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Effect of Calcium on the Membrane Potential of *Amphiuma* Red Cells

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Summary. An increase in extracellular Ca concentration causes the membrane of giant red cells of the salamander, *Amphiuma means*, to undergo a marked, transient hyperpolarization. This hyperpolarization is caused by an increase in K permeability of the membrane as judged from the K sensitivity of the membrane potential and from the rate of K loss under influence of raised extracellular Ca concentration. At constant external pH, the induction of hyperpolarization by increased extracellular Ca has a relatively well-defined threshold concentration. Furthermore the phenomenon is of an "all or none" type with most of the cells having membrane potential values either in the normal range (about −15 mV) or in the range −40 to −70 mV. Shortly after suspension in Ringer's with 15 mM Ca, most if not all of the individual cells are hyperpolarized. Upon continued exposure (5–20 min) to the higher Ca concentration the membrane potential returns to the normal value in a fashion compatible with an "all or none" response. The observed Ca effect is sensitive to the pH of the suspending medium. At pH 6.2 the response is absent whereas the hyperpolarization is markedly stronger at pH 8.2 than at pH 7.2. It is argued that a reliable transport number for K under influence of Ca cannot be estimated from the slope of membrane potential *vs.* log (extracellular K concentration). This is probably related to the fact that the membrane potentials of the cells in the population do not stay constant in time. The above phenomenon is compared with the Ca-induced K permeability in poisoned human red cells or red cell ghosts. It is important to note that the cells employed in the present study are neither poisoned nor mechanically disrupted. This study emphasizes that the role of Ca in regulating cell membrane permeability to K seems to be a general feature.

In a number of different cell types calcium seems to influence membrane permeability to other ions. This mechanism has been extensively studied in human red cells where a metabolic depletion of ATP is prerequisite to a Ca-dependent, rapid loss of KCl from the cells (Gárdos, 1958, 1959; Lepke & Passow, 1960). From such experiments it was suggested that the enhanced K permeability was the direct result of an increase in intracellular Ca (Lew, 1970, 1974; Romero & Whittam, 1971).

This notion received strong support from experiments in which Ca was incorporated into human red cell ghosts (Blum & Hoffman, 1971, 1972). When Ca was present on the inside of the membrane, the concentrations necessary to produce an increased K permeability were much lower than those needed when Ca was added to the suspending medium of ghosts or poisoned red cells. The present authors have reported that giant red cells of the salamander, *Amphiuma means*, hyperpolarized as a consequence of a preceding micropuncture (Lassen, Pape, Vestergaard-Bogind & Bengston, 1974). This hyperpolarization was ascribed to a temporary increase in K permeability resulting from the entry of Ca before resealing of the membrane after the micropuncture. These experiments thus give independent support to the contention that increasing Ca on the inside of the cell membrane causes a rise in the relative K permeability.

All of the experiments mentioned above suffer from a common weakness if one attempts to identify the Ca-induced increase in K permeability with a physiological regulation mechanism for cell permeability. In the case of ATP depletion, the cells were exposed to a variety of strong metabolic poisons, some of which may have a direct effect on the cell membrane. Furthermore, the presence of normal intracellular ATP concentration may be necessary to maintain functional integrity of the membrane. In experiments with human red cell ghosts, metabolic poisons are usually not present, but the membrane has been made temporarily leaky to the cell contents (even to macromolecules like hemoglobin). The absence of ATP may play a role also in this case. The experiments by the present authors (Lassen *et al.*, 1974) in which a Ca-dependent hyperpolarization was seen following mechanical damage to the *Amphiuma* red cell membrane might also be unrepresentative of the response of a nonperturbed cell to a rise in intracellular Ca.

The simplest possible approach to accelerate Ca influx into cells with the aim of increasing intracellular and/or membrane-bound Ca is to increase the extracellular concentration of Ca in the suspending medium. The present work demonstrates that *Amphiuma* red cells respond to raised extracellular Ca concentrations in a characteristic fashion. At pH 7.2, a rise in extracellular Ca from the normal 1.8 to 6 mM elicits a transient hyperpolarization of the cell membrane that can be ascribed to an increase in relative K permeability. This response which is totally reversible and appears to be of an "all or none" nature, has a threshold concentration for Ca that is pH dependent. These experiments are performed on normal cells without metabolic poisoning or mechanical disruption of the membrane prior to potential measurements and thus con-

stitute an indication that Ca ions may be of immediate importance for the regulation of red cell permeability to K ions.

Parts of the experiments have been presented in preliminary form elsewhere (Lassen, Pape & Vestergaard-Bogind, 1973, 1975).

Materials and Methods

Red cells from anesthetized specimens of *Amphiuma means* were used in all experiments. Blood was drawn by puncture of the atria of the heart and immediately diluted in large volumes of Ringer's. Unless otherwise stated, the blood cells were used within 3 hr. Further details about blood sampling and conditions for maintaining the stock of animals have been given in a previous paper (Lassen *et al.*, 1974).

After withdrawal of the blood, the cells were washed twice in "normal" Ringer's and stored at 17°C until use. In the experiments where the Ca concentration of the medium was changed, a small sample of the blood was centrifuged and the supernatant discarded. The cells were then carefully resuspended in 50–100 volumes of the new medium. The instant at which the new medium was added was taken as zero-time for exposure to the given Ca concentration. The suspension was then recentrifuged and the cell pellet resuspended to give a hematocrit of 0.1–0.5 per cent, which is suitable for introduction into the chamber for microelectrode measurements. The time interval from the change in suspension medium until the first potential measurements could be obtained was typically 60–80 sec.

The compositions of incubation media are given in Table 1. Media having K and/or Ca concentrations different from the "normal" Ringer's were held at constant osmolarity by appropriate changes of the Na concentrations. This also led to minor changes in Cl concentrations in the case of the $CaCl_2$ addition in exchange for NaCl. These changes in Cl concentration are without measurable influence on the membrane potential in the present type of experiments. In the experiments at different pH values, the media were

Table 1. Composition of incubation media[a]

mM	"Normal" Ringer's	15 mM Ca Ringer's
Na^+	118	95
K^+	2.5	2.5
Cl^-	124	128
Ca^{++}	1.8	15.0
MOPS[b]	10	10

[a] All media contained 1 g of bovine serum albumin (Povite Corp., Amsterdam) per liter. Media with different K concentrations were prepared by replacing NaCl with KCl in equimolar amounts. Media with Ca concentrations between 1.8 and 15 mM were prepared by interpolation between the concentrations given in the Table.

[b] Morpholinopropane sulfonic acid (Sigma Chemical Co., St. Louis, Mo.) titrated to the desired pH (normally 7.2) with 1 M NaOH.

buffered with 8 mM morpholinopropane sulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.) and 2 mM tris (hydroxymethyl) aminomethane (Tris) (Sigma Chemical Co.).

Determinations of extra- and intracellular concentrations of Na, K, and Cl were performed as previously described (Lassen *et al.*, 1974).

For control of Ca activity in Ringer's a Ca-sensitive microelectrode filled in the tip with Ca-dioctylphenylphosphate, Di-*n*-octylphosphonate and polyvinylchloride (Christoffersen & Johansen, *in preparation*) was employed. This type of electrode has a nearly linear response in emf to the logarithm of the Ca concentration in the range 1 to 15 mM of Ca (at constant ionic strength).

Membrane potentials of the *Amphiuma* red cells were measured by means of conventional microelectrodes. The electrodes were firmly attached to an electromechanical transducer which gave a movement of less than 2 μm thus requiring the tip of the microelectrode to be gently pressed against the cell membrane before releasing advancement of the electrode. This close contact with the cell membrane often resulted in d–c potential change of 1–3 mV. For this reason the reference potential level was taken as the potential with the electrode tip in free fluid. The membrane potential was recorded as the most negative potential after penetration of the cell membrane (within 200 μsec after the sharp potential change when the microelectrode pierced the membrane). As discussed in greater detail elsewhere (Lassen, Nielsen, Pape & Simonsen, 1971; Lassen *et al.*, 1974) the recorded potentials did not stay at the most negative value, but decayed within a few msec to a stable value. In the present work the difference between the most negative potential ("peak value") and the reference potential were transferred *via* an A/D converter with a conversion time of 6 μsec to a digital computer (PDP 8/e, Digital Equipment Corporation, Maynard, Mass.) and stored on magnetic tape for subsequent treatment of the data (Bengtson & Lassen, *in preparation*). In this way the rate of consecutive potential measurements was limited only by the skill of the experimenter in moving the microelectrode tip from one cell to another. A rapid succession of potential measurements were of importance when following the time-dependent changes in membrane potential caused by Ca.

Results

Fig. 1*A* shows superimposed oscilloscope tracings from a number of micropunctures of *Amphiuma* red cells suspended in "normal" Ringer's having an extracellular Ca concentration of 1.8 mM. The obtained membrane potentials, as indicated by the negative peak values, show a nearly normal distribution around a mean value of −15 mV in a large number of experiments. This potential is close to the equilibrium potential for Cl (Lassen *et al.*, 1974) and does not change appreciably during several hours of incubation. If cells from the same batch are transferred to a Ringer's containing 15 mM Ca, the potential tracings obtained within the first 180 msec after resuspension show a marked change. As seen in Fig. 1*B* it is obvious that some of the cells have hyperpolarized. A prominent feature of the response to the high external Ca concentration is that the potentials distribute themselves in two distinct classes: one with values of −40 to −70 mV and another with values close to the potential of control cells (Fig. 1*A*).

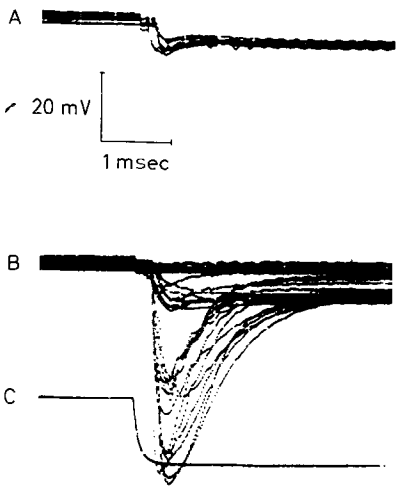

Fig. 1. Oscilloscope tracings of membrane potential recordings in *Amphiuma* red cells. Traces indicated by "*A*" are obtained on micropuncture of cells in "normal" Ringer's. The sudden drop in potential indicates that the membrane has been penetrated by the electrode tip. The potential reaches a peak negative value within less than 200 μsec. This value is taken as the membrane potential of the cell with reference to the potential measured with the electrode tip in free solution. Several traces are superimposed by use of a storage oscilloscope. The slow d-c drift observed both in trace *A* and trace *B* does not affect the value of the recorded membrane potential. Trace *B* shows consecutive recordings from an experiment where cells were transferred to a 15 mM Ca Ringer's. Three distinct types of recordings are seen: a) unsuccesful penetrations are indicated by the heavy horizontal band of sweeps, b) potential recordings in the normal range, resembling those in part *A* of the Figure, c) large potential drops upon penetration indicating hyperpolarization of the membrane. Traces indicated by "*C*" monitor the high voltage pulse for the piezoelectric transducer that advances the electrode. Bars indicate the vertical deflection sensitivity and the sweep speed for traces *A* and *B* (*C* not calibrated). pH 7.2, temperature 17 °C

Fig. 2 shows the distribution of the potential values obtained in a typical experiment in which cells were exposed to 15 mM Ca. The fraction of hyperpolarized cells is a function of the time after transference of the cells from normal Ringer's to 15 mM Ca Ringer's. As seen in the upper left-hand histogram, most of the cells are hyperpolarized shortly after exposure to the higher Ca concentration. About 10 min after exposure to 15 mM Ca, most of the measured potentials are within the normal range. Although the number of hyperpolarized cells decreases during the initial 10-min period, it should be noted that the measured potentials still are grouped around the same values as during the initial phase. The immediate implication of this type of pattern is that membrane potentials are most stable in either of the two groups. This indicates the existence of an "all or none" phenomenon. Furthermore, this type of distribution indicates that the transition time from the hyperpolarized

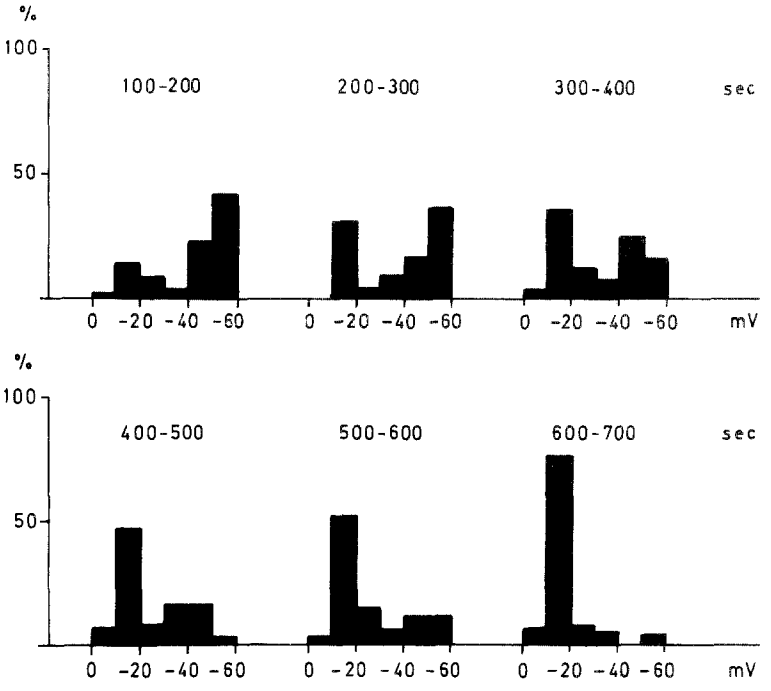

Fig. 2. Time dependence of membrane potentials of *Amphiuma* red cells after suspension in 15 mM Ca Ringer's, pH 7.2 (17°C). Above each of the histograms is indicated the time interval in seconds after exposure to 15 mM Ca. The abscissae represent intervals of 10 mV. The ordinates show the relative fraction of measured potentials in each interval in per cent of the total number of observations in each of the histograms. Despite the maintained incubation in 15 mM Ca, the fraction of hyperpolarized cells decreases in time

state to the state with normal potential must be relatively short as compared with the time "spent" in either of the two states.

As demonstrated above, hyperpolarization of the *Amphiuma* red cell membrane vanishes in time despite the maintained presence of the high extracellular Ca concentration. The period from exposure to 15 mM Ca until the hyperpolarization is no longer observed may vary considerably between individual experiments although the general pattern is always the same. The times for disappearance of the hyperpolarization vary from a few minutes to about 20 min (in rare cases) despite apparently identical experimental conditions. Both in this respect and in the determination of fluxes there is more variability between individual cell batches of *Amphiuma* red cells than observed with human red cells. In view of the variability whose cause is not clear, pooling the results from several experiments for time-frequency distribution plots of membrane potential would not be meaningful. However, within the first 3 min after

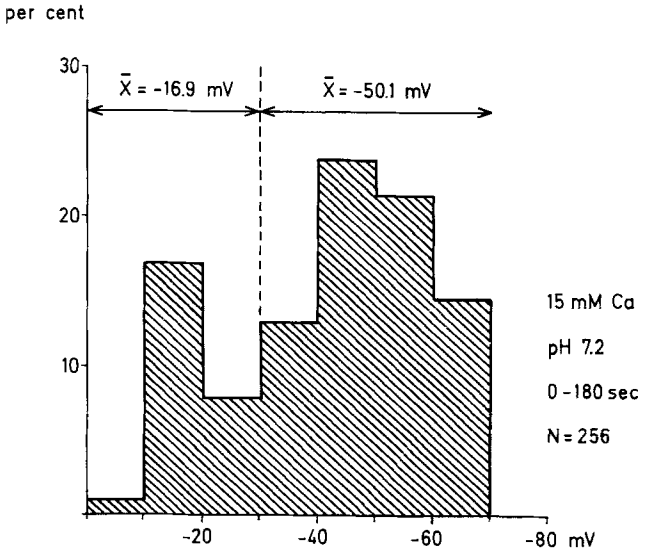

Fig. 3. Distribution of the membrane potentials of *Amphiuma* red cells obtained within 180 sec after suspension in 15 mM Ca Ringer's (pH 7.2, 17°C). The abscissa represents intervals of 10 mV and the ordinate shows the relative fraction of measured potentials in each interval. The distribution shows the two maxima as discussed in the text. The mean values ($\bar{x}$) of the potentials below and above an arbitrary line of division are indicated in the Figure

exposure to 15 mM Ca, there is only a small variation between the individual experiments. Fig. 3 shows the compiled results from a number of experiments in which only measurements within the first 3 min after onset of incubation in 15 mM Ca Ringer's are included. A distribution having two dominant potential values is again apparent. Also in this type of plot it should be noted that no measurements were obtained within about 60 sec after exposure to 15 mM Ca. This lag period represents the necessary handling time and the time needed for sedimentation of cells to the bottom of the experimental chamber where they are accessible for micropuncture. In some experiments a retropolation to the start of incubation in 15 mM Ca was possible and indicated that it is most likely that all of the cells become hyperpolarized upon exposure to 15 mM Ca.

Despite continued incubation the cells apparently adapt to the high Ca concentration and regain their normal potential. This does not mean that these cells permanently have lost their ability to respond to Ca. If cells which have been incubated in 15 mM Ca long enough for hyperpolarization to disappear are washed in normal Ringer's and then suspended

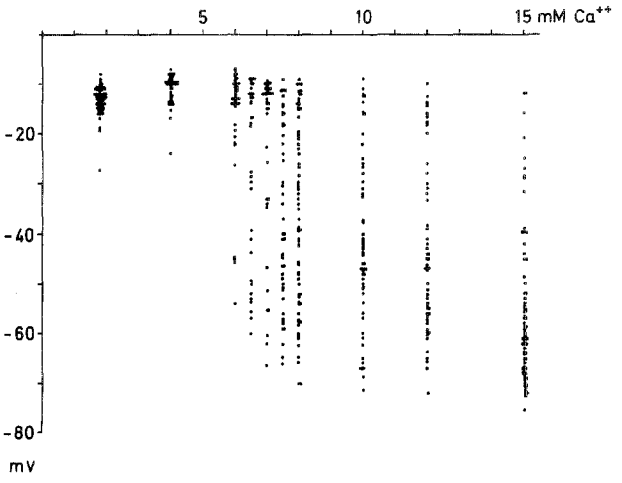

Fig. 4. Membrane potentials of *Amphiuma* red cells obtained within 180 sec after suspension in media with different Ca concentrations. The individual potential values are indicated as separate points. The abscissa shows the Ca concentration of the medium (mM) and the ordinate shows the potential values in mV. As seen from the data the threshold concentration for hyperpolarization is 6 mM Ca. Above this concentration a further increase in Ca in the medium does not significantly increase the maximal hyperpolarization but does increase the fraction of hyperpolarized cells. pH 7.2, 17°C

in 15 mM Ca Ringer's the same pattern of hyperpolarization is seen again.

Since a spontaneous hyperpolarization is not seen in normal Ringer's (1.8 mM Ca) it is of interest to see if the Ca dependence of the apparent "all or none" response exhibits a threshold. Fig. 4 shows the potentials obtained within the first 3 min after suspending in a Ringer's solution having the indicated Ca concentrations. In the case of the control cells in normal Ringer's, the cells were merely washed in the same medium before transference to the chamber for microelectrode measurements. Whereas the potentials in 4 mM Ca are unchanged with respect to the control, there is a marked fraction of hyperpolarized cells at a Ca concentration of 6 mM. With increasing concentrations of Ca, the potential values for the hyperpolarized part of the population are essentially the same, but the higher the Ca concentration, the larger the fraction of hyperpolarized cells.

A number of organic anions used in buffers as substitutes for chloride in biological experiments have a marked tendency to form chelates with Ca, thereby markedly reducing the Ca activity (Christoffersen & Skibsted, 1975). The present experiments were performed in Ringer's containing 10 mM of the organic anion morpholinopropane-sulfonate (MOPS) as

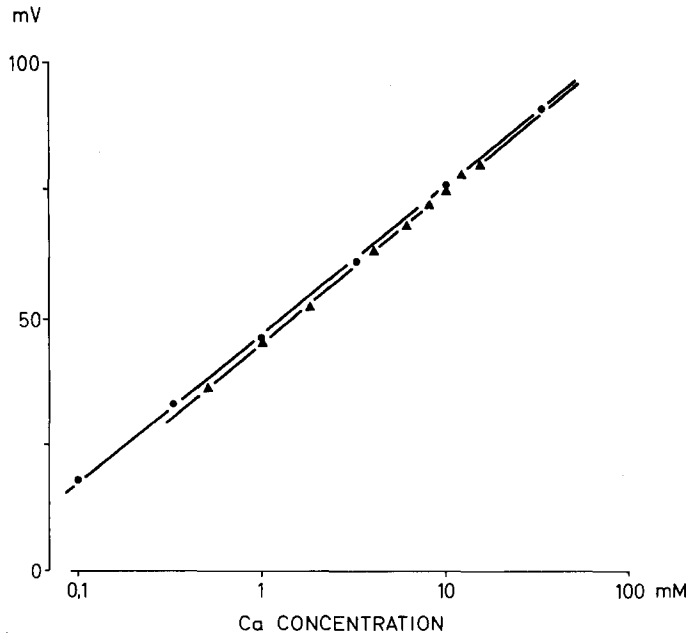

Fig. 5. Potentials recorded with a Ca-sensitive electrode as a function of the Ca concentration of the medium surrounding the electrode tip. Abscissa: Ca concentration in mM (log scale); ordinate: potential in mV. Circles indicate values from standard $CaCl_2$ solutions (in NaCl) and triangles indicate MOPS containing media as employed in the present study

a buffer. The apparent Ca concentration threshold for induction of hyperpolarization might be misinterpreted if MOPS was able to bind significant amounts of Ca. Fig. 5 shows potentials recorded by a calcium-sensitive electrode in MOPS-containing Ringer's and in ($NaCl + CaCl_2$) solutions with a constant ionic strength of 0.1 M. As seen from the Figure, the potentials recorded in the two sets of solutions are essentially identical. Therefore a binding of Ca by MOPS in the employed concentration range can be excluded.

It is important to know to what extent there is a parallellism between the effects of Ca on the *Amphiuma* red cell membrane and on poisoned human red cells or red cell ghosts. In the latter case, the marked effect of extracellular Ca at physiological pH is not seen at values below 6.0 to 6.5 (Knauf, Riordan, Schuhmann & Wood-Guth, 1975). Fig. 6 shows the results of an experiment performed at three different pH values and two concentrations of Ca, 1.8 (control) and 6 mM. The cells were equilibrated for more than one hour at each pH value in normal Ringer's (with 1.8 mM Ca) before transference to the higher Ca concentration at the same pH. It is obvious that the higher the pH, the larger the

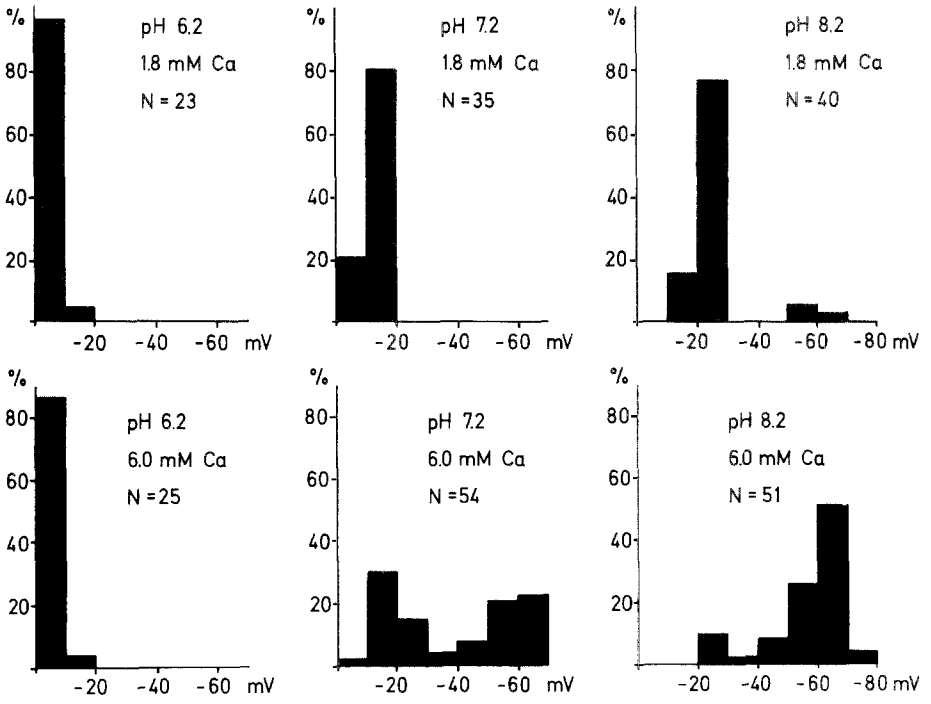

Fig. 6. Effect of pH on the membrane potential of *Amphiuma* red cells at two Ca concentrations: 1.8 and 6 mM. The experiments were performed at pH 6.2, 7.2 and 8.2. Abscissae represent intervals of 10 mV and ordinates indicate the relative fraction (per cent) of the observed potentials. The number of measurements are indicated in each histogram. The hyperpolarizing effect of 6 mM Ca is absent at pH 6.2 and is enhanced at pH 8.2 (with respect to pH 7.2). 17°C

potentials recorded in normal Ringer's. This is in accordance with the findings of Hoffman and Lassen (*see* Lassen, 1972) and probably reflects a shift in Donnan potential at the different pH values. The finding of a few hyperpolarized cells in normal Ca concentration at pH 8.2 is not readily explained since the cells were exposed to the same conditions during both the prolonged preincubation and the measurement of potentials.

The results from experiments where cells were transferred from 1.8 to 6 mM Ca at the pH values at which they were equilibrated are shown in the lower half of Fig. 6. At pH 6.2 there is no sign of hyperpolarization. At pH 7.2, this "suprathreshold" Ca concentration produces a marked hyperpolarization as previously shown (Fig. 4). In 6 mM Ca Ringer's, pH 8.2, the least negative group of potentials are in the range -20 to -30 mV corresponding to those seen in normal Ringer's, pH 8.2., but the fraction of hyperpolarized cells is significantly larger than at

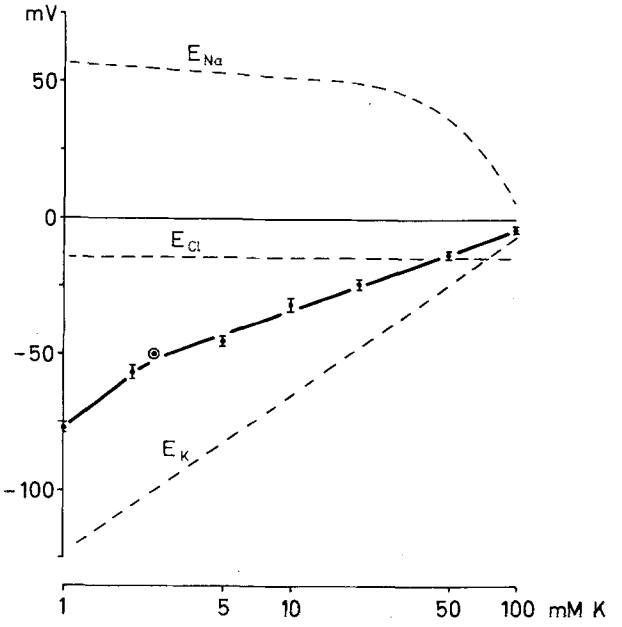

Fig. 7. Dependence of the Ca-induced hyperpolarization of the *Amphiuma* red cell membrane on the K concentration of the medium. Abscissa: external K concentration in mM (log scale); ordinate: potential in mV. The points on the solid line are mean values from experiments at different K concentrations indicated ± 1 SEM (0–120 sec after exposure to 15 mM Ca). The encircled point shows the potential of the hyperpolarized fraction of cells in Fig. 3. The equilibrium potentials for Na, K, and Cl are indicated as dotted lines. pH 7.2, 17 °C

pH 7.2. This indicates that the effect of Ca on the *Amphiuma* red cell membrane potential at different pH values is qualitatively similar to the pH effects on induced K loss from poisoned human red cells and ghosts (Knauf *et al.*, 1975).

The similarities between the Ca-induced hyperpolarization of the *Amphiuma* red cell membrane and the Ca-induced K loss from human red cells suggest that the hyperpolarization might be due to a selective increase in K permeability of the cell membrane. The equilibrium potential for K ions across the membrane of *Amphiuma* red cells suspended in normal Ringer's is about -90 mV (Lassen *et al.*, 1974). A K permeability increase would thus readily account for the membrane potentials observed during Ca-induced hyperpolarization. A change in the extracellular K concentration would then be expected to influence the magnitude of the hyperpolarization. Fig. 7 shows the mean potential values after exposure to 15 mM Ca as a function of the K concentration in the medium (log scale). At K concentrations above 5–10 mM there is a straight

Table 2. Intracellular concentrations of Na, K, and Cl in cells suspended in "normal" and 15 mM Ca Ringer's

	"Normal" Ringer's		15 mM Ca Ringer's	
mM (liter cell water)$^{-1}$	$t=0$ (min)	$t=5$ (min)	$t=0$ (min)	$t=5$ (min)
Na^+	10.6	10.6	10.3	12.6
K^+	136	135	134	133
Cl^-	67	68	71	72

Concentrations are mean of two or more determinations. pH 7.2, 17 °C.

line relationship of 31 mV/decade between the measured potential values and the log of the external K concentration. At the lowest K concentrations the curve bends downwards and approaches a slope of 58 mV/decade. The values in Fig. 7 are all obtained within the first two minutes after exposure to 15 mM Ca as the potentials obtained at 2 mM and especially at 1 mM K had a strong tendency to become less negative after the first few minutes. This time instability of the membrane potential of the hyperpolarized group of cells at 1 and 2 mM K is considered further in the discussion. The equilibrium potentials for K, Na and Cl are indicated in the Figure.

As shown above (Figs. 1–3), the effect of raising the extracellular Ca is transient. It is therefore reasonable to question whether there is a rapid, marked change of the intracellular concentrations of diffusible ion species, in particular K. As the membrane potential change induced by high extracellular Ca is sensitive to the K gradient across the membrane, loss of a substantial fraction of the intracellular K would lead to decreased hyperpolarization. However, Table 2 shows that the cellular concentrations of K, Na, and Cl are nearly identical in normal Ringer's and in 15 mM Ca Ringer's and therefore the disappearance of the highly negative group of potentials cannot be accounted for by a change in E_K. This is consistent with the above-mentioned observation of the reappearance of normal hyperpolarization when cells in 15 mM Ca are washed in normal Ringer's and resuspended in 15 mM Ca.

Fig. 8 shows the results of experiments performed in order to determine the net cellular K loss that may accompany the hyperpolarization. With a cytokrit of 0.25, the extracellular pool of K is about 20 times smaller than the intracellular pool. A small K loss from the cells would thus give a larger fractional change in the extracellular K concentration. Furthermore, ouabain in a concentration of 10^{-4} M was added to prevent

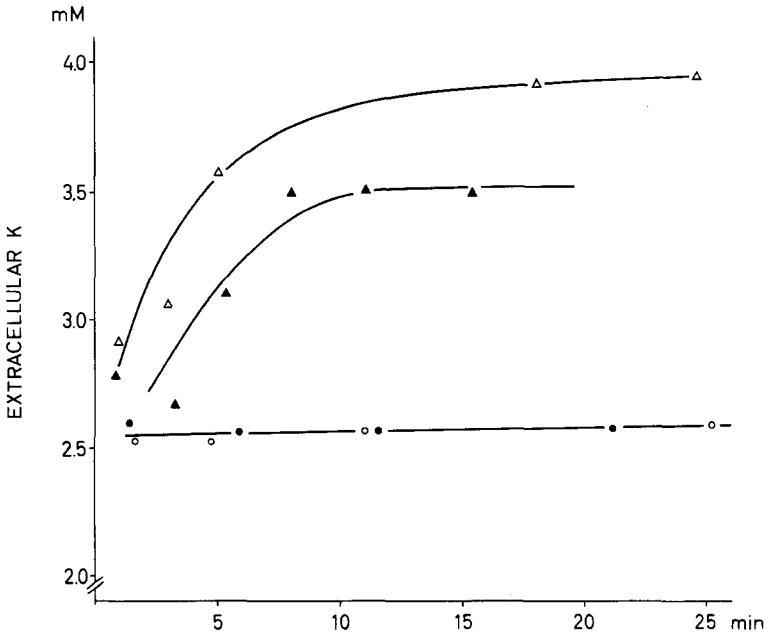

Fig. 8. Extracellular K concentrations of suspensions of ouabain (10^{-4} M) poisoned *Amphiuma* red cells as a function of time. Abscissa: time in minutes after suspension of cells in 15 mM Ca Ringer's with ouabain (triangles) or resuspension in "normal" Ringer's with ouabain (circles). Ordinate shows the extracellular K concentration in mM. Open and closed symbols refer to two parallel experiments. After an initial rapid K loss from the cells upon suspension in 15 mM Ca, the rate of K loss is essentially the same in 15 mM Ca as in normal Ringer's. Cell volume fraction approximately 0.25, pH 7.2, 17°C

reaccumulation of K. Contrary to the findings on human red cell ghosts (Blum & Hoffman, 1971, 1972) neither ouabain nor oligomycin block the Ca-induced hyperpolarization of the *Amphiuma* red cell membrane (Lassen *et al.*, 1974, and *unpublished results*). Fig. 8 shows the results of two experiments on the same batch of blood. These findings are representative of those from a number of similar experiments. Upon suspension of the cells in 15 mM Ca Ringer's there is an accelerated K loss which lasts about 10 min. Following this interval of time, the K losses from control cells and cells in 15 mM Ca follow parallel courses. The average initial net K flux in nine experiments was 0.84 ± 0.44 (SEM) mM K (liter of cells $\times$ min)$^{-1}$. The corresponding net K fluxes in ouabain containing control media were 0.01 ± 0.002 (SEM, $n = 11$) mM K (liter of cells $\times$ min)$^{-1}$. This means that the rate of K loss is transiently increased about two orders of magnitude. As even slight hemolysis might lead to a marked increase in extracellular K concentration, only data from experiments without visible hemolysis were included. There was a large

scatter of the time intervals during which the K loss was accelerated from experiment to experiment (from 2 to 18 min). The total extra K loss due to increased extracellular Ca concentration averaged about 5 mM (liter of cells)$^{-1}$ or less than 4% of the intracellular K.

Discussion

The present study has shown that an increase in extracellular calcium ion concentration can induce a transient hyperpolarization of the *Amphiuma* red cell membrane. This change in membrane potential is closely related to an increase in the potassium conductance of the membrane relative to the conductances of other ions. As shown in Fig. 8 there is a small increase in net KCl loss from the cells during the period of hyperpolarization. Thus, this phenomenon seems to be equivalent to the well-documented Ca-induced net KCl loss from poisoned human red cells and red cell ghosts (Gárdos, 1958, 1959; Lepke & Passow, 1960; Lew, 1970; Blum & Hoffman, 1971, 1972; Romero & Whittam, 1971; Riordan & Passow, 1973). But whereas the conditions under which Ca is able to produce a measurable increase in the K permeability of human red cells are "highly artificial" (Riordan & Passow, 1973), an effect of Ca on *Amphiuma* red cells can be demonstrated without poisoning or disrupting the cells.

Raising the extracellular Ca concentration above 6 mM results in a typical pattern of membrane hyperpolarization of the *Amphiuma* red cell. Under influence of Ca the potential values are distributed in two main groups, one around the potential of the normal unperturbed cell and one in the range of -40 to -60 mV. With increasing time after exposure to the higher Ca concentrations, the potentials do not undergo a continuous slow decay from the hyperpolarized values, but seem to "jump" back to the normal potential as seen in Fig. 2. This behavior resembles the metastable response of excitable tissues since the cells can only have membrane potentials at intermediate values for short periods of time. This resemblance is further strengthened by the present finding of an apparent "threshold concentration" for Ca (Fig. 4).

Although direct evidence for an "all or none" response to Ca in human red cells or red cell ghosts has not as yet been available, Passow (Passow & Tillmann, 1955; Passow, 1970) suggested such a mechanism for the action of Pb^{++} on K permeability of human erythrocytes. In a subsequent study Riordan and Passow (1971) extended the notion of an "all or none" response to the effect of Ca on human erythrocyte

ghosts. The basic argument is that at a given submaximal concentration of Pb^{++} or Ca^{++}, the KCl loss ceases after some time whereas the initial time constant for KCl loss (using the loss at $t=\infty$ as reference) is nearly independent of concentrations of Ca above 0.5–1 mM. From these and similar observations Riordan and Passow (1973) and Lew (1974) conclude that an "all or none" response to Ca is the most likely explanation for the kinetics of KCl efflux. According to their hypothesis, as the Ca concentration is raised, more and more cells are "recruited" for the Ca-induced KCl loss. Colombe and Macey (1974) has questioned this interpretation. In experiments with Ca present in the hemolyzing medium, ghosts were prepared with different internal (and external) Ca concentrations. When the internal Ca concentration in ghosts was raised from 0.1 to 1 mM the "normalized" rate constants for KCl loss were markedly increased. This is in contrast with the findings of Lew (1974) and Riordan and Passow (1971).

Both in studies on human red cells and in the present study, the increase in K permeability after exposure to Ca is a function of time also. At present it is not clear whether the time needed for disappearance of the Ca-induced increase in K permeability is also a function of the absolute Ca concentration. Before this question is resolved the interpretation of the experiments of Colombe and Macey (1974) remains open. But the present experiments, which by nature are able to show effects on individual cells in a mixed population, give strong independent support to the postulate that the effect of Ca on K conductance is of an "all or none" type.

The evidence for an "all or none" effect of Ca on the K permeability of intact *Amphiuma* erythrocytes reveals nothing about the mechanism of the interaction. It is likely, however, that the Ca concentration at the inward facing side of the cell membrane determines the K permeability (*see* e.g. Blum & Hoffman, 1972). Upon exposure of *Amphiuma* red cells to a larger external Ca concentration, the Ca gradient across the membrane is increased. This may cause a net influx of Ca into the cells. Even with a low permeability of the red cell membrane to Ca (Lew, 1974) a small entry will change the internal concentration from its normally very low value (Lichtman & Weed, 1972). The entering Ca will interact with "receptors" on the inside of the membrane, thus giving rise to a larger K permeability. As the equilibrium potential for K is about -90 mV (Lassen *et al.*, 1974) compared to the normal membrane potential of about -15 mV, a hyperpolarization will ensue. This increase in the electric field will add strongly to the tendency of the

divalent Ca to enter the cells, thus amplifying the Ca-induced K permeability increase. Thus a regenerative response seems likely from simple physical arguments.

If Ca uptake into depleted human red cells is equivalent to the Ca effect observed in *Amphiuma* cells, the findings of Lew (1974) have specific bearing on a regenerative response. Lew reports that Ca uptake is maximal and constant in the external K concentration range of 0.02–4 mM K. At higher K concentrations, the Ca uptake is inhibited with a K_i (inhibitor constant) of 40–50 mM (external K). Thus the Ca uptake is inhibited as the equilibrium potential for K becomes smaller resulting in a smaller electric driving force across the membrane even with an initial similar Ca-induced K permeability increase. Although Ca has an effect even at raised external K concentrations in the present experiments (*see* Fig. 7) this is probably due to the fact that the employed Ca concentration of 15 mM is well above the threshold concentration of 4–6 mM.

There is a distinct difference between the present experiments and those on human red cell ghosts reported by Knauf *et al.* (1975). The hyperpolarizing effect of 15 mM Ca in the *Amphiuma* red cells is present also in K-free media (*unpublished experiments*) whereas Knauf *et al.* (1975) find that a low external K concentration is necessary to elicit a Ca-induced K loss. In their experiments the K concentration necessary to give half maximal stimulation is 0.7 mM. The same authors furthermore find it unlikely that the membrane potential *per se* plays a role in the Ca-induced K loss since the increase in K permeability (tracer exchange) can be seen with equal K concentrations on both sides of the ghost membrane. To evaluate the possible importance of a regenerative response of Ca in *Amphiuma* red cells, additional experiments are needed comparing membrane potential and Ca uptake at different external K concentrations.

If the membrane potential in a given experimental period is invariant with time it is possible to estimate the transference number for K (T_K) from the slope of membrane potential *vs.* log $[K]_0$ as shown in Fig. 7. Provided that the intracellular concentrations remain essentially constant during the period of observation (Table 2), the following expression can be used to calculate T_K (for derivation, *see* Brown, Walker & Sutton, 1970; Christoffersen, 1973)

$$\left[\frac{\delta V_m}{\delta \log K_0}\right]_{Na_0,\, Cl_0} = 0.058\, T_K \qquad (1)$$

where V_m is the membrane potential, and the subscripts denote the extracellular phase. At the lowest concentrations the slope of the curve shown in Fig. 7 is close to 58 mV per decade, suggesting a transference number of 1.0. Such a transference number means that all current across the membrane is carried by K. If this is true, then the membrane potential should equal the equilibrium potential for K at each external K concentration. It was mentioned in the results section that the potentials measured in the low K solutions were unstable in time, not only in the usual sense of an apparent "all or none" response, but in sliding from very negative values in the first few minutes after exposure to 15 mM Ca. Thus, there must have been a small inward current during the period of potential sampling from different cells. This contradicts the underlying assumption for the derivation of Eq. (1); therefore the slope of the curve cannot be used to determine the value of T_K in this case. At K concentrations above 10 mM, the slope of the experimental line in Fig. 7 is about 31 mV per decade. This would, based on Eq. (1), correspond to a T_K of 0.53. This value for T_K might seem reliable since the potentials did not slide towards less negative values in this range of K concentrations within the first two minutes after exposure to 15 mM Ca. However, with constant P_{Na} and P_{Cl} this relatively low value for T_K would require that the curve deviates markedly upward at the low concentration end when employing a constant field approximation. As seen from the data presented in Fig. 7, this is not the case. According to Knauf *et al.* (1975) and Lew (1974) higher concentrations of external K inhibit the Ca evoked KCl efflux. Such an effect would be expected to reduce the slope of the curve at the high concentrations, eventually giving rise to the experimentally observed linear relationship.

As seen from Fig. 6 the Ca-induced hyperpolarization varies with pH of the external medium. Knauf *et al.* (1975) show that at pH values below 6.0–6.5 it is not possible to induce a net KCl loss from red cell ghosts by Ca. This is consistent with the present experiments where no hyperpolarization is seen with 6 mM Ca at pH 6.2. At pH 8.2 the Ca effect is potentiated to a degree where even the normal Ca concentration of 1.8 mM induces hyperpolarization in some cells. The mechanism behind this pH dependence of Ca-induced hyperpolarization is not clear, but it may be related to the pH-dependent Ca binding to red cell membranes as described by Forstner and Manery (1971). In the case of the nerve membrane there is an antagonism between the effect of Ca^{++} on ion conductances and the effect of raising the H^+ concentration, i.e. lowering the pH (Frankenhaeuser & Hodgkin,

1957; Hille, 1968; Gilbert & Ehrenstein, 1969; Christoffersen, 1973; Schrager, 1974). This antagonism has been ascribed to a competition between Ca^{++} or H^{+} in titrating the surface charges of the membrane. Schrager (1974) furthermore proposes that protonation of a histidine group (on the inside of the membrane) may determine the ease with which the potassium gates open and close.

In the present experiments, as in studies on human red cells and red cell ghosts, the Ca-induced increase in K permeability is transient despite the maintained presence of Ca in the extracellular medium. Lew (1974) finds that Ca uptake always levels off with time, even though the average intracellular Ca concentration is two orders of magnitude below the external one. Thus a time-dependent rectification of Ca movement (*see also* Porzig, 1972) may be connected with the disappearance of hyperpolarization or net KCl loss.

It has been implicit in the above discussion that the primary effect of Ca on the membrane potential of *Amphiuma* red cells was a hyperpolarization. Due to the experimental technique employed in the present experiments it was not possible to follow the potential changes within the first minute after exposure to a high external Ca concentration. Therefore it cannot be excluded that the primary event is a short burst of inward Ca current corresponding to the early Na or Ca current in various excitable tissues. The hyperpolarization described in the present paper would then correspond to the after-hyperpolarization in nerve.

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