidin, 1974), the time for a pore of diameter 10 Å to be refilled is approximately, $t = \bar{x}^2/2D = 10^{-14}/2 \times 10^{-9} = 5 \times 10^{-6}$ sec.

Results

Effect of Ouabain

As described (Kinosita & Tsong, 1977*a*), hemolysis of the red blood cell occurs when it is submitted to a transient suprathreshold electric field. This effect has been reported to be a function of the magnitude of the electric field and of its duration. As shown in Fig. 1, the presence of ouabain, up to a concentration of 110 μM, in the suspending medium did not affect the shape and the position of the hemolysis curve against the electric field for a given pulse duration of 30 μsec. Neither did the substitution of K^+ ion for Na^+ ion in the external medium change the normal shape of the hemolysis curve. Associated with this electric field-induced hemolysis, intracellular K^+ ion has been shown to leak out (Kinosita & Tsong, 1977*b*). When ouabain was present in the extracellular buffer, the process also appeared unaffected; so was the penetration of Na^+ ion into the voltage-treated cells. However, when the conductivity change associated with the electric perforation of the membrane was measured, ouabain was found to have a large effect on the kinetic signal. The signal of the voltage-induced membrane conductivity was greatly reduced. This is shown in Fig. 2.

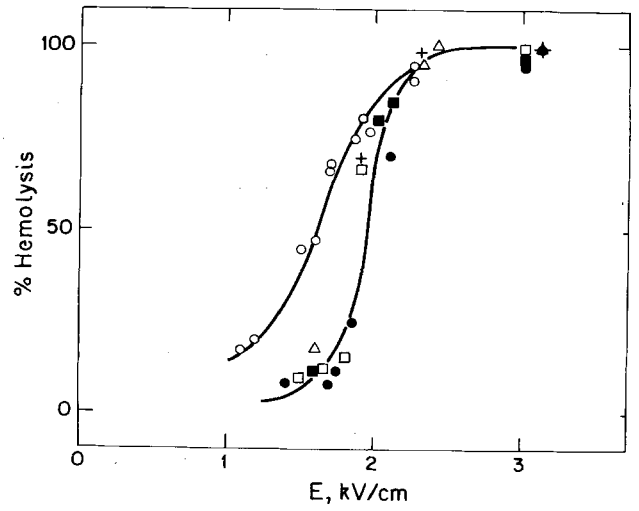

Fig. 1. Extent of induced hemolysis *vs.* the intensity of the applied electric field. The pulse duration was 30 μsec. The temperature was 25 °C. The lysing medium was 0.15M NaCl. After pulsation, the cells were kept for 20 hr at 4 °C in the lysing medium; then the suspension was centrifuged and the supernatant was analyzed for its hemoglobin content (absorption at 410 nm). One hundred percent value was obtained by hypotonic lysis. *Symbols for different media:* ●, Na^+/sucrose ratio of 10:90%; ■, Na^+/K^+/sucrose of 5:5:90, and 110 μM ouabain; △, K^+/sucrose of 10:90; +, K^+/sucrose of 10:90, and 110 μM ouabain; □, Na^+/sucrose of 10:90, and 110 μM ouabain; ○, Na^+/sucrose of 10:90, and Cu^{++}-phenanthroline treatment

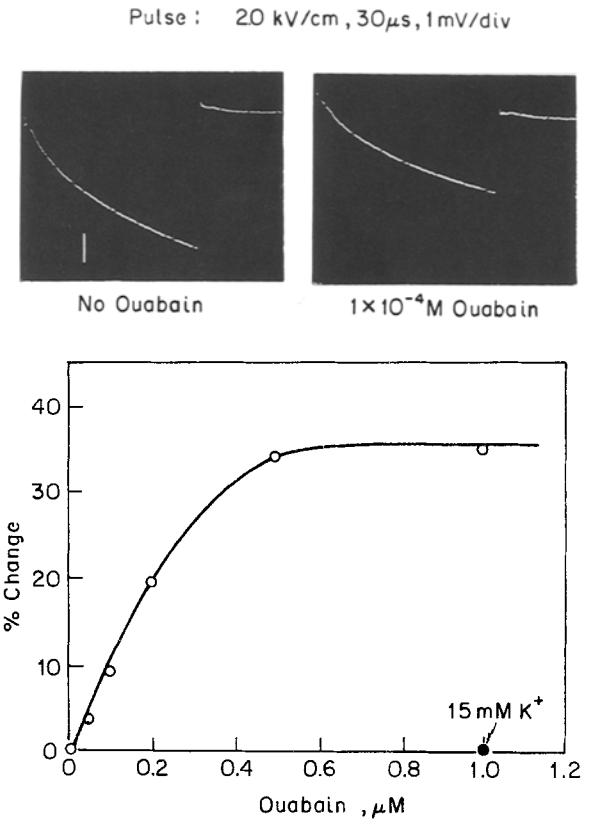

Fig. 2. Time courses of conductivity increase in erythrocyte suspensions when a high square voltage wave was applied to the sample. Ordinate is conductivity change. The bottom curve gives the magnitude of the decrease in the conductivity change at the end of a 30 μsec (E=2.0 kV/cm) as a function of the ouabain content in the pulsation buffer. The pulsation medium was 10% isotonic NaCl, 90% isotonic sucrose, 7 mM phosphate buffer; pH=7.4. The hematocrit index was 0.2. The temperature was 25 °C. The attenuation constant, a (Kinosita & Tsong, 1979) was slightly different for the two oscillographs. When the medium contained 15 mM K^+ instead of Na^+, no effect of ouabain was detected

Moreover, the effect of ouabain was found to be a function of the concentration of the added glycoside and appeared to be saturable (Fig. 2). The concentration for the half-maximum effect is about 0.15 μM. And, if K^+ ion was present in the external medium during the pulsation, the effect of ouabain was completely suppressed. The maximal suppression of the conductivity signal was 35% at a low ionic strength medium (NaCl/sucrose=10:90%).

To examine whether the threshold potential of the membrane perforation was affected by the presence of ouabain, we have used a voltage pulse with a gradually increasing field strength (Kinosita & Tsong, 1979). The effect of ouabain in suppressing the conductivity changes remained the same as observed for the squared wave form, and there was no change in the threshold potential. The threshold potential of pore formation was consistently found

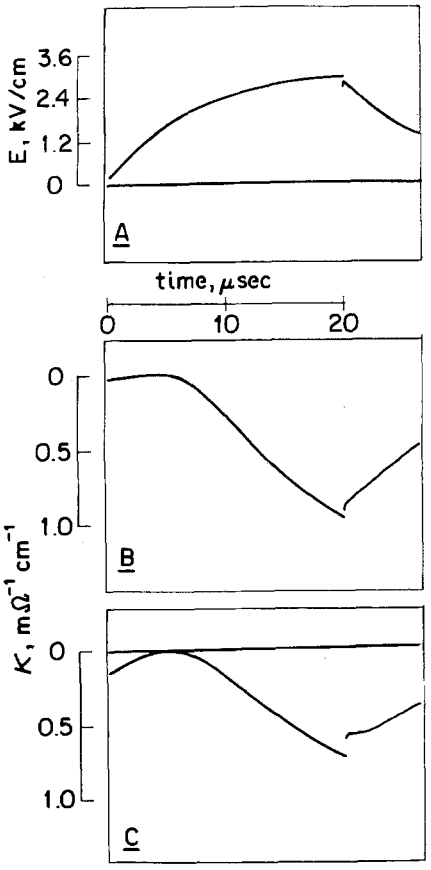

Fig. 3. Effect of ouabain on conductivity change under a gradually increasing electric field. *Upper curve:* Wave form of the electric fields. *Middle curve:* Changes in conductivity. The red blood cells at a hematocrit index of 0.2 were suspended in a mixture of Na^+/sucrose 10:90%. The temperature was set at 37 °C. *Bottom curve:* Changes in conductivity with added ouabain. The conditions were the same as in the preceding experiment. The concentration of ouabain was 10 μM

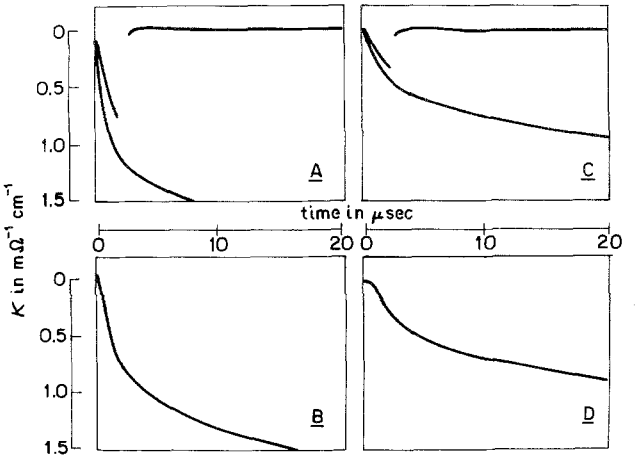

Fig. 4. Effects of ouabain on conductivity changes associated to repeated electric pulses. The electric field intensity was 2.9 kV/cm; the temperature was 37 °C. The hematocrit index was 0.2 in a mixture Na^+/sucrose 10:90%. When present, the ouabain was at a concentration of 10 μM (expts. *A* and *C*). (*B* and *D*): Changes in conductivity when only one pulse (duration 25 μsec) was applied to the sample. (*A* and *C*): A short pulse (duration 2 μsec) was first applied, and after a few seconds a longer one (duration 25 μsec) was applied. (*See* text for details)

to have a value of 1.8 kV/cm (or 0.8 V of transmembrane potential). This is shown in Fig. 3. Since no change in the shape of the conductivity response curves was seen (the change was confined to a 35% reduction in the amplitude), the effect of ouabain was not limited to certain kinetic phases, but rather had a uniform effect on the perforation kinetics.

Another set of experiments was performed to see whether a completely reversible condition could be found for the pore opening-closing process. It has been known that voltage-perforated red blood cells spontaneously reseal by incubation at 37 °C. However, the resealing was too slow to be considered fully reversible. If the Na^+/K^+ channels were the site of perforation, one might hope to find a condition where the opening and closing process could be completely reversible. When a channel is open, a strong transmembrane current is bound to occur, and this strong current may generate a large local heating that could denature a protein. To avoid such a severe local heating, we used a short voltage pulse to perforate the membrane, and the closing of pores was checked by a longer pulse after a few seconds of intermission. The result of the double-pulse experiment is shown in Fig. 4.

In oscillograph *A*, two conductivity curves are seen. The first pulse with a width of 2 μsec generated the short conductivity curve. After a few seconds of intermission a second pulse with a width of 25 μsec was applied. If the opened channels were closed reversibly within this few second intermission, the second conductivity curve would have the same shape and amplitude as seen for a single 25 μsec pulsation of a fresh sample in oscillograph *B*. Although partial resealing occurred within this few-second period, a complete resealing was not obtained. The conductivity curve generated by the 25-μsec pulse was slightly larger (25–28%) for the pretreated cells than that for the fresh cells, and the cells treated with a 2-μsec pulse eventually lysed without a proper resealing procedure. In oscillographs *C* and *D*, it is shown that although ouabain reduced the amplitude of the conductivity curve, as usual, it did not improve the reversibility of the treated cells. The amplitude of the membrane conductance was again 28% larger for the 2-μsec pulse-treated cells than for the fresh cells.

The rate of swelling of RBC submitted to a transient electric field could be measured by changes in light scattering of the suspension after the voltage pulsation (Kinosita & Tsong, 1977*b*). When ouabain

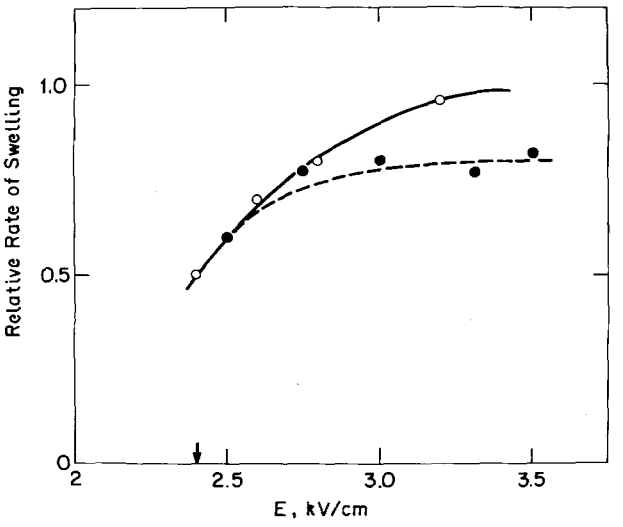

Fig. 5. Effect of ouabain and cross linking on the rate of swelling of pulsed erythrocytes. The ratio of the rate of swelling of treated erythrocytes to that of untreated samples is plotted *vs.* the magnitude of the applied electric field. The rate of swelling was obtained from light scattering measurements. The pulsation medium was a mixture of Na^+/sucrose 10:90%, the swelling medium, a mixture of 90:10%. The pulse duration was 7 μsec. The temperature was 25 °C. The isotonicity of both buffers was maintained by use of isotonic NaCl (0.15 M) and sucrose (0.272 M) mixtures. ○, the pulsation medium contained 40 μM ouabain; ●, red cells treated with Cu^{++}-phenanthroline cross linking. Arrow indicates the threshold voltage of 7 μsec pulsation

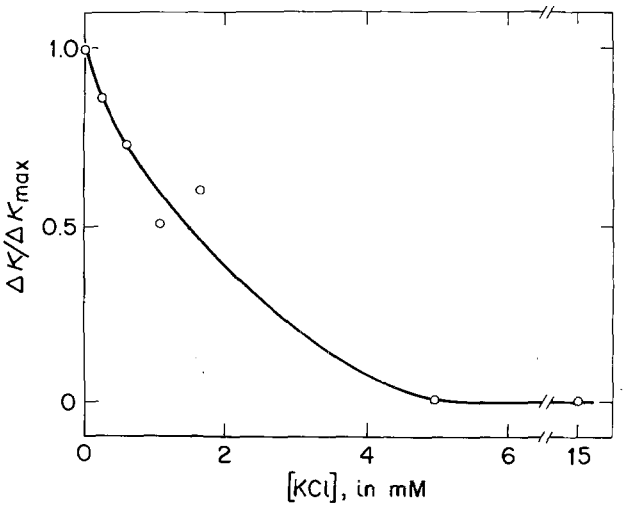

Fig. 6. Effect of K^+ ion on the ouabain-induced decrease in conductance change. The magnitude of the field was 3.2 kV/cm, pulse duration 30 μsec, temperature 25 °C, hematocrit index 0.2, and ouabain concentration 50 μM. The concentration of $Na^+ + K^+$ was 15 mM, but the buffer contained 7 mM Na phosphate; pH = 7.4. The isotonicity of the medium was maintained by use of 90% of 0.272 M sucrose

was present in the pulsation medium, the rate of swelling decreased (Fig. 5). This effect of ouabain was a function of the strength of the applied field. The smaller the field the larger was the effect of ouabain. If the pulsation voltage was kept constant, the shorter the pulse width the larger was the effect. The decrease in the swelling rate was also observed when melezitose was substituted for sucrose in the swelling buffer.

Suppression of the Effect of Ouabain

The effect of ouabain in inhibiting part of the voltage-induced membrane conductance was strongly reduced or even completely suppressed if K^+ ion was present in the extracellular buffer (Figs. 2 and 6). This effect of K^+ ion was present even at very low concentrations (Fig. 6). The ability of K^+ ion to compete with ouabain binding to Na^+/K^+ ATPase of erythrocyte membranes is known from physiological studies of this enzyme (Harris & Kellermeyer, 1972). The suppression of the effect of ouabain by K^+ ion in the voltage pulsation experiment is thus consistent with the physiological studies. These results suggest that that portion of the membrane conductance signal which was inhibited by ouabain apparently resulted from the opening of Na^+/K^+ ATPase channels by the external field.

To examine further the above interpretation, we carried out cross linking experiments. Cu^{++} and 1–10 phenanthroline mixture is known to cross link Na^+/K^+ ATPase. The equilibrium hemolysis of cross linked red cells appeared to be very similar to the untreated cells except that the curve was slightly shifted to lower voltage (Fig. 1). The hematocrit index, that measures the effective volume of cells, was not significantly altered by the treatment of the cross linking reagent for 1 hr.

However, the membrane conductance associated with the voltage pulsation decreased progressively during the cross linking reaction. After one hour of reaction with the cross linking reagent the conductance signal lost roughly 30% of its amplitude, and a plateau was obtained at longer incubation times, as shown in Fig. 7. Cu^{++} and 1–10 phenanthroline when used separately did not have the observed effect.

N-methylmaleimid is a relatively nonspecific sulfhydryl group reagent. Mere addition of this reagent to the medium did not affect the membrane conductance signal. However, if the red cells were preincubated with this sulfhydryl reagent for 1 hr (2 mg/ml) specific cross linking by Cu^{++}-phenanthroline was

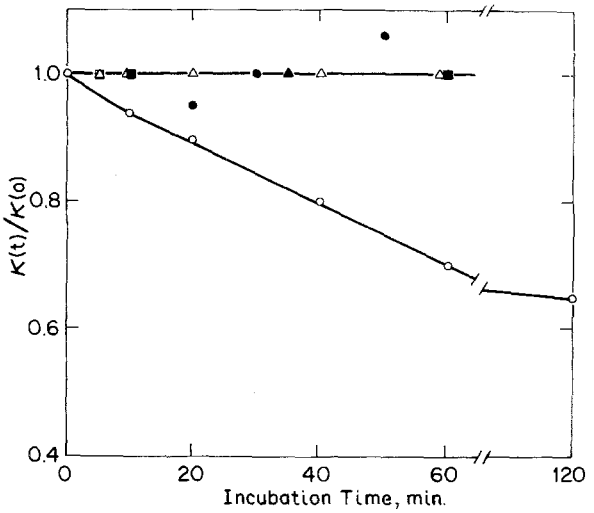

Fig. 7. Relative decrease in the electric field-induced conductance change as a function of the incubation time. The electric field strength was 2.8 kV/cm, pulse duration 30 μsec, temperature 25 °C, and hematocrit index 0.2. *Symbols used:* ○, the medium contained 200 μM phenanthroline, 100 μM $CuSO_4$, Na^+/sucrose ratio of 10:90%; ●, 200 μM phenanthroline, 100 μM $CuSO_4$, 100% isotonic NaCl; △, 2 mg/ml N-methylmaleimid, Na^+/sucrose of 10:90%; ▲, 100 μM $CuSO_4$, Na^+/sucrose of 10:90%; □, pretreatment by N-methylmaleimid as in △, followed by treatment as in ○

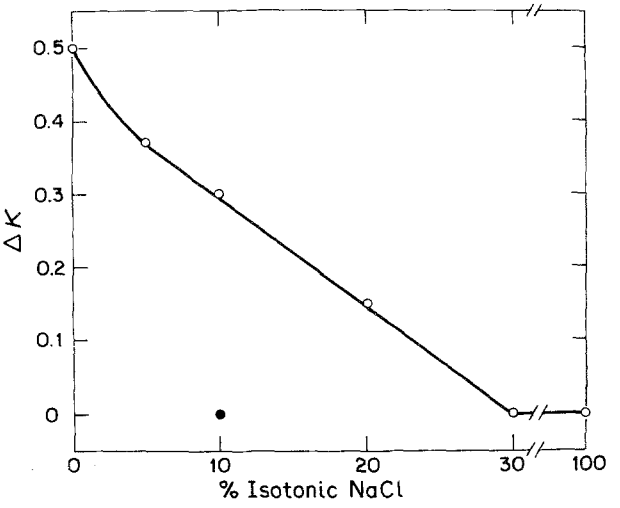

Fig. 8. Effect of ionic strength on the ouabain-induced decrease in conductance change. The magnitude of the field was 3.7 kV/cm, pulse duration 30 μsec, temperature 25 °C, hematocrit index 0.2, and ouabain concentration 50 μM. The isotonicity of the buffer was kept constant by use of mixtures of 0.15 M NaCl and 0.272 M sucrose. In a control experiment, isotonic choline chloride was used instead of sucrose to keep the isotonicity constant (filled circle)

prevented, and the membrane conductance signal was no more inhibited by the cross linking reagent. EDTA (1 mM) also completely removed the effect of cross linking reagent. The rate of swelling of the pulsed cross-linked cells was reduced as compared with untreated cells. This reduction in rate was a function of the applied field, the smaller the field, the larger the effect. Cu^{++}-phenanthroline cross-linked red cell membranes appeared to have an unchanged SDS electrophoresis gel pattern. This indicates that the cross linking experiment described here was highly specific, and the cross linking of about 200 Na^+/K^+ ATPase molecules (Harris & Kellermeyer, 1972) in the red cell membrane was not detectable by the SDS electrophoresis of the membrane proteins.

Effect of Ionic Strength

Kinosita and Tsong (1977*b*) have reported that the size of voltage-induced pores is larger for red cells treated in a low ionic strength medium than cells treated in high ionic strength medium. Interestingly, we have observed that the effect of ouabain in inhibiting the voltage-induced membrane conductance did not exist in high ionic media. Figure 8 shows that the ouabain suppressible membrane conductance decreased to zero at NaCl concentration of 45 mM. Substitution of sucrose by ionic species, choline also eliminated the ouabain suppressible conductance signal. No modification of the membrane conductance was observed for the Cu^{++}-phenanthroline cross-linked red cells if the voltage pulsation was done in a high ionic strength medium. All these experiments suggest that the site(s) of channel opening are variable according to the ionic strength of suspending medium, and at a high ionic medium Na^+/K^+ ATPase could no longer be opened by the externally applied electric field. This is consistent with the observation of Donlon and Rothstein (1969) that cation permeability of erythrocytes is extremely low in high ionic media, but is greatly enhanced in low ionic media. They have found a sharp change in the membrane cation permeability (K^+) around 30–45 mM NaCl in the external medium.

Discussion

Ionic transport mechanisms of the cell membrane are generally electrogenic, i.e., the rate of transport or the opening and closing of these channels depend on the potential difference across the cell membrane. Voltage pulsation of a cell suspension can generate a large transmembrane potential (Kinosita & Tsong, 1977*a*). The experiments presented in this communication consistently show that at a low ionic medium a voltage pulse exceeding a threshold potential can

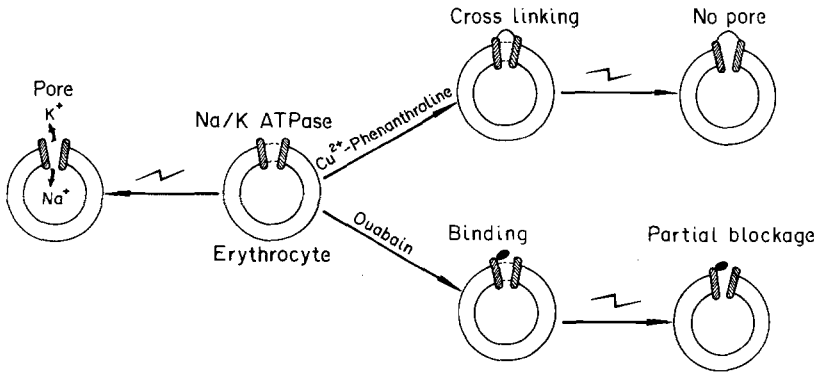

Fig. 9. Proposed mechanism of the opening of the Na^+ K^+ ATPase channels by an externally applied electric field. (*See* text for details)

force open Na^+/K^+ ATPase channels of the erythrocyte membrane. The presence of voltage-induced Na^+/K^+ ATPase channels was demonstrated by using the Na^+/K^+ transport inhibitor, ouabain, and a specific cross linking reagent to this enzyme.

Ouabain is known to be very specific in its action, and it blocks the active transport of Na^+ and K^+ ions across the erythrocyte membrane (Harris & Kellermeyer, 1972). Its binding sites are on the exocytoplasmic side. More precisely, the binding occurs at the large polypeptide (α chain) of the Na^+/K^+ ATPase (Forbush & Hoffman, 1979). Our result indicates that the effect of ouabain on the voltage-induced membrane conductance of erythrocytes are related to its action on the ATPase. The dose-response curve of the conductance decrease *vs.* the drug concentration (Fig. 2) gave a half-effect concentration of 0.15 μM, which is, within experimental uncertainty, identical to the inhibition constant for the ATPase (Harris & Kellermeyer, 1972). K^+ ion is a competitive inhibitor of the ouabain effect (Figs. 2 and 6), as it is for the ATPase activity (Harris & Kellermeyer, 1972). From our result we can conclude that roughly 35% of the voltage-induced pores in a low ionic medium are related to the ATPase. Since another 65% of pores occurred at as yet unidentified sites, it is not surprising that the critical field, as defined by Kinosita and Tsong (1977*b*) was not altered by this drug as shown in Fig. 1. Neither were the hemolysis and the K^+ leakage inhibited by the presence of ouabain.

The complex of cupric ion and 1–10 phenanthroline is known to induce cross linking between neighboring sulfhydryl groups (Kobashi, 1968). In an organized system such as a membrane, cross linking can occur only intramolecularly, or between associated polypeptides. This was used to demonstrate the oligomeric structure of a large number of the red blood cell membrane proteins (Steck, 1972; Wang & Richards, 1974). Only few sulfhydryl groups are available on the outer surface (Rothstein et al., 1974). Under our experimental conditions only these exocytoplasmic groups were able to cross link; none of the cytoplasma-exposed groups could react. This is shown by our electrophoresis studies which showed that band 3 (Fairbanks et al., 1971) was not significantly affected by the cross linking reaction. It has been shown that all the sulfhydryl groups of the anion transport protein, the major component of band 3, are on the cytoplasmic side of the erythrocyte membrane (Rao, 1979) and that a very effective cross linking of band 3 was obtained when ghosts were reacted with the cupric ion phenanthroline complex (Steck, 1972). On the other hand, a nonpenetrating SH reagent, pCMBS, was shown to inhibit the Na^+ active transport across the erythrocyte membrane (Harris & Kellermeyer, 1972). This suggests that some of the sulfhydryl groups available on the outer layer of the red blood cell are at the Na^+/K^+ pump sites. Cross linking of the polypeptidic subunits of the Na^+/K^+ ATPase by the cupric ion-phenanthroline complex has already been described (Kyte, 1975; Giotta, 1976). Under such conditions the Na^+/K^+ ATPase appears to be a very potent target for the cross linking reaction on the whole red blood cell. Since the amount of Na^+/K^+ ATPase per erythrocyte is very small, around 200 molecules per cell (Harris & Kellermeyer, 1972; Rothstein et al., 1974), no change is expected for the electrophoresis pattern of reacted and unreacted cells, as was the case in our experiment.

The experiments using ouabain inhibition and cross linking of the ATPase can be best summarized in Fig. 9. At a low ionic medium the Na^+/K^+ ATPase channels are accessible from the external medium. A voltage pulse that generates a transmembrane potential can break a weak interaction between the two major subunits of the enzyme and thus renders the channel freely permeable to K^+ and Na^+ ions. If the enzyme is cross linked, voltage treatment would still break the weak bonding, but pore formation is now restricted. Similarly, partial blockage of the pore formation can be accomplished by ouabain action.

The above picture must be taken as tentative, and is applicable only in a low ionic condition. At a high ionic medium it is believed that the ATPase is not perforable by the voltage pulsation. As mentioned, Donlon and Rothstein (1969) have shown that K^+ transport rate of erythrocyte is extremely low at high ionic strength, but dramatically increases in a low ionic medium. Previous experiments have already noted differences between pores induced in the high and in the low ionic strength buffers (Kinosita & Tsong, 1977*a*). Our experiments have demonstrated that different kinds of pores can be generated by a voltage pulse, and at high ionic content, few, if any of these pores are related to the ATPase.

What are then the nature of the pores generated in a high ionic medium, or the other 65% of pores in a low ionic medium? Although lipid matrix could be a site of a dielectric breakdown (*see*, e.g., Benz, Beckers & Zimmermann, 1979), we submit that pores that occur in the lipid matrix should be highly reversible and are unlikely to have a long lifetime, as was found for pores in the red cell membranes[2]. Consequently, further studies of these unidentified voltage-perforable sites are needed. One might also mention that Kinosita and Tsong (1979) have estimated the number of pores per cell to be small, in the order of several hundreds.

A recent study by Hoffman, Kaplan and Callahan (1979) has demonstrated that the Na^+/K^+ pump of red blood cells is electrogenic. The operation of this pump can generate several mV of transmembrane potential. Our result is consistent with their observation. Although the voltage required for the opening of these channels is much higher, the voltage pulses used are very short, only in microseconds, and it is known that the threshold voltage depends rather strongly on the pulse width (Kinosita & Tsong, 1977*b*).

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References

Benz, R., Beckers, F., Zimmermann, U. 1979. Reversible electrical breakdown of lipid bilayer membranes: A charge-pulse relaxation study. *J. Membrane Biol.* **48**:181

Donlon, J.A., Rothstein, A. 1969. The cation permeability of erythrocytes in low ionic strength media of various tonicities. *J. Membrane Biol.* **1**:37

Edidin, M. 1974. Rotational and translational diffusion in membranes. *Annu. Rev. Biophys. Bioeng.* **3**:179

Fairbanks, G., Steck, T.L., Wallach, D.F.H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606

Forbush, B., Hoffman, J.F. 1979. Evidence that ouabain binds to the same large polypeptide chain of dimeric Na,K ATPase that is phosphorylated from P_i. *Biochemistry* **18**:2308

Giotta, G.J. 1976. Quaternary structure of $(Na^+ + K^+)$ dependent adenosine triphosphatase. *J. Biol. Chem.* **251**:1247

Harris, J.W., Kellermeyer, R.W. 1972. The Red Cell. p. 474. Harvard University Press, Cambridge

Hoffman, J.F., Kaplan, J.H., Callahan, T.J. 1979. The Na:K pump in red cells is electrogenic. *Fed. Proc.* **38**:2440

Kinosita, K., Jr., Tsong, T.Y. 1977*a*. Hemolysis of human erythrocytes by a transient electric field. *Proc. Nat. Acad. Sci. USA* **74**:1923

Kinosita, K., Jr., Tsong, T.Y. 1977*b*. Voltage-induced pore formation and hemolysis of human erythrocytes. *Biochim. Biophys. Acta* **471**:227

Kinosita, K., Jr., Tsong, T.Y. 1977*c*. Formation and resealing of pores of controlled sizes in human erythrocyte membrane. *Nature (London)* **268**:438

Kinosita, K., Jr., Tsong, T.Y. 1978. Survival of sucrose-loaded erythrocytes in the circulation. *Nature (London)* **272**:258

Kinosita, K., Jr., Tsong, T.Y. 1979. Voltage-induced conductance in human erythrocyte membranes. *Biochim. Biophys. Acta* **554**:479

Kobashi, K. 1968. Catalytic oxydation of sulfhydryl groups by *o*-phenoanthroline copper complex. *Biochim. Biophys. Acta* **158**:239

Kyte, J. 1975. Structural studies of sodium and potassium ion-activated adenosine triphosphatase. *J. Biol. Chem.* **250**:7443

Rao, A. 1979. Disposition of the band 3 polypeptide in the human erythrocyte membrane. *J. Biol. Chem.* **254**:3503

Riemann, F., Zimmerman, U., Pilwat, G. 1975. Release and uptake of haemoglobine and ions in red blood cells induced by dielectric breakdown. *Biochim. Biophys. Acta* **394**:449

Rothstein, A., Knauf, P.A., Cabantchick, Z.I., Balshin, M. 1974. The location and chemical nature of drug 'targets' within the human erythrocyte membrane. *In:* Drug and Transport Process. B.A. Callingham, editor. p. 53. University Park Press, Baltimore

Sale, A.J.H., Hamilton, W.A. 1968. Effects of high electric fields on microorganisms. III. Lysis of erythrocytes and protoplasts. *Biochim. Biophys. Acta* **163**:37

Steck, T.L. 1972. Cross-linking of the major proteins of the isolated erythrocyte membrane. *J. Mol. Biol.* **66**:295

Tsong, T.Y., Kingsley, E. 1975. Hemolysis of human erythrocyte induced by a rapid temperature jump. *J. Biol. Chem.* **250**:786

Tsong, T.Y., Tsong, T.T., Kingsley, E., Siliciano, R. 1976. Relaxation phenomena in human erythrocyte suspensions. *Biophys. J.* **16**:1091

Wang, K., Richards, F.M. 1974. An approach to nearest neighbor analysis of membrane proteins. Application of the human erythrocyte membrane of a method employing cleavable cross-linkages. *J. Mol. Biol.* **249**:8005

Weber, K., Osborn, M. 1975. Proteins and sodium dodecyl sulfate: Molecular weight determination on polyacrylamide gels and related procedures. *In:* The Proteins. H. Neurath and R.L. Hill, editors. p. 180. Academic Press, New York

[2] *See* footnote 1, p. 134.

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