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EFFECT OF EXTRACELLULAR Ca^{2+}, K^+ AND OH^- ON ERYTHROCYTE MEMBRANE POTENTIAL AS MONITORED BY THE FLUORESCENT PROBE 3,3'-DIPROPYLTHIODICARBOCYANINE

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Changes in fluorescence intensity of thiodicarbocyanine, DiS-C_3(5), were correlated with direct microelectrode potential measurements in red blood cells from *Amphiuma means* and applied qualitatively to evaluate the effects of extracellular Ca^{2+}, K^+ and pH on the membrane potential of human red cells. Increasing extracellular [Ca^{2+}] from 1.8 to 15 mM causes a K^+-dependent hyperpolarization and decrease in fluorescence intensity in *Amphiuma* red cells. Both the hyperpolarization and fluorescence change disappear when the temperature is raised from 17 to 37°C. No change in fluorescence intensity is observed in human red cells with comparable increase in extracellular Ca^{2+} in the temperature range 5–37°C. Increasing the extracellular pH, however, causes human red cells to respond to an increase in extracellular Ca^{2+} with a significant but temporary loss in fluorescence intensity. This effect is blocked by EGTA, quinine or by increasing extracellular [K^+], indicating that at elevated extracellular pH, human erythrocytes respond to an increase in extracellular Ca^{2+} with an opening of K^+ channels and associated hyperpolarization of the plasma membrane.

Introduction

In 1974, Hoffman and Laris [1] described the use of carbocyanine dye as a fluorescent probe to measure the electric potential of the red cell membrane. Shortly thereafter, Sims et al. [2] examined the spectral characteristics and fluorescent efficiency of a large group of cyanine dyes applied to the measurement of membrane potential. They investigated the mechanism responsible for the changes in fluorescence intensity which occurred in conjunction with potential changes and demonstrated that the response was a function of the partition of the probe between intra- and extracellular phases. On hyperpolarization, a shift in distribution takes place due to the movement of the probe into the cell. The increased concentration of cell-associated dye results in binding of the dye molecules, with subsequent loss in fluorescent efficiency. The increase in fluorescence intensity seen on depolarization was then explainable simply as the reverse effect.

Hladky and Rink [3] undertook a detailed investigation of the mechanism by which the fluorescent dye DiS-C_3(5) responds to the red cell membrane potential. They concluded that the amount of dye which enters the cell and is bound in the cytoplasm is a direct indicator of the membrane potential. Tsien and Hladky [4] reported that hemoglobin is the most important dye-binding site in whole red cells, with hemoglobin and inner membrane surfaces equally important sites in red

Abbreviations: DiS-C_3(5), 3,3'-dipropylthiodicarbocyanine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

cell ghosts. In either case, the amount of cell-bound dye is reflected in the fluorescence of the suspension, which can then be used to assess membrane potentials under the proper circumstances.

In most of the studies correlating fluorescence intensity with membrane potentials it has been necessary to calculate membrane potentials from assumed permeabilities and the measured equilibrium concentrations of the principle ions. Direct measurement of membrane potentials with microelectrodes has been possible in only a limited number of cell types due to the associated technical difficulties [5]. Because of its size, the giant red cell of *Amphiuma means* is amenable to direct microelectrode measurement of the membrane potential. It was, therefore, of interest to correlate fluorescence intensity measurements with directly measured membrane potentials.

In addition, the effect of increased extracellular Ca^{2+} on the membrane potential of *Amphiuma* red cells has been examined in this laboratory [6–8]. These studies have shown that an increase in extracellular Ca^{2+} from the normal 1.8 mM to 15 mM causes an increase in potassium permeability and concomitant hyperpolarization in normal *Amphiuma* red cells. This effect resembles the Ca^{2+}-induced potassium permeability increase in depleted or metabolically poisoned human red cells first described by Gárdos [9]. Using the potential-sensitive fluorescent probes should make it possible to determine whether otherwise unperturbed human red cells respond to increased extracellular concentrations of Ca^{2+} with a comparable hyperpolarization and, if so, which parameters are involved. The investigation described here was undertaken with this purpose in mind.

TABLE I

COMPOSITION OF INCUBATION MEDIA

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

	Amphiuma red cell/ Ringer's solution (mM)	Human red cell/ Ringer's solution (mM)
Na^+	118	142
K^+	2.5	2.0
Cl^-	124	148
Ca^{2+}	1.8	2.0
MOPS [a]	10	10

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

Materials and Methods

Red cells

Amphiuma red cells were obtained from anaesthetized animals by cardiac puncture immediately before experiments and suspended in large volumes of 'normal' *Amphiuma* red cell/Ringer's solution (see Table I). The cells were then washed twice and resuspended in Ringer's solution at a final hematocrit of 9%. During experiments, the cells were incubated with constant agitation at 17°C. Human red cells were obtained from healthy adults and processed in essentially the same manner using human red cell Ringer's (Table I). The final stock blood suspension with a hematocrit of 9% was incubated at 37°C with constant agitation.

Solutions were prepared in steam-cleaned glassware using doubly distilled water and analytic grade chemicals.

Membrane potentials

Membrane potentials were measured with conventional 2.5 M KCl-filled electrodes with a tip diameter of 0.2 μm. The electrodes were mounted in a holder attached to a piezoelectric electromechanical transducer, allowing rapid advancement of the microelectrode. As described previously [6], the membrane potential is taken as the peak of the potential change in immediate relation to the penetration of the cell membrane with the microelectrode tip.

Fluorescence measurements

Fluorescence measurements were made with an Aminco-Bowman spectrofluorometer containing a xenon lamp equipped with a magnetic arc stabilizer. Fluorescence excitation was carried out at a constant wavelength of 622 nm and emission intensity measured at wavelengths corresponding to peak outputs determined spectroscopically prior to each run. In the case of Ringer's solution containing fluorescent dye, this was most commonly in the range 670–675 nm. Addition of cells caused a

shift in the peak emission wavelength to approximately 680–684 nm. No effort was made to obtain corrected wavelengths. All values of fluorescence intensity as well as changes in intensity (ΔI_f) reported in this study are based on the peak values. The change in fluorescence intensity ΔI_f is given by

$$\Delta I_f = \frac{I_f^0 - I_f^P}{I_f^0} \cdot 100$$

where I_f^0 = fluorescence intensity before perturbation and I_f^P the corresponding intensity after perturbation. Cuvette solutions consisted of 2500 μl of the appropriate Ringer's solution, 25 μl DiS-$C_3(5)$ to give a final dye concentration in the cuvette of $3 \cdot 10^{-6}$ M, and 150 μl 9% red cells to give a final hematocrit of 0.5%. Constant mixing during measurement of fluorescence intensity was accomplished with both an overhead mechanical fly and bottom magnetic fly. Temperature within the cuvette was held constant within ±0.5°C by means of a Hetofrig Thermostat (Birkerød, Denmark) and monitored continuously. Cells were added to the cuvette containing Ringer's solution plus dye and temperature and fluorescence intensity were stable within 4 min. Membrane potentials were then perturbed by addition of the appropriate agents. In the case of potential-fluorescence correlation studies, 25 μl of valinomycin solution were added to give a final valinomycin concentration of $4 \cdot 10^{-6}$ M. To examine the effect of increased extracellular Ca^{2+}, 25 μl of 1.5 M $CaCl_2$ were added to give a final $[Ca^{2+}]_0$ of 15 mM. In order to determine whether the slight increase in tonicity (approx. 10%) affected the fluorescence response, cells were added to Ringer's solution containing 15 mM Ca^{2+} and adjusted to normal tonicity. The magnitude of the change in fluorescence intensity was identical. In all cases, control fluorescence measurements were made on solutions without cells to make sure that addition of the various agents such as valinomycin, etc. did not, in themselves, give rise to changes in fluorescence characteristics.

Fluorescent dye

The fluorescent dye, DiS-$C_3(5)$, used in this study was the kind gift of Dr. A.S. Waggoner. Solutions were prepared by dissolving weighed amounts of the dye in absolute ethanol. Final concentration of ethanol in the cuvette was not greater than 0.5%.

Results

Fig. 1 shows the emission spectra of Ringer's solution containing $3 \cdot 10^{-6}$ M DiS-$C_3(5)$ before and after addition of *Amphiuma* red cells. The spectra show a shift in wavelength as well as reduction in intensity on addition of red cells. The emission peak at 622 nm represents 90° scattering of the excitation beam. Some characteristic intensity measurements at various extracellular K^+ concentrations are reproduced in Fig. 2. Fig. 3 is a plot of microelectrode membrane potential measurements as a function of extracellular $[K^+]$ in the presence of $4 \cdot 10^{-6}$ M valinomycin. The change in fluorescence intensity as a function of extracellular $[K^+]$, in the presence of valinomycin, was investigated and results are shown in Fig. 4.

Combining the data shown in Figs. 3 and 4 results in the correlation shown in Fig. 5. It is clear from Fig. 5 that the correlation between ΔI_f and membrane potential is not linear. This finding is consistent with the results of similar correlation studies between fluorescence intensity and calculated values of membrane potential [3,4,10,11]. The resting membrane potential ($\Delta I_f = 0$) is −13 mV, in good agreement with the value of −13.8

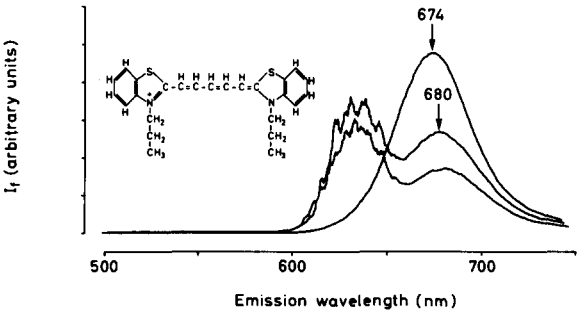

Fig. 1. Emission spectra of DiS-$C_3(5)$ at excitation wavelength 622 nm. Upper trace is emission spectrum for DiS-$C_3(5)$ in normal *Amphiuma* red cell/Ringer's solution, pH 7.2, 17°C. Middle trace is emission spectrum after addition of *Amphiuma* red cells and lower trace after addition of $4 \cdot 10^{-6}$ M valinomycin. Inset shows structure of DiS-$C_3(5)$. Ordinate: fluorescence intensity in arbitrary units. Abscissa: emission spectrum wavelength in nm.

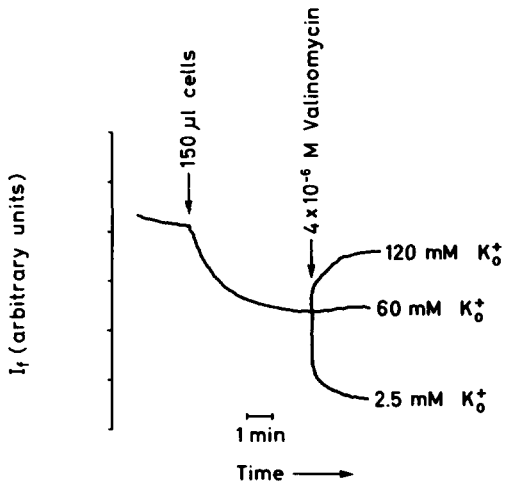

Fig. 2. Typical trace of fluorescence intensity at peak emission wavelength. 0.5% *Amphiuma* red cells in normal *Amphiuma* red cell/Ringer's solution, pH 7.2, 17°C, plus $3 \cdot 10^{-6}$ M DiS-$C_3(5)$ and various extracellular K^+ concentrations. The individual traces have been superimposed for representational purposes so that initial fluorescence intensities at the instant of valinomycin addition are identical. Ordinate: arbitrary fluorescence intensity units. Abscissa: time (min).

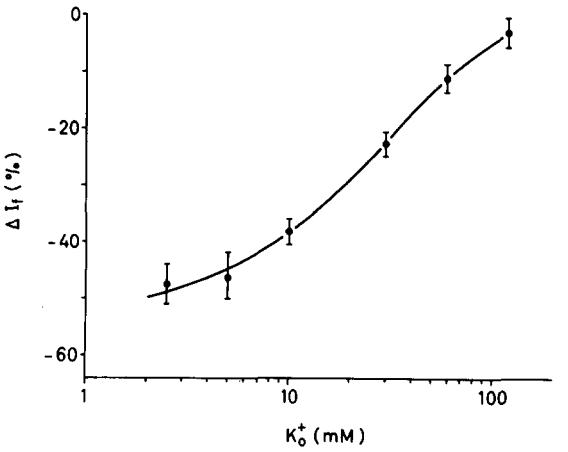

Fig. 4. Change in fluorescence intensity as a function of extracellular K^+ concentration. 0.5% *Amphiuma* red cells in normal *Amphiuma* red cell/Ringer's solution, pH 7.2, 17°C plus $3 \cdot 10^{-6}$ M DiS-$C_3(5)$ and $4 \cdot 10^{-6}$ M valinomycin. Ordinate: ΔI_f in %. Abscissa: extracellular K^+ concentration in mM.

mV obtained by microelectrode measurements in *Amphiuma* red cells [7].

Notwithstanding the lack of a linear relationship between ΔI_f and V_m, there is an obvious qualitative correlation in the change in fluorescence intensity which accompanies a measured change in membrane potential. To test this qualitative correlation further, the change in fluorescence intensity when *Amphiuma* red cells were exposed to elevated extracellular Ca^{2+} levels was examined. As reported by Lassen et al. [7], raising the extracellular Ca^{2+} concentration to 15 mM results in a temporary hyperpolarization of the plasma membrane of *Amphiuma* red cells. The time course of fluorescence intensity after addition of $CaCl_2$ to the cuvette containing *Amphiuma* red cells and fluorescent probe is shown in Fig. 6, and demonstrates a significant loss in intensity. The reduction in intensity which results from the increase in extracellular Ca^{2+} is prevented if the cells are suspended in 100 mM K^+/Ringer's solution or one containing 0.5 mM EGTA. Based on these results, a comparable study was carried out

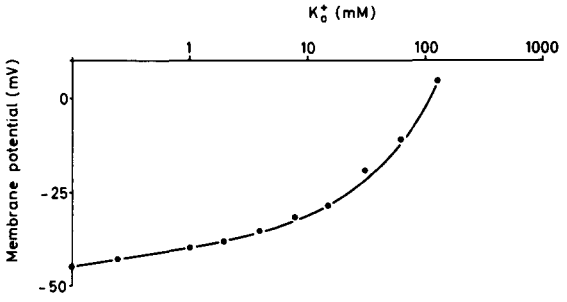

Fig. 3. Microelectrode potential measurements as a function of extracellular K^+. *Amphiuma* red cells in normal *Amphiuma* red cell/Ringer's solution, pH 7.2, 17°C plus $3 \cdot 10^{-6}$ M DiS-$C_3(5)$ and $4 \cdot 10^{-6}$ M valinomycin. Ordinate: membrane potential (V_m) in mV. Abscissa: extracellular K^+ concentration in mM.

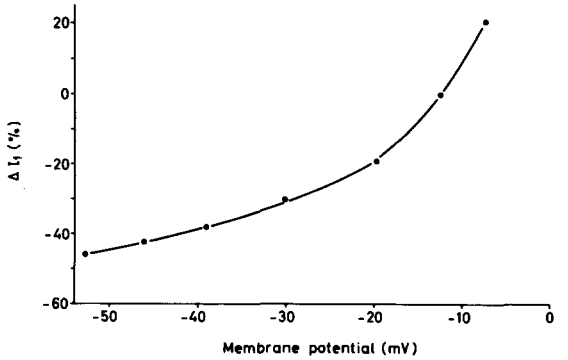

Fig. 5. Correlation between ΔI_f and V_m in *Amphiuma* red cells, pH 7.2, 17°C. Ordinate: ΔI_f in %. Abscissa: V_m in mV.

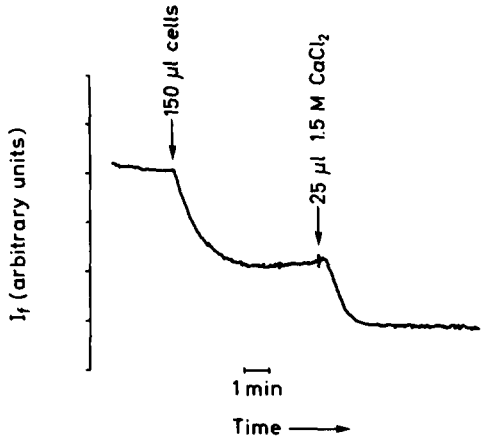

Fig. 6. Effect of increase in extracellular Ca^{2+} on fluorescence intensity in *Amphiuma* red cells. $CaCl_2$ was added to a cuvette containing 0.5% *Amphiuma* red cells in normal *Amphiuma* red cell/Ringer's solution, pH 7.2, 17°C and $3 \cdot 10^{-6}$ M DiS-$C_3(5)$. Final concentration of extracellular Ca^{2+} was 15 mM. Ordinate: fluorescence intensity in arbitrary units. Abscissa: time (min).

on normal human red cells. No change in fluorescence intensity was observed on raising the extracellular Ca^{2+} concentration to as high as 30 mM. Having observed that raising the incubation temperature of *Amphiuma* red cells to 37°C caused the Ca^{2+}-induced hyperpolarization to disappear,

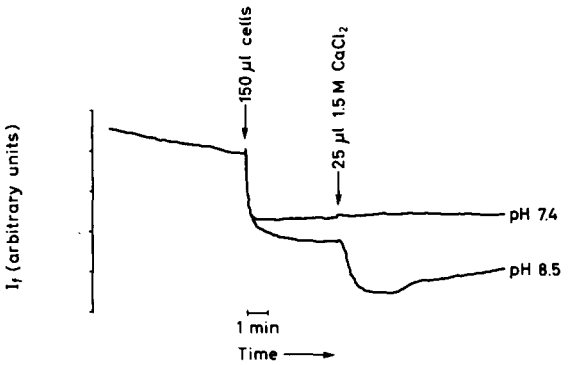

Fig. 7. Effect of increased extracellular Ca^{2+} on fluorescence intensity in human red cells. $CaCl_2$ was added to a cuvette containing 0.5% human red cells in normal Ringer's solution, pH 7.4, 37°C, and Ringer's solution adjusted to pH 8.5. DiS-$C_3(5)$ concentration in both cases was $3 \cdot 10^{-6}$ M. Final extracellular Ca^{2+} concentration was 15 mM. The traces have been superimposed for representational purposes so that initial fluorescence intensity at the instant of Ca^{2+} addition are identical. Ordinate: fluorescence intensity in arbitrary units. Abscissa: time (min).

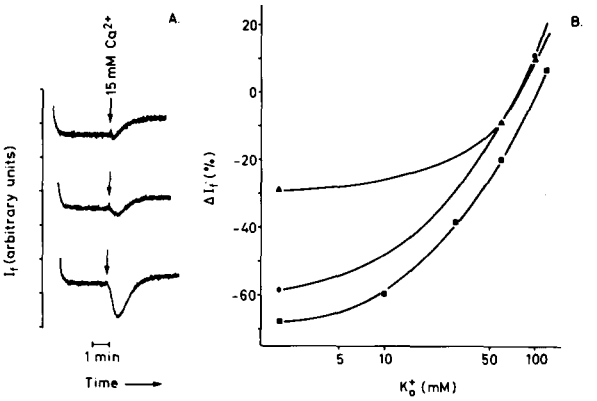

Fig. 8. Ca^{2+}-induced changes in fluorescence intensity as a function of extracellular K^+ concentration. A, representative experimental traces of fluorescence intensity on addition of Ca^{2+} to a suspension of 0.5% human red cells in Ringer's solution, pH 8.5, 37°C containing various extracellular K^+ concentrations: 100 mM (upper trace), 60 mM (middle trace) and 2 mM (lower trace). Ordinate: fluorescence intensity in arbitrary units. Abscissa: time (min). B, comparison of the effect of extracellular K^+ on changes in fluorescence intensity produced by addition of $4 \cdot 10^{-6}$ M valinomycin, pH 7.4 (■), $4 \cdot 10^{-6}$ M valinomycin, pH 8.5 (●), and 15 mM Ca^{2+}, pH 8.5 (▲). Red cell concentrations were 0.5% in all cases. Ordinate: ΔI_f in %. Abscissa: extracellular K^+ concentration in mM.

it seemed possible that the normal 37°C incubation temperature for human red cells was the reason for the lack of fluorescence response to increased extracellular Ca^{2+}. Therefore, the response to increased extracellular Ca^{2+} at lower incubation temperatures was investigated. No Ca^{2+}-induced change in fluorescence intensity was observed in normal human red cells in the temperature range 5–37°C.

Hoffman and Laris [1] noted that suspending red cells at higher extracellular pH resulted in a loss in fluorescence intensity. Furthermore, we have observed that in the case of *Amphiuma* red cells increasing the extracellular pH potentiated the response to extracellular Ca^{2+} as measured by microelectrodes. With these observations in mind, the response of human red cells to increased $[Ca^{2+}]$ in the presence of elevated extracellular pH was examined. Fig. 7 shows superimposed representative traces of the time-course of fluorescence intensity at pH 7.4 and 8.5. Prior to the addition of $CaCl_2$ the cells in pH 8.5 Ringer's solution show a lower equilibrium intensity than those at pH 7.4,

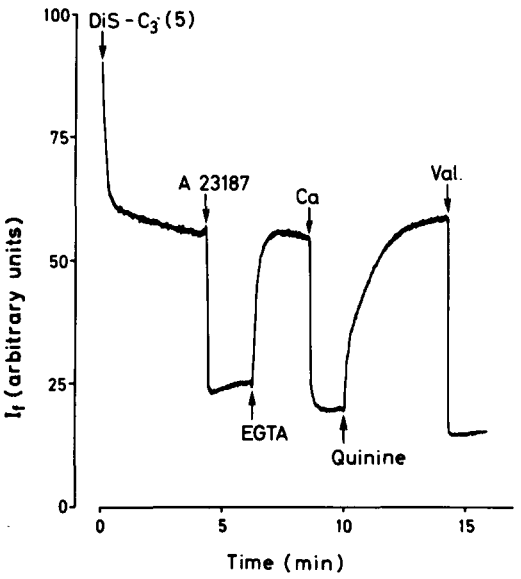

Fig. 9. Typical experimental fluorescence trace showing the effect of various agents. 0.5% human red cells in normal Ringer's solution, pH 7.4, 37°C containing $3 \cdot 10^{-6}$ M DiS-$C_3(5)$. Ordinate: fluorescence intensity in arbitrary units. Abscissa: time (min).

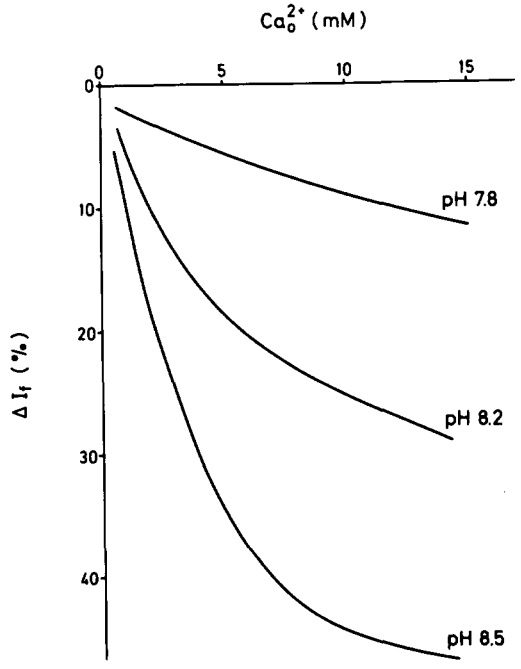

Fig. 10. Change in fluorescence intensity as a function of extracellular Ca^{2+} concentrations at various extracellular pH values. 0.5% human red cells in Ringer's solution adjusted to the appropriate pH and containing $3 \cdot 10^{-6}$ M DiS-$C_3(5)$. Ordinate: ΔI_f in %. Abscissa: extracellular Ca^{2+} concentration in mM.

indicative of a greater amount of cell bound dye at elevated extracellular pH [3]. On addition of 15 mM Ca^{2+} to the cells in pH 8.5 Ringer's solution, the fluorescence intensity undergoes a further marked reduction which is temporary. The magnitude of the change in fluorescence intensity on elevation of extracellular calcium is dependent on extracellular $[K^+]$, as seen in Fig. 8. The resting membrane potential ($\Delta I_f = 0$) is identical in the presence of either 15 mM Ca^{2+} or valinomycin and has a calculated value of -17.8 mV, based on an intracellular potassium concentration of 155 mM. The hyperpolarization due to the increased extracellular pH alone corresponds to the change in membrane potential under similar circumstances discussed by Hladky and Rink [3]. As a further check on whether the observed Ca^{2+}-induced changes in fluorescence intensity represented changes in potassium permeability, the effects of various agents such as Ca^{2+}-ionophore A23187, EGTA and quinine were investigated. The results of a typical experiment are shown in Fig. 9. The fact that final addition of valinomycin elicited a full response indicated that the cells were still viable. The change in fluorescence intensity as a function of extracellular $[Ca^{2+}]$ at various extracellular pH is shown in Fig. 10 and demonstrates that a Ca^{2+}-induced change in fluorescence intensity is already evident at pH 7.8.

Discussion

The hyperpolarization of *Amphiuma* red cells in the presence of increased extracellular Ca^{2+} demonstrated by microelectrode measurements is reflected in fluorescence intensity measurements in a population of cells under comparable conditions. The average stable ΔI_f measured with DiS-$C_3(5)$ 4 min after a population of *Amphiuma* red cells were exposed to 15 mM Ca^{2+}, was 33%. Based on the correlation shown in Fig. 5, the associated membrane potential is -33 mV. Lassen et al. [7] reported a distribution of membrane potentials in the first 3 min after exposure to 15 mM Ca^{2+} which results in a calculated weighted potential of approximately 35–40 mV. Since it is precisely this type of weighted potential that the fluorescent probe is expected to exhibit, the correspondence between 'microscopic' single cell electrode poten-

tial measurements and 'macroscopic' fluorescence intensity measurements is quite good. Although there is good quantitative agreement between the two techniques in the initial phase of the response to high extracellular Ca^{2+}, there is a difference in the later phase. Whereas the hyperpolarization measured with microelectrodes in *Amphiuma* erythrocytes is transient so that the cells have regained their normal resting potential within 5–10 min after exposure to an increase in extracellular Ca^{2+}, the change in fluorescence intensity is constant over a period of as long as 10–20 min.

A study of membrane potential in *Amphiuma* red cells measured with microelectrodes in the presence of DiS-C_3(5) has shown that the probe potentiates and prolongs the hyperpolarizing effect of increased extracellular Ca^{2+} in these erythrocytes [12]. Furthermore, in fluorescence studies an increase in extracellular K^+, or addition of quinine or EGTA to the cuvette after hyperpolarization (loss in intensity) caused an immediate increase in intensity to pre-perturbation levels. This suggests that the prolonged decrease in intensity reflects a continuing hyperpolarization. While it is likely that the action of the probe is localized to either the intracellular Ca^{2+} transport system or potassium permeability control mechanism, the possibility that increased extracellular Ca^{2+} causes a retention of the intracellularly bound dye cannot be ruled out at this time.

Based on the qualitative response of the fluorescent probe DiS-C_3(5) to perturbations of the plasma membrane potential, it is apparent that the reaction of human erythrocytes to elevated extracellular Ca^{2+} is not identical to that of *Amphiuma* red cells. The hyperpolarization and increase in passive K^+ permeability which occurs when normal *Amphiuma* red cells are exposed to increased extracellular Ca^{2+} concentrations does not occur in human red cells under normal physiological conditions. The disappearance of this phenomenon when the incubation temperature of *Amphiuma* erythrocytes is raised from 17 to 37°C does not, in itself, provide a simple explanation for this difference, since lowering the incubation temperature of human red cells does not lead to hyperpolarization at normal pH when the extracellular Ca^{2+} concentration is increased. However, when the pH of the incubation medium is raised, there is a comparable hyperpolarization and K^+ permeability increase in human red cells, which is clearly transient, in the presence of increased extracellular Ca^{2+}.

Lassen et al. [13] measured $^{45}Ca^{2+}$ and $^{42}K^+$ isotope fluxes in intact human red cells and observed that increased extracellular Ca^{2+} caused an increase in intracellular $^{45}Ca^{2+}$ and a transient increase in $^{42}K^+$ flux in cells suspended at elevated extracellular pH. These findings, together with the results of the present study, support the conclusion that a Ca^{2+}-dependent transient increase in potassium permeability occurs in otherwise unperturbed human erythrocytes exposed to an increase in extracellular OH^-. It is probable that the related changes in intracellular pH result in an increased transmembrane Ca^{2+} transport which in turn triggers the K^+ permeability system. It is also possible that potentiation of the 'Ca^{2+} effect' involves, at least in part, the external surface of the membrane. There are a number of indications that this may be the case. We have observed that the Ca^{2+}-induced hyperpolarizations measured with microelectrodes in *Amphiuma* red cells and with the fluorescent probe DiS-C_3(5) in human red cells, although transient, do not undergo oscillations as might be expected. Once the cells have returned to the non-hyperpolarized state, they must be resuspended in normal Ringer's solution (2 mM Ca^{2+}) before they regain their capacity to rehyperpolarize on elevation of extracellular Ca^{2+}. Reduction of extracellular [K^+] from the normal 2 mM causes a proportionately more rapid depolarization after the Ca^{2+}-induced hyperpolarization in human red cells (unpublished data). This finding is similar to the observations of Simon [14] concerning the effects of extracellular [K^+] on the Ca^{2+}-induced changes in DiS-C_3(5) fluorescence in red cell ghosts. Thus, the pH-mediated, Ca^{2+}-induced increase in potassium permeability in otherwise unperturbed human erythrocytes is not only associated with an increase in intracellular Ca^{2+}, but may be partially under the control of a process localized at the outer surface of the red cell membrane.

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