J. Membrane Biol. 18, 125–144 (1974)

Calcium-Related Hyperpolarization of the *Amphiuma* Red Cell Membrane Following Micropuncture

U. V. Lassen, L. Pape, B. Vestergaard-Bogind and O. Bengtson

University of Copenhagen, Zoophysiological Laboratory B,
13 Universitetsparken, DK 2100 Copenhagen Ø, Denmark

Received 8 March 1974

Summary. The erythrocyte of *Amphiuma means* was chosen as a model for elucidation of membrane properties of red cells because the large size of this cell permitted direct measurements of plasma membrane potential. In the 30-sec period following micropuncture and withdrawal of the electrode the plasma membrane reseals and hyperpolarizes to a value of about −50 mV. The hyperpolarization is followed by a gradual return to the unperturbed potential of −15 mV. The magnitude of the hyperpolarization is strongly reduced by an increase in extracellular K concentration and is therefore related to an increase in relative K permeability. The transference number for K is calculated to have a maximal value of about 0.6. However, it is not yet clear whether the hyperpolarization can be solely attributed to a rise in K permeability, or whether there is a concomitant decline in Cl permeability as well. The magnitude of the hyperpolarization is unaffected by the presence of either ouabain or oligomycin.

Extracellular Ca is prerequisite to the observed hyperpolarization and presumably acts by permitting the membrane to seal, and having entered the cell during the leak period, causes an increase in the relative K permeability. This permeability change resembles the Ca-induced rise in K permeability seen in metabolically depleted human red cells and red cell ghosts. An important difference, however, is that the *Amphiuma* red cells used in the present study are neither poisoned nor metabolically depleted so that the Ca effect is not prevented by the presence of cellular ATP as seems to be the case in human erythrocytes. The transient nature of the hyperpolarization may be related to the active transport of Ca out of the cell.

Evidence of the rapid exchange of labeled chloride across the red cell membrane (Tosteson, 1959; Dalmark & Wieth, 1970) suggested that the membrane potential is equal to the equilibrium potential for chloride across the plasma membrane. However, it is well established both from direct measurements of membrane resistance (Hoffman & Lassen, 1971; Lassen, Pape & Vestergaard-Bogind, 1973) and from indirect estimates using valinomycin to increase the membrane conductance to K (Hunter, 1971;

Tosteson, Gunn & Wieth, 1973) that the chloride conductance is several orders of magnitude lower than calculated from the rate of $^{36}Cl^-$ exchange. This means that chloride ions are not necessarily in equilibrium under all experimental conditions and that measurements of membrane potential in red cells may give valuable information about the properties of the membrane that cannot be derived simply from considering the chloride distribution.

Measurement of the membrane potential in most single cells in suspension is technically difficult and the results obtained must be interpreted with caution (Lassen, Nielsen, Pape & Simonsen, 1971). Penetration of the cell membrane with a microelectrode tip seems as a rule to give rise to a leak which causes a change in the original potential. In red cells, which have a very high membrane resistance, it does not seem possible to obtain a sufficiently good sealing of the membrane around a microelectrode tip to allow stable potential measurements. The rate of discharge of the potential after impalement of the cell is a function of the total membrane capacitance of the individual cell membrane. Since plasma membranes seem to have similar specific capacitances (*see* Katz, 1966) cells must be expected to have a total membrane capacitance which is proportional to the surface area. For these reasons giant red cells of the salamander *Amphiuma means* were chosen for studies of membrane potential and related properties of erythrocytes (Hoffman & Lassen, 1971). In this study it proved possible to obtain a measure of the membrane potential before major changes due to discharge had taken place. During these investigations of the membrane potential it was incidentally observed that if a given cell was impaled by a microelectrode and the electrode withdrawn after 1 to 2 sec, a second micropuncture within 1 min revealed a marked hyperpolarization of the membrane.

In the present paper it is demonstrated that the hyperpolarization following micropuncture of an *Amphiuma* red cell is related to a marked increase in relative K permeability of the cell membrane. This characteristic change is transient and requires Ca ions in the suspending medium. The phenomenon bears a close resemblance to the Ca-induced K loss from metabolically depleted red cells (Gárdos, 1958) and red cell ghosts, as well as possibly to the effect of lead on the red cell membrane (*see* Riordan & Passow, 1973). But in contrast to these effects, the K permeability increase described in the present paper is transient despite the continued presence of Ca ions in the suspension medium. In addition, aspects of the hyperpolarization give indirect evidence for a very low chloride conductance of the red cell membrane.

Part of the material has been presented in short form elsewhere (Lassen, 1972; Lassen *et al.*, 1973).

Materials and Methods

Animals and Collection of Cells

The red cells used for all experiments were taken from specimens of *Amphiuma means* weighing 500 to 2000 g. The animals were obtained from Carolina Biological Supply Company, Burlington, N.C. and shipped air freight to Denmark. The stock of animals was maintained in running tap water tanks at a temperature of 15 to 17 °C and were fed strips of beef-heart once a week. In this way more than 95% of the population have survived for periods of up to three years in captivity. One hour prior to collection of blood by puncture of the atria of the heart, the animals were anaesthetized by immersion in water with 5 g per liter of MS 222-Sandoz (Sandoz Ltd., Basel). Blood was drawn into a disposable syringe containing cold Ringer's without anticoagulant. The sample (up to 8 ml blood from one animal) was immediately diluted in 100 ml cold, stirred Ringer's to avoid coagulation. The cells were rapidly washed two times in Ringer's and subsequently stored at 16 to 17 °C until used (usually less than 2 hr). If the initial centrifugation showed hemolysis of the cells, the sample was discarded. Individual animals were bled at intervals of no less than 2 months.

The composition of the various media used for incubation is shown in Table 1. For experiments with different K concentrations the change in K content was compensated for by equivalent changes in Na concentrations. Ouabain (g-Strophantin extra pure, Merck, Darmstadt) containing Ringer's was prepared by dissolving the substance in the medium under stirring at room temperature for 12 to 14 hr before use. Oligomycin containing Ringer's was made by addition of 10 ml per liter Ringer's of a 5 mg/ml oligomycin (Sigma, lot 115 B-1580, St. Louis, Mo.) in pure ethanol. Phloretin (K & K Laboratories Inc., Plainview, N.Y.) used in a final concentration of 0.25 mM, was added to the Ringer's in a similar fashion (100× dilution of a 25 mM solution in ethanol). Since 1% ethanol in the control Ringer's did not influence the measured membrane potentials, ethanol was not routinely added to the controls. Other chemicals used were analytical grade.

For determination of cellular concentrations of Na and K, 100 μliters of a cell suspension (cell volume fraction about 0.25) were transferred to a centrifuge tube and centrifuged for 10 sec at 2,000 to 4,000× *g*. Fifty μliters of the supernatant were removed for extracellular determinations and the cells washed twice in 1,300 μliters isotonic Tris-Cl, pH 7.2. Finally, the cells were hemolyzed with 50 μliters saponin (0.01%) and the protein precipitated by addition of four volumes of 6% perchloric acid. The concentrations of Na and K were determined by emission-flame-photometry (Eppendorff, Hamburg, Germany).

Table 1. Composition of incubation media

mM	"Normal Ringer's"	K Ringer's	Ca-free Ringer's
Na^+	118	13.7	118
K^+	2.5	104	2.5
Cl^-	124	124	121
Ca^{++}	1.8	1.8	0
MOPS[a]	10	10	10

[a] Morpholinopropane sulphonic acid (Sigma Chemical Co., St. Louis, Mo.) titrated to pH 7.2 with 1 M NaOH.

All media contained 1 g of bovine serum albumin (Povite Corp., Amsterdam) per liter.

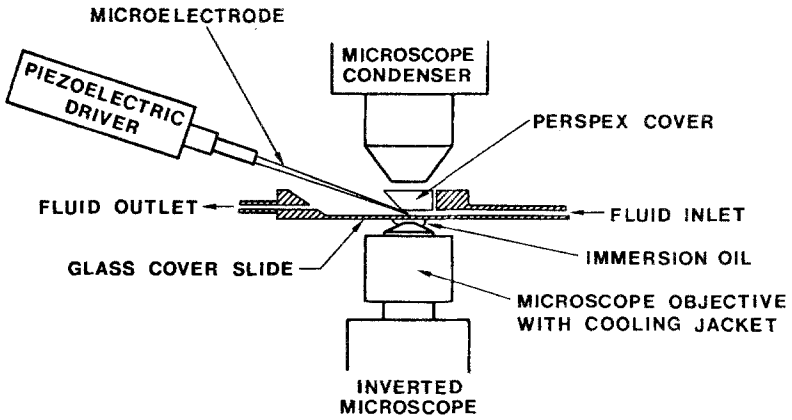

Fig. 1. Schematic drawing of experimental set-up. The cells are allowed to precipitate on the bottom of the chamber located above the objective of an inverted microscope. The microelectrode is attached to a tubular piezoelectric electro-mechanical transducer and is positioned by a micromanipulator. The temperature of the chamber and its contents is maintained constant by means of a cooling jacket surrounding the microscope objective and is measured by means of a small thermistor (not shown). The inlet and outlet permit rapid rinsing of the chamber and introduction of cell suspensions

The chloride content of cellular and extracellular phases was measured on deproteinized samples by automatic coulometric titration (CMT 10, Radiometer, Copenhagen). For determination of cellular chloride, a 200-µliter sample of cell suspension was centrifuged and 125 µliters of the extracellular phase removed. The cells in the remaining fluid were lysed by addition of 50 µliters of saponin solution (0.01 %) and then deproteinized with 1,000 µliters of perchloric acid (6 %).

Cellular concentrations of chloride were calculated on the basis of the determined cell volume fraction and a predetermined average value for cellular water content of 76.9 wt/vol %.

Fig. 1 shows the experimental set-up for microelectrode measurements of the membrane potential. The chamber containing the cell suspension was 0.3 mm in height and located above the objective of an inverted microscope. The temperature of the chamber was kept constant at 17 °C by heat transmission through the immersion oil of the objective to a cooling jacket surrounding the objective. The chamber could be flushed for rinsing and for introduction of cell suspensions.

The microelectrode was advanced through the cell membrane by means of a piezoelectric transducer which gave a movement of about 2 µm within 50 µsec on application of 400 V. Technical details of the electronics will be given elsewhere (Bengtson & Lassen, *in preparation*).

Conventional microelectrodes with tip diameters of 0.2 µm were filled with water by zonal distillation (Zeuthen, 1971) followed by exchange of the water with 3 M membrane-filtered KCl. In the experiments where Ca injection was attempted, 30 mM $CaCl_2$ was added to the KCl. The microelectrodes had tip potentials of less than 5 mV.

Results

A typical potential trace from a micropuncture of an *Amphiuma* red cell suspended in Ringer's at pH 7.2 is shown in Fig. 2A. As the cell membrane

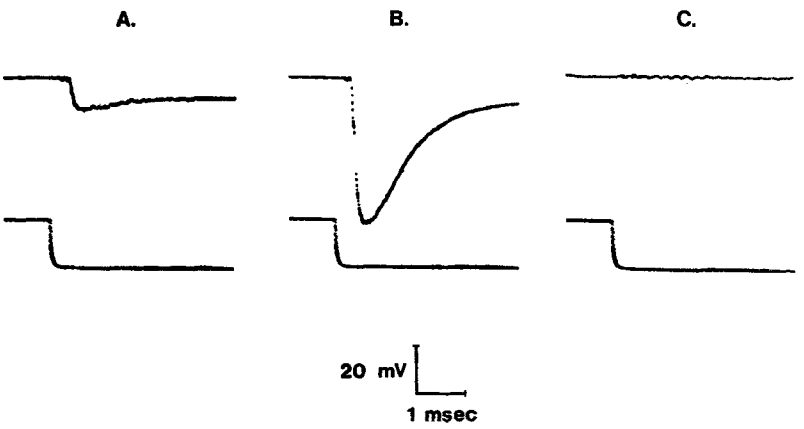

Fig. 2. *Frame A* shows a typical recording of the membrane potential of *Amphiuma* red cells. The top trace indicates the potential shifts upon penetration of the membrane. The sudden drop in potential indicates that the membrane has been penetrated by the electrode. The potential reaches a minimal value within less than 200 μsec which is taken as the membrane potential of the cell. The subsequent decay of the potential to a less negative value is ascribed to a leak around the electrode. The lower trace monitors the driving voltage for advancement of the microelectrode. "Normal Ringer's", pH 7.2, 17 °C. *Frame B.* Potential recording on penetration of a cell which had been micropunctured 30 sec in advance. The electrode was withdrawn 1 sec after the first puncture. During the following period the cell membrane undergoes a marked hyperpolarization as indicated by the large potential drop recorded by the microelectrode. Other experimental data as in frame *A*. *Frame C.* Potential trace where the microelectrode is advanced in Ringer's without contacting a cell. Other data as in frame *A*

is penetrated, there is a sharp change in potential. A peak value of about −15 mV is reached in less than 200 μsec after the sudden break in the trace. From this peak value the potential gradually achieves a stable level of some −8 mV in a few milliseconds. For reasons discussed in an earlier publication (Lassen *et al.*, 1971), the peak value of the potential is considered a measure of the membrane potential before it was perturbed by impalement of the cell. One second after the puncture, the electrode tip was withdrawn from the cell and after an additional 30 sec the same cell was again punctured with the result shown in Fig. 2B. The membrane potential was now markedly more negative as indicated by the magnitude of the potential peak. Under the present experimental conditions such highly negative potentials were only observed in cells which had been previously punctured. Even marked deformation of the cell membrane with the microelectrode tip did not elicit a hyperpolarization unless the membrane was actually pierced. Fig. 2*C* is a recording with the electrode advanced in the Ringer's without making contact with cells. The actual movement of the electrode occurred about 100 to 400 μsec after

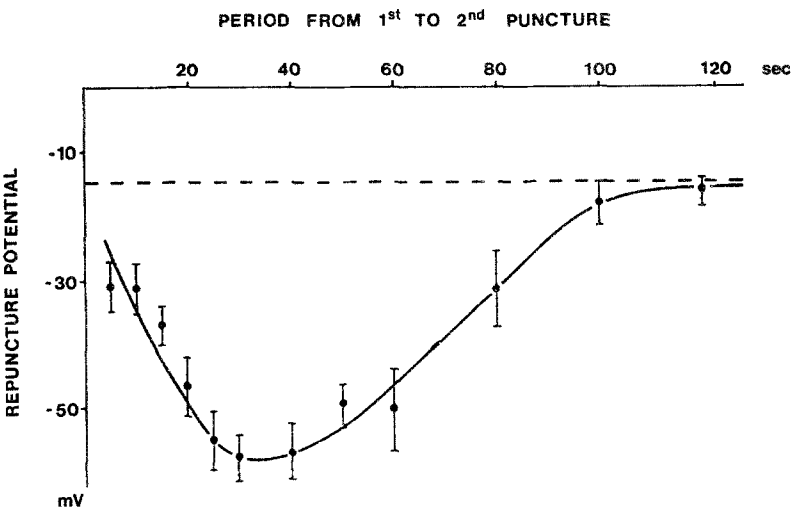

Fig. 3. Time course of the hyperpolarization induced by micropunture of the *Amphiuma* red cell membrane. The individual cells were punctured and the electrode withdrawn after 1 sec. The same cell was subsequently repunctured after a time lapse indicated on the abscissa. Each point represents a large number of cells, none of which was micropunctured more than twice. Abscissa: time in sec; ordinate: membrane potential in mV. Values of the membrane potentials are given ± 1 SEM. "Normal Ringer's", pH 7.2, 17 °C

termination of the artifact. There is no sign of an electrokinetic response from the electrode. The same is true if the piezoelectric transducer is activated with the electrode tip still in the cell and thus within the protein-rich cytoplasm.

To clarify the nature of the potential changes seen on repeated micropuncture of the same cell, the variation in membrane potential was studied as a function of time after the initial puncture. This presented certain difficulties because, as is obvious, micropuncture severely influences the properties of the cell membrane. Thus it was not possible to obtain meaningful results by merely leaving the electrode tip in the cell for extended periods. Therefore the timecourse of the hyperpolarization was followed in a large population of cells by puncturing each cell twice. The initial (unperturbed) membrane potential was measured by the first puncture which at the same time initiated the process leading to hyperpolarization. The second puncture, at a given time interval after the first, provided a measure of the subsequent change in membrane potential. The results are shown in Fig. 3 and represent the time course of the membrane potential following the initial puncture. The abscissa represents the interval between the first ($t=0$) and second puncture, and the ordinate shows the recorded second or repuncture poten-

Table 2. Cellular concentrations of Na, K and Cl ions and their corresponding equilibrium potentials

	mM per liter cell water[a]	Equilibrium potential[b] (mV)
Na	9.5±0.5	+ 64
K	129 ±0.8	−100
Cl	58.6±1.8	− 19

[a] Cells were incubated in "Normal Ringer's", pH 7.2 and cellular concentrations given ±1 SEM.

[b] Equilibrium potentials are calculated on the basis of cellular and extracellular ("Normal Ringer's") concentrations.

tial. It is apparent that the hyperpolarization reaches a maximum at about 30 to 40 sec after the initial puncture. From this point there is a gradual return to the original membrane potential in less than 2 min.

The *Amphiuma* red cell has a high potassium and low sodium concentration as have red cells from many other species including most mammals. Table 2 shows the concentrations of K, Na and Cl in the cells and the corresponding equilibrium potentials in the Ringer's solution normally employed. The values for the intracellular concentrations are calculated under the assumption of uniform distribution of ions in the total cell water. Equal activity coefficients in the extracellular and intracellular water phases have been assumed in calculation of the equilibrium potentials. If the hyperpolarization described above is caused by changes in passive permeabilities, only the K ions with an equilibrium potential more negative than the potential of the unperturbed cell membrane could have a net movement in a direction (out of the cell) which would render the membrane potential more negative. In this regard it is worth noticing that since the hyperpolarization never exceeds the Nernst potential for K, a downhill movement is possible.

If the hyperpolarization is associated with changes in the relative membrane permeability for K, this should be revealed by variation of the external K concentration (in exchange for Na). Fig. 4 shows the effects of various K concentrations in the medium on the initial membrane potential and potential on repuncture after 30 sec. Whereas the change in K concentration has no significant effect on the initial membrane potential, the potential after 30 sec shows a strong K dependence. The decline in hyperpolarization with increasing K concentration in the medium is maximally about 38 mV per 10-fold concentration change.

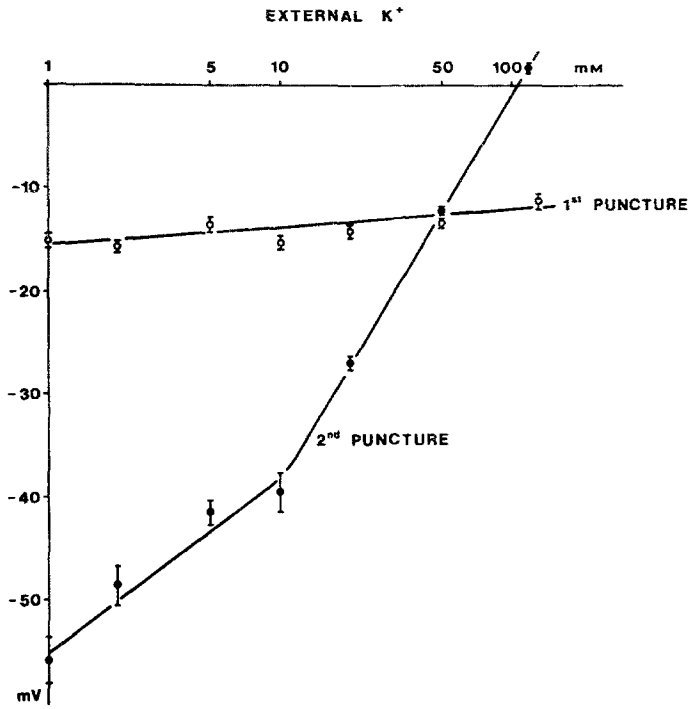

Fig. 4. Membrane potential as a function of the logarithm of the external K concentration. The curve marked "1st puncture" represents the membrane potentials of cells which had not previously been subjected to micropuncture. There is a negligible depolarization with increasing K concentration. The potentials recorded on 2nd puncture, 30 sec after the first, show a hyperpolarization comparable to that in Figs. 2 and 3. The potential recorded on the second puncture is markedly reduced as the K concentration of the medium is raised. In these experiments changes in K concentration were compensated for by an equal and opposite variation in the Na concentration of the medium. Abscissa: external K concentration (log scale); ordinate: membrane potential in mV. The mean potential values are indicated ± 1 SEM. pH 7.2, 17 °C

From the experiments described above, it is evident that micropuncture of *Amphiuma* red cells results in a transient increase in the relative K permeability of the membrane to an extent where the K gradient across the membrane is a major factor in determining the magnitude of the membrane potential.

A closer examination of the early phase of the hyperpolarization was made in order to determine how soon after the initial puncture the membrane potential began to change. A plot of the potentials sampled from 2 to 15 sec after the initial puncture is shown in Fig. 5. Two seconds after the initial puncture the peak potential is about -8 mV and is equal to the stable junction potential seen during the initial puncture (*see* Fig. 2). From this

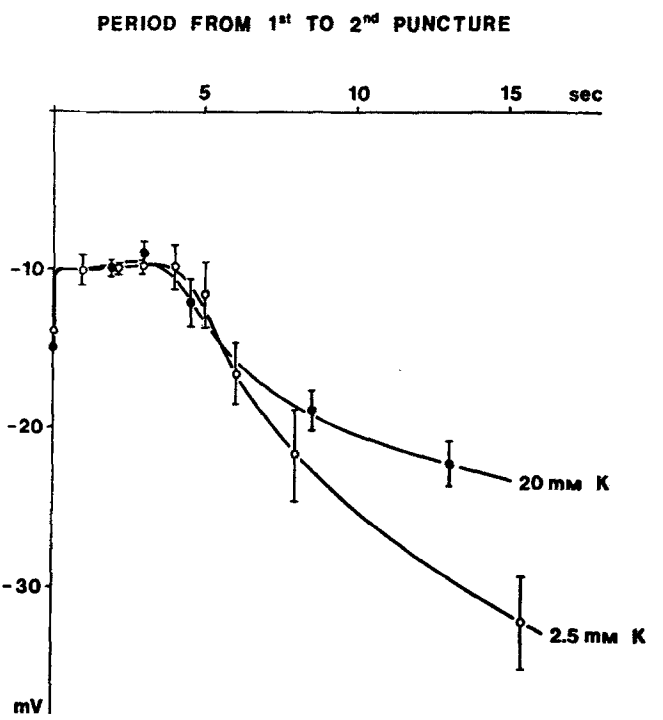

Fig. 5. Initial time course of the potential following micropuncture. The experimental procedure is the same as in Fig. 3, but in these experiments special care was taken to obtain measurements shortly after the initial micropuncture. After the very rapid depolarization to about -10 mV, the membrane potential stays essentially constant for about 5 sec. This lag period before the hyperpolarization begins is independent of the K concentration of the medium (2.5 or 20 mM) and thus of the final potential level achieved after 30 sec (*see* Figs. 3 and 4). Abscissa: time in sec; ordinate: membrane potential in mV. The mean potential values are indicated ± 1 SEM. pH 7.2, 17 °C

value, at which the cell appears to be electrically leaky, the membrane potential begins to change to a more negative value after about 5 sec. This indicates that the hole in the membrane produced by the initial penetration has sealed sufficiently to allow recharging of the membrane capacitance. Therefore it is reasonable to assume that triggering of the mechanism responsible for the hyperpolarization occurs between penetration of the membrane and its subsequent resealing. The possibility that KCl leaking from the electrode was responsible for the change in potential was ruled out, however, because the hyperpolarization measured with NaCl or Na-acetate-filled electrodes was essentially identical with that measured with normal KCl-filled electrodes.

It has been demonstrated that the concentration of Ca in red cells is extremely low (Lichtman & Weed, 1972). This low intracellular concentra-

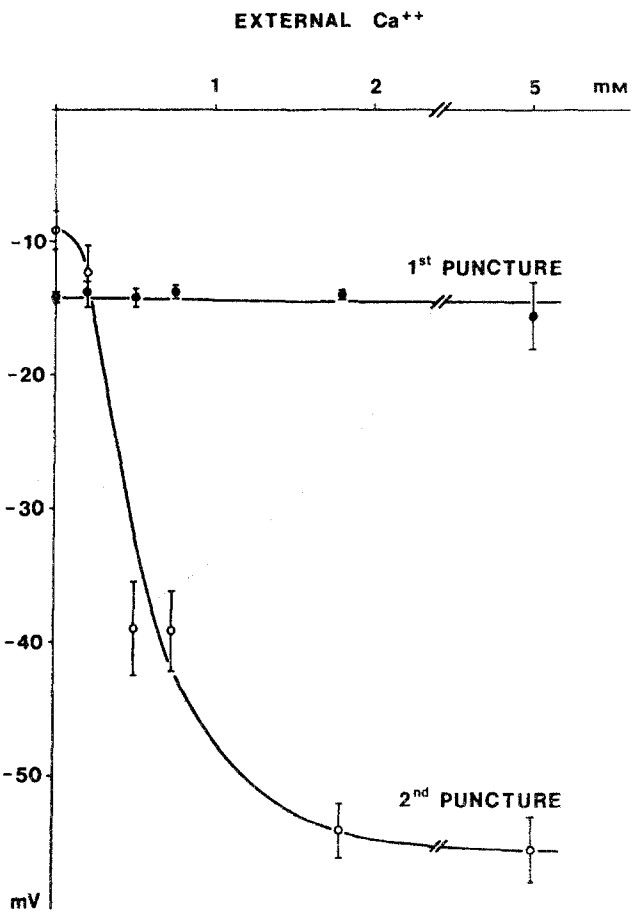

Fig. 6. Effect of external Ca concentrations on initial membrane potentials and potentials recorded 30 sec after the initial micropuncture. Abscissa: Ca concentration of medium in mM; ordinate: membrane potential in mV. Mean potential values are indicated ± 1 SEM. pH 7.2, 17 °C

tion is maintained by a metabolically driven Ca pump that counterbalances the constant leakage of Ca into the cells (Schatzmann & Vincenzi, 1969). As shown by a number of authors (*see* Riordan & Passow, 1973), when poisoned red cells or ATP-depleted red cell ghosts are incubated in a medium containing Ca, there is an increased cellular uptake of Ca and an associated marked loss of cellular KCl. Although this Ca-dependent increase in membrane permeability for K ions has not as yet been demonstrated in cells with a normal ATP content (and without further additions to the medium), it is conceivable that the transient increase in K permeability following micropuncture in the *Amphiuma* red cell is related to the influx

Table 3. Effects of Mg, phloretin, and picrate on membrane potentials measured in Ca-free Ringer's

Condition	Membrane potential	
	First puncture (mV)	30-sec Repuncture (mV)
Control	-11.7 ± 0.5	-7.8 ± 0.8
2×10^{-3} M Mg	-13.6 ± 1.8	-8.0 ± 1.4
2.5×10^{-4} M phloretin	-44.0 ± 2.2	-5.8 ± 0.9
2.5×10^{-4} M phloretin+2×10^{-3} M Mg	-38.7 ± 1.5	-6.6 ± 0.3
2.5×10^{-4} M phloretin+2×10^{-2} M Mg	-38.4 ± 2.1	-9.3 ± 0.2
3×10^{-3} M picrate+2×10^{-2} M Mg	-38.9 ± 3.9	-2.7 ± 0.9

Values for membrane potentials are given ±1 SEM.

of a substantial amount of Ca prior to the resealing of the membrane after the initial puncture. If this were the case, the hyperpolarization as described should be absent in cells suspended in Ca-free media. Fig. 6 shows the hyperpolarization measured 30 sec after the initial puncture as a function of Ca in the medium. Between the normal Ca concentration of 1.8 and 5 mM the response is constant. But at concentrations below 0.4 mM there is no sign of hyperpolarization regardless of the time interval between the first and the second puncture. On the contrary, a small depolarization of about 5 mV with respect to the initial puncture is constantly observed. Ringer's without $CaCl_2$ and with 1 mM EDTA or EGTA gave identical results. Thus the potentials obtained upon the second puncture in low Ca or Ca-free media were not significantly different from the stable potential level measured during micropuncture. This stable potential is considered to be a composite diffusion potential between the cytoplasm and the external medium at the site of damage in the cell membrane (*see* Lassen *et al.*, 1971). Under these circumstances, the depolarization of the membrane to the stable value might be due to the absence of Ca ions which may play a role in the sealing of the cell membrane. Magnesium accelerates resealing of human red cell ghosts after osmotic lysis (Hoffman, 1962; Bodemann & Passow, 1972). However, as shown in Table 3, addition of $MgCl_2$ did not produce signs of resealing of the *Amphiuma* red cell membrane in Ca-free media.

As further evidence that Ca is required for resealing of the membrane, repuncture potentials were measured in the presence of either picrate or phloretin. These substances cause the *Amphiuma* red cell membrane to undergo strong hyperpolarization even in the absence of Ca in the medium

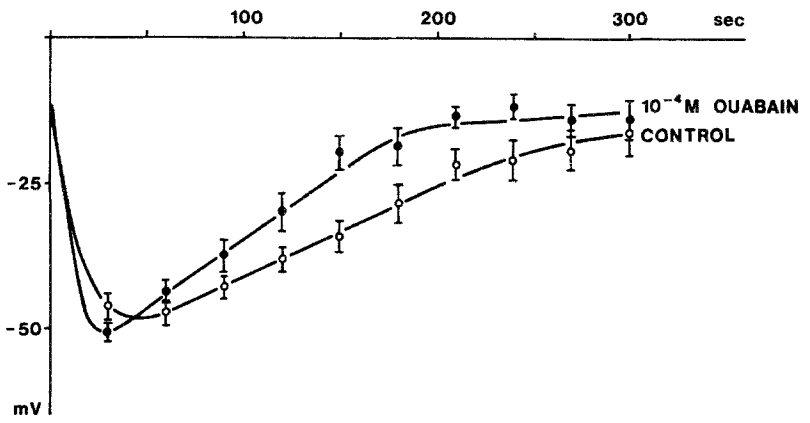

Fig. 7. Effect of repeated punctures at intervals of 30 sec on individual cells. Each cell was punctured a total of 11 times. Control cells were measured in "normal Ringer's". Ouabain-treated cells were preincubated 5 to 10 min in 10^{-4} M ouabain. Abscissa: time in sec; ordinate: membrane potential in mV. Mean potentials ± 1 SEM. pH 7.2, 17 °C

(Vestergaard-Bogind, Pape & Lassen, 1973; Vestergaard-Bogind & Lassen, 1974). If resealing could take place in spite of the lack of Ca, then the initial hyperpolarization produced by picrate or phloretin should be present on repuncture. The results of this experiment are shown in Table 3. As demonstrated by the hyperpolarization seen on repuncture, the membrane is apparently capable of resealing in the presence of phloretin or picrate provided the Ringer's solution contains Ca. On the other hand, although the first puncture in Ca-free Ringer's containing either phloretin or picrate shows the expected highly negative value for the membrane potential, resealing apparently does not occur as judged from the small negative potential measured on repuncture.

If the hyperpolarization induced by micropuncture is associated with an increase in K permeability, there should be a concomitant loss of K ion from the cell. This should be manifest as a time-dependent change in the magnitude of the hyperpolarization. A method for estimating the integrated K loss during the leak period and subsequent hyperpolarization would be to record the membrane potentials obtained with repetitive punctures of the same cell over an extended period. Single cells were punctured every 30 sec for 5 min so that each cell was subjected to a total of 11 consecutive micropunctures. This procedure proved experimentally difficult and only cells which did not show signs of mechanical damage after 5 min were included in the data presented in Fig. 7. Each point on the graph represents an average

Table 4. Effect of ouabain and oligomycin on micropuncture-induced hyperpolarization

Condition	Membrane potential	
	First puncture (mV)	30-sec Repuncture (mV)
Control	-17.1 ± 0.7	-51.4 ± 2.4
10^{-4} M ouabain	-14.2 ± 0.6	-47.8 ± 2.5
Oligomycin 50 μg/ml	-14.6 ± 0.2	-51.1 ± 3.1

Values for potential given ± 1 SEM.

of at least five cells. The cells show a slow decline in hyperpolarization with time, consistent with a possible decrease in cellular K. In addition to the loss of K ion, penetration of the membrane can be expected to result in entry of Na through the leak. This in turn should accelerate the rate of active transport across the cell membrane tending to restore the original ion gradient. Thus, repetitive punctures in the presence of ouabain may alter the rate with which the original ion concentrations are reestablished. Addition of 10^{-4} M ouabain to the Ringer's solution accelerated the disapprearance of the hyperpolarization, most likely due to blocking of the Na/K pump.

Blum and Hoffman (1971, 1972) have reported that both ouabain and oligomycin inhibit the Ca-induced KCl loss in human red cell ghosts. It was proposed that this was due to a direct interference with the mechanism leading to the increased K permeability. Therefore, it was of interest to investigate the effect of ouabain and oligomycin on the magnitude of hyperpolarization following micropuncture. Table 4 is a survey of the 30-sec repuncture potential in normal Ringer's and in the presence of either ouabain or oligomycin. It is clear that neither of the two substances influence the 30-sec hyperpolarization.

A more direct proof that Ca on the inside of the membrane is responsible for the high relative K permeability that leads to hyperpolarization would be to inject Ca into the cell in a Ca-free medium. Such experiments have been attempted using microelectrodes filled with 3 M KCl and 30 mM $CaCl_2$ as shown in Table 5. After penetration of the cell membrane, a slight positive pressure was applied to the contents of the electrode until a drop in the electrode resistance indicated that the diffusion zone in the region of the electrode tip was dominated by the strong electrolyte solution. After 1 to 5 sec of injection, suction was applied and the electrode was withdrawn from the cell. The cell was then repunctured 30 sec later. These experiments gave

Table 5. Membrane potentials measured in Ca-free Ringer's using $CaCl_2$-containing electrodes

Electrode solution	Membrane potential	
	First puncture (mV)	30-sec Repuncture (mV)
30 mM $CaCl_2$, 3 M KCl	−12	− 8
	−10	−18
	−13	−40
	−12	−14
	− 8	−40
	− 9	−16
	−10	−16
	− 8	−46
	−12	−18
	− 7	−22
3 M KCl (control)	−14	− 4
	−10	− 5
	− 9	− 7
	−10	− 6
	−14	− 7
	−14	− 8
	−11	− 5
	−12	− 5
	−14	− 7
	−11	− 5

A slight positive pressure was applied to the contents of the electrode when the tip was inside the cell. The above data are values from a typical experiment.

highly variable results, partly because the cells frequently burst. However, some of the cells actually hyperpolarized in response to the electrodes with $CaCl_2$. This was never observed in Ca-free Ringer's without $CaCl_2$ in the electrode. But despite the precautions taken, the possibility that a minor amount of $CaCl_2$ was deposited outside the cells cannot be excluded.

Discussion

The present work has shown that micropuncture of the *Amphiuma* red cell membrane is followed by a period in which the membrane is first depolarized, then transiently hyperpolarized. This hyperpolarization depends on the presence of Ca in the medium despite the fact that the membrane

potential of the unperturbed cell is independent of the external Ca concentration.

The effect of calcium may be related to a change in intracellular concentration of that ion. Lichtman and Weed (1972) have demonstrated that essentially all of the detectable Ca in human red cells is present in the membrane. This supports the suggestion by other authors (Romero & Whittam, 1971; Blum & Hoffman, 1972; Porzig, 1972) that the intracellular concentration of Ca is extremely low, although no exact figures are presently available. Ca in the cytoplasm is furthermore bound by complex formation to ATP and other metabolites (*see* Riordan & Passow, 1971). The low intracellular Ca concentration is maintained by an active transport mechanism which is probably closely related to a Ca-activated ATPase (Schatzmann & Vincenzi, 1969). Fluoride poisoning of human red cells leads to a net KCl-loss from the cells (Wilbrandt, 1940) which was shown to be dependent on the presence of Ca in the medium (Gárdos, 1958). Furthermore, the K permeability increase resulting in KCl loss can be evoked by a rise in the intracellular concentration of Ca as demonstrated by Blum and Hoffman (1972) and Riordan and Passow (1971) in experiments where Ca was incorporated into human red cell ghosts.

By parallel reasoning, the hyperpolarization following micropuncture of the *Amphiuma* red cell may be related to the entry of Ca ions through the hole in the membrane caused by the penetration of the microelectrode. Based on the duration of the initial depolarization of the membrane (Fig. 5) the lag period before resealing is about 5 sec. Considering the large inward gradients for Ca, it is likely that Ca ions diffuse into the cell during this period of mechanical damage. The resultant increase in intracellular Ca concentration may then lead to an increase in K permeability. The experimental attempts to inject Ca into cells (Table 5) appear to support such an explanation, although there is a scatter in these results which is primarily ascribable to technical difficulties.

As shown in Fig. 4, the potential recorded upon the initial puncture is nearly independent of the external K concentration. In contrast to this, the membrane potentials obtained 30 sec after the first puncture are markedly influenced by changes in the K concentration of the media. The change in membrane properties evoked by the initial puncture shifts the membrane potential in the direction of the equilibrium potential for K (assuming a relatively constant internal K concentration). This in turn means that the relative K conductance must be larger than that of the unperturbed cell. The observed phenomenon bears a resemblance to the Ca-induced KCl loss in metabolically depleted human red cells.

Although the obvious mode for checking the proposed course of events is the determination of Ca uptake and K permeability increase, a direct experimental verification is not possible in individual cells as dealt with in this study. However, it is possible to estimate the transference number of K (T_K) from the slope of the membrane potential at 30 sec after the initial puncture *vs.* log [K_0] (Fig. 4). T_K is defined by $T_K = g_K/g_m$ where g_K is the K conductance and g_m is the total conductance of the membrane. It can be shown (Brown, Walker & Sutton, 1970; Christoffersen, 1973) that

$$\left[\frac{\partial V_m}{\partial E_K}\right]_{E_{Na},\, E_{Cl}} = T_K. \tag{1}$$

V_m is the membrane potential and E_K, E_{Na} and E_{Cl} are the equilibrium potentials for K, Na and Cl, respectively. Provided that the intracellular concentrations remain constant, Eq. (1) can be written:

$$\left[\frac{\partial V_m}{\partial \log [K]_0}\right]_{[Na]_0,\,[Cl]_0} = 0.058\, T_K. \tag{2}$$

These equations are derived for zero net current across the membrane ($dV_m/dt = 0$). This condition is fulfilled at the peak hyperpolarization obtained about 30 sec after the initial micropuncture (*see* Fig. 3).

The slope of V_m *vs.* log $[K]_0$ (Fig. 4) in the concentration range 10 to 100 mM is 35 mV per decade, corresponding to a T_K of 0.60. This number is surprisingly high. For example in human red cells, the ratio between tracer fluxes of K and Cl is 10^{-5} (*see* Gunn, Dalmark, Tosteson & Wieth, 1973), giving an apparent maximal T_K of 10^{-5}.

Use of data in Fig. 4 in Eq. (1) or (2) is not obviously permissible, since the variation of $[K]_0$ is brought about by substitution for $[Na]_0$. Thus, E_{Na} does not stay constant. However, E_{Na} changes markedly only at the highest concentrations of $[K]_0$ (lowest $[Na]_0$). The linear relationship between membrane potential and $\log [K]_0$ in the concentration interval 10 to 100 mM $[K]_0$ supports the assumption that the changes in E_{Na} are insignificant in the present context.

The presence of an efficient carrier exchange system for chloride in the red cell membrane gives rise to a ratio between the conductances for K and for Cl which is closer to unity than is expected from tracer flux data. In the present type of experiments it has not been possible as yet to determine

either the normal membrane conductance or the conductance in relation to the induced potential changes. This point will be further discussed in a following paper (Lassen *et al., in preparation*). It is possible that a rise in intracellular Ca not only increases the K conductance but also decreases the Cl conductance as described for snail giant neurons (Christoffersen, 1973). Such a decrease in Cl conductance would lead to further hyperpolarization.

The effect of Ca on the K permeability in red cells seems to be an all or none phenomenon (Lassen *et al.*, 1973; Riordan & Passow, 1973). The period required to reach maximal hyperpolarization as shown in Fig. 3 may reflect the time needed to produce an increase in P_K. If, however, the rise in P_K after sealing of the cells is very rapid the initial rate of change in potential can be used as an indirect measure of the total membrane resistance (Lassen, Pape & Vestergaard-Bogind, 1974). This leads to a hypothetical value of $10^7\ \Omega\ cm^2$ which can be compared to the specific resistance of black lipid membranes made from sheep red cell lipid (Andreoli, Tieffenberg & Tosteson, 1967) of 1 to $3 \times 10^8\ \Omega\ cm^2$.

If P_K undergoes a large increase, the cells would be expected to lose K at a rate that is partly determined by the permeabilities of other ions, e.g. Cl. The results of repeated repunctures of the same cell (Fig. 7) indicate that it is possible to obtain hyperpolarization "cycles" several times, even in the presence of ouabain which would be expected to prevent restoration of eventual losses of intracellular K. Thus, only a minor fraction of K appears to be lost from the cells during the micropuncture and the temporary hyperpolarization of the membrane. How much of the K loss responsible for the slow decline in hyperpolarization occurs during the first few seconds, when the membrane is leaky, is not readily assessible. But it is interesting to note that in ouabain-treated cells the magnitude of the hyperpolarization declines more rapidly than in control cells. This may indicate that the K loss is in the range of the maximal transport rate of the active Na/K transport. Stimulation of the active pump is expected since Na is likely to enter the cells during the period of leakiness. According to the experiments by Riordan and Passow (1973), a rise in cellular Na concentration may by itself lead to a smaller Ca-induced increase in P_K. If a similar mechanism is involved in the present experiments, the integrated K loss over one cycle of induced hyperpolarization would be small compared to the cellular content of K.

Blum and Hoffman (1971, 1972) found that the Ca-induced increase in P_K was partly inhibited by ouabain or oligomycin. This was taken as an indication of a common site for the specific Ca effect on P_K and the normal

Na/K pump system. Lew (1971*a*, *b*) questioned this interpretation and presented experimental evidence supporting the thesis that ouabain (and oligomycin), in his experiments, acted to prevent ATP-hydrolysis by the pump, thus leaving traces of ATP in the cells which prevented further entry of Ca. In the present study it has proven possible to induce a Ca-related increase in P_K despite the lack of metabolic depletion. This may be due to a relatively large initial influx of Ca and a subsequent rise in cellular Ca activity sufficient to elicit the increase in P_K. The inability of ouabain and oligomycin to inhibit the micropuncture-induced hyperpolarization supports the explanation by Lew.

The transient nature of the hyperpolarization in the present experiments may be due to a gradual removal of excess Ca from the cytoplasm. This may result from a pumping of Ca out of the cells or a Ca accumulation in the mitochondria (which are present in this nucleated red cell). Further experiments are needed to distinguish between these two possibilities.

This work was supported by the Danish Science Research Council (511-1704-72. 511-2650-73). The skillful technical assistance of Kirsten Abel, Pia Anthonsen, and Annelise Honig is gratefully acknowledged.

References

Andreoli, T. E., Tieffenberg, M., Tosteson, D. C. 1967. The effect of valinomycin on the ionic permeability of thin lipid membranes. *J. Gen. Physiol.* **50**:2527

Blum, R. M., Hoffman, J. F. 1971. The membrane locus of Ca-stimulated K transport in energy depleted human red blood cells. *J. Membrane Biol.* **6**:315

Blum, R. M., Hoffman, J. F. 1972. Ca-stimulated K transport in human red cells: Localization of the Ca-sensitive site to the inside of the membrane. *Biochem. Biophys. Res. Commun.* **46**:1146

Bodemann, H., Passow, H. 1972. Factors controlling the resealing of the membrane of human erythrocyte ghosts after hypotonic hemolysis. *J. Membrane Biol.* **8**:1

Brown, A. M., Walker, I. L., Sutton, R. B. 1970. Increased chloride conductance as the proximate cause of hydrogen ion concentration effects in *Aplysia* neurons. *J. Gen. Physiol.* **56**:559

Christoffersen, G. R. J. 1973. Chloride conductance and the effect of extracellular calcium concentration on resting neurons in the snail, *Helix pomatia*. *Comp. Biochem. Physiol.* **46***A*:371

Dalmark, M., Wieth, J. O. 1970. Chloride and sodium permeabilities of human red cells. *Biochim. Biophys. Acta* **219**:525

Gárdos, G. 1958. The function of calcium in the potassium permeability of human erythrocytes. *Biochim. Biophys. Acta* **30**:653

Gunn, R. B., Dalmark, M., Tosteson, D. C., Wieth, J. O. 1973. Characteristics of chloride transport in human red blood cells. *J. Gen. Physiol.* **61**:185

Hoffman, J. F. 1962. The active transport of sodium by ghosts of human red blood cells. *J. Gen. Physiol.* **45**:837

Hoffman, J. F., Lassen, U. V. 1971. Plasma membrane potentials in *Amphiuma* red cells. Abstract, *XXV Int. Congr. Physiol. Sci.*, Munich

Hunter, M. J. 1971. A quantitative estimate of the non-exchange-restricted chloride permeability of the human red cell. *J. Physiol.* **218**:49P

Katz, B. 1966. Nerve, Muscle, and Synapse. p. 47. McGraw-Hill, New York

Lassen, U. V. 1972. Membrane potential and membrane resistance of red cells. *In*: Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status. M. Rørth and P. Astrup, editors. p. 291. Munksgaard, Copenhagen

Lassen, U. V., Nielsen, A.-M. T., Pape, L., Simonsen, L. O. 1971. The membrane potential of Ehrlich ascites tumor cells. Microelectrode measurements and their critical evaluation. *J. Membrane Biol.* **6**:269

Lassen, U. V., Pape, L., Vestergaard-Bogind, B. 1973. Membrane potential of *Amphiuma* red cells: Effect of calcium. *In*: Erythrocytes, Thrombocytes, Leukocytes. E. Gerlach, K. Moser, E. Deutsch and W. Wilmanns, editors. p. 33. Thieme, Stuttgart

Lassen, U. V., Pape, L., Vestergaard-Bogind, B. 1974. A possible value for the resistance of the *Amphiuma* red cell membrane. *In*: Comparative Biochemistry and Physiology of Transport. K. Bloch, L. Bolis and S. E. Luria, editors. North Holland Publishing Co., Amsterdam (*In press*)

Lew, V. L. 1971*a*. On the ATP dependence of the Ca^{2+}-induced increase in K^+ permeability observed in human red cells. *Biochim. Biophys. Acta* **233**:827

Lew, V. L. 1971*b*. Effect of ouabain on the Ca^{2+}-dependent increase in K^+ permeability in depleted guinea pig red cells. *Biochim. Biophys. Acta* **249**:236

Lichtman, M. A., Weed, R. I. 1972. Divalent cation content of normal and ATP-depleted erythrocytes and erythrocyte membranes. *Nouv. Rev. fr. Hémat.* **12**:799

Porzig, H. 1972. ATP-independent calcium net movements in human red cell ghosts. *J. Membrane Biol.* **8**:237

Riordan, J. R., Passow, H. 1971. Effects of calcium and lead on potassium permeability of human erythrocyte ghosts. *Biochim. Biophys. Acta* **249**:601

Riordan, J. R., Passow, H. 1973. The effects of calcium and lead on the potassium permeability of human erythrocytes and erythrocyte ghosts. *In*: Comparative Physiology. L. Bolis, K. Schmidt-Nielsen and S. H. P. Maddrell, editors. p. 543. North Holland Publishing Co., Amsterdam

Romero, P. J., Whittam, R. R. 1971. The control by internal calcium of membrane permeability to sodium and potassium. *J. Physiol.* **214**:481

Schatzmann, H. J., Vincenzi, F. F. 1969. Calcium movements across the membrane of human red cells. *J. Physiol.* **201**:369

Tosteson, D. C. 1959. Halide transport in red cells. *Acta Physiol. Scand.* **46**:19

Tosteson, D. C., Gunn, R. B., Wieth, J. O. 1973. Chloride and hydroxyl conductances in sheep red cell membranes. *In*: Erythrocytes, Thrombocytes, Leukocytes. E. Gerlach, K. Moser, E. Deutsch and W. Wilmanns, editors. p. 62. Thieme, Stuttgart

Vestergaard-Bogind, B., Pape, L., Lassen, U. V. 1973. Membrane potential of *Amphiuma* red cells: Effect of some substituted phenols. *In*: Erythrocytes, Thrombocytes, Leukocytes. E. Gerlach, K. Moser, E. Deutsch and W. Wilmanns, editors. p. 37. Thieme, Stuttgart

Vestergaard-Bogind, B., Lassen, U. V. 1974. Membrane potential of *Amphiuma* red cells: Hyperpolarizing effect of phloretin. *In*: Comparative Biochemistry and Physiology of Transport. K. Bloch, L. Bolis and S. E. Luria, editors. North Holland Publishing Co., Amsterdam (*In press*)

Wilbrandt, W. 1940. Die Abhängigkeit der Ionenpermeabilität der Erythrocyten vom glykolytischen Stoffwechsel. *Pflüg. Arch. Ges. Physiol.* **243**:519

Zeuthen, T. 1971. A method to fill microelectrodes by local heating. *Acta Physiol. Scand.* **81**:141