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Membrane Potentials Associated with Ca-Induced K Conductance in Human Red Blood Cells: Studies with a Fluorescent Oxonol Dye, WW 781

Jeffrey C. Freedman and Terri S. Novak
Department of Physiology, State University of New York, Upstate Medical Center, Syracuse, New York 13210

Summary. A divalent anionic dye, bis-[3-methyl-1-*p*-sulfophenyl-5-pyrazolone-(4)]-pentamethine oxonol (WW 781) is a rapidly responding fluorescent indicator of KCl diffusion potentials induced in human red blood cells with valinomycin, gramicidin, and with the Ca ionophore A 23187 in the presence of external Ca. WW 781 has a sensitivity of 0.13% ΔF/mV, a detection limit of 10 mV, a response time of less than 1 sec, and exhibits a decrease in fluorescence intensity upon hyperpolarization without detectable shifts in absorption or emission peaks. This dye does not perturb the normal resting potential, and unlike the slow permeant cyanine dyes, does not inhibit Ca-induced K conductance in human red blood cells. However, WW 781 does stimulate Ca-induced unidirectional Rb efflux. With Ca plus A 23187, the initial rapid change in dye fluorescence is sensitive to $[Ca]_o$ and to [A 23187], is reversible with excess EGTA, and is inhibited by quinine, oligomycin, and by trifluoperazine. A biphasic dependence of hyperpolarization on K_o is evident at pH 6, where the ionic selectivity of activation is K, Rb > Cs > Na and that of conductance is K, Rb > Cs. Conditions were defined which permitted continuous monitoring of E_m for at least 10 min, and the time dependence of the Ca-induced potentials was characterized. Since the properties of the Ca-induced changes in dye fluorescence correlate well with the known characteristics of Ca-induced K permeability, we conclude that WW 781 is a useful indicator of changes in E_m, provided that sufficient controls are employed to separate direct effects of Ca on dye fluorescence from the effects of E_m on fluorescence.

Key words red blood cells · membrane potential · calcium · Ca ionophore A 23187 · oxonol · fluorescence

Introduction

Human red blood cells exhibit a phenomenon, known as the "Gardos effect," in which Ca induces a loss of K from the cells (Gardos, 1959). The increase in K permeability may reach 1000 times normal, with a high selectivity for K over Na (Passow, 1963; Kregenow & Hoffman, 1972), and occurs when intracellular Ca reaches micromolar levels (Blum & Hoffman, 1972). At normal internal and external [K] and [Na], the result is a net efflux of KCl down the electrochemical gradient and shrinkage of the cells. While the metabolic dependency and the kinetic characteristics of Ca-induced K fluxes in red cells have been studied in some detail (for reviews *see* Passow, 1963, 1981; Lew & Ferriera, 1977, 1978; Hoffman et al., 1980), the associated changes in membrane potential E_m have not been satisfactorily described. Previous results (Goldinger & Hoffman, 1976; Simons, 1979) using the fluorescent potentiometric indicator, diS-C_3(5) (Hoffman & Laris, 1974; Sims et al., 1974) were consistent with suggestions (Glynn & Warner, 1972; Hoffman & Knauf, 1973; Gardos, Szasz & Sarkadi, 1977) that Ca-induced K permeability in human red cells is mediated by a conductive pathway. A quantitative analysis of the relationship between E_m, as measured with fluorescent dyes, and Ca-induced K fluxes in red cells has been somewhat complicated by the finding that diS-C_3(5), the cationic cyanine dye most frequently used for monitoring E_m of red cells, is itself a potent selective inhibitor of the Gardos effect (Simons, 1976*a*, 1979). For some purposes, the rate of development of inhibition of diS-C_3(5) is sufficiently slow so as to permit an approximate assessment of the Ca-dependent voltages (Hoffman et al., 1980); however, for continuous monitoring of E_m during the course of net KCl efflux induced by elevated internal Ca, a rapidly responding and noninhibitory optical potentiometric indicator would clearly be desirable.

Extrinsic fluorescent potentiometric indicators give two types of signals – slow redistribution signals and fast signals (for reviews, *see* Cohen & Salzberg, 1978; Waggoner, 1979; Freedman & Laris, 1981; Cohen & Hoffman, 1982). "Slow" dyes are permeant, bear a delocalized charge, give signals as large as 1.7% ΔF/mV[1] in red blood cells (Freedman & Hoffman, 1979*b*), and redistribute between the bulk intracellular and extracellular solutions in accordance with an altered membrane

[1] % ΔF stands for percentage change in fluorescence.

potential; the potentiometric optical signal arises from altered quantum yields of fluorescence due to interactions of the dye with cellular constituents (Sims et al., 1974). The best "fast" dyes bear a localized charge which reduces their permeance across cell membranes. Some, such as WW 781 (dye XXV of Gupta et al., 1981) also bear a delocalized charge. Fast dyes have exhibited fluorescence changes approaching 0.1% ΔF/mV in nerve and muscle, and are thought to interact with the cell membrane, giving optical signals due to the effect of E_m on the binding of dye to the membrane, or on the orientation, state of aggregation, or optical properties of dye associated with the membrane. Most studies of suspensions of cells and organelles have utilized slow dyes (*see* Freedman & Laris, 1981), although the use of a fast dye with a sufficiently large signal would offer some advantages.

In this paper, we evaluate the utility of a fast dye, a divalent negatively charged oxonol dye, denoted WW 781, as a fluorescent indicator of red cell membrane potentials. The use of this particular dye derived from positive reports in tests of its ability to track action potentials in squid giant axons (Cohen et al., 1977; Gupta et al., 1981, dye XXV), and subsequent studies of excitation-contraction coupling in skeletal muscle, (Baylor, Chandler & Marshall, 1981) and of the spread of activation in cardiac muscle (Dillon & Morad, 1981). In experiments attempting to monitor E_m of sarcoplasmic reticulum vesicles during Ca pumping, dyes other than WW 781 were preferred because the net accumulation of millimolar Ca by the vesicles was large enough to alter the surface potential and thereby affect dye binding and fluorescence independently of E_m (Beeler, Farmen & Martonosi, 1981). In this paper, we describe the ability of WW 781 to monitor KCl diffusion potentials induced by the addition of ionophores to human red blood cells, and then characterize E_m under a variety of experimental conditions when internal Ca, or Ca_c, is elevated by exposure of the cells to the Ca ionophore A 23187 in the presence of external Ca, or Ca_o. Preliminary accounts of parts of this research have been presented on several occasions (Freedman & Hoffman, 1979*c*; Freedman, 1980; Freedman & Novak, 1981).

Materials and Methods

Preparations of Cells, Membranes, and Media

Blood from healthy human donors was drawn by venipuncture into heparinized tubes and immediately centrifuged at 13,800 × *g* for 3 min at 4 °C. The plasma and buffy coat were aspirated and discarded, and the packed cells were then washed three or four times by centrifugation, each time resuspending in about 5 vol of a chilled wash solution containing 145 mM NaCl, 5 mM KCl, and 5 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), adjusted to pH 7.35 at 22 °C. When low $[K]_o$ was desired, the cells were washed and suspended in media containing 150 mM NaCl and 5 mM HEPES. For experiments with low $[Na]_o$, the cells were washed and suspended in media containing 150 mM choline Cl and 5 mM HEPES. In each case, the cells were then adjusted to 50% hematocrit in the cold wash solutions and kept on ice for use on the same day.

When $[Ca]_o$ was varied, all glassware was washed with acid. Ca was buffered with EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetra-acetic acid), or in later experiments with HEDTA (N-hydroxyethylethylene-diamine triacetic acid). When $[K]_o$ was varied, the sum of external [KCl] and either choline Cl or [NaCl] was kept constant. In order to vary pH, a series of 1.2% hematocrit suspensions (15 ml each) was prepared at pH 7.2 (37 °C), and these were titrated to the desired end point at 37 °C with 0.3 M NaOH or HCl using an automatic burette (Radiometer ETS 822, The London Company, Cleveland, Ohio). Unless otherwise indicated, the values for the pH of the suspensions as given in the Figure legends refer to 37 °C, the temperature at which the cuvettes were thermostatically controlled. The pH of suspensions in the cuvettes was monitored with miniature glass and reference electrodes (MI-506 and MI-402, Microelectrodes, Inc., Londonderry, New Hampshire).

White ghosts were prepared by the method of Dodge, Mitchell and Hanahan (1963), and inside-out and right-side-out vesicles by the method of Steck (1974). To prepare resealed ghosts (Hoffman, Tosteson & Whittam, 1960; Lepke & Passow, 1972), cells were washed as described above and lysed by stirring at pH 6.0 at 1 °C for 10 min in hypotonic media containing 15 mM KCl, 4 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 5 mM HEPES (1 ml cells/10 ml lysis solution). Isotonicity was then restored by adding 125 mM KCl from a 3 M stock, and the pH was adjusted to 7.4 at 37 °C with NaOH. The ghosts were then incubated at 37 °C for 50 min, washed 4 times in the isotonic cell wash solution, adjusted to about 50% HCT, and were kept on ice for use on the same day.

Electrolyte Analysis

Cell Na, K and water contents were determined as previously described (Freedman & Hoffman, 1979*a*). Cl ratios were determined with ^{36}Cl (0.1 μCi/ml) in supernatants and in trichloroacetic acid extracts of weighed aliquots of packed cells, with parallel determinations of water content. At low K_o (0.01 to 10 mM), the actual [K] of the media and cell supernatants were determined when desired by flame photometry, using a LiCl internal standard. The actual total $[Ca]_o$ of the media was checked by atomic absorption spectroscopy and found to be within 10% of the desired values. Concentrations of ionized Ca^{2+} in media containing EGTA or HEDTA were estimated approximately by the equations of Raaflaub (1956) with the constants used by Simons (1976*b*).

Fluorescence and Absorption

Changes in the fluorescence of red cell suspensions containing the oxonol dye, WW 781, were determined either with a 90° differential fluorescence accessory of a split beam absorption spectrophotometer (Aminco DW-2, American Instrument Co., Silver Springs, Md.) used for Fig. 2, 3 and 6–9, or with a

single beam photon counting fluorimeter with a 500 W Xenon lamp, double excitation and single emission monochromators, and a cooled Hamamatsu R 928 photomultiplier tube (SLM 8000 S, SLM Instruments, Inc., Urbana, Ill.) used for Fig. 1, 4 and 11. With the DW-2, matched deflecting mirrors are used to bring the excitation light ($\lambda ex = 615$ nm; 8 nm bandpass) from a 120 W tungsten-iodide lamp into the sides of the reference and sample cuvettes, and a 3-mm thick cut-off filter (RG 645, Schott Optical Glass Inc., Duryea, Pa.) is placed between the cuvettes and the photomultiplier tube (Hamamatsu R 562) at right angles to the excitation beam. The reference cuvette contained sufficient dye in 3 ml of the hydrophobic solvent 2-octanol to match approximately the fluorescence of the sample before the initial membrane potential of the cells was perturbed. Except in preliminary experiments where cell and dye concentrations were systematically varied, the sample cuvette contained 3 ml of isotonic solution (composition as indicated in Figure and Table legends), to which was added 75 μl of 50% hematocrit suspension (final concentration 1.2% HCT) followed by 8 µl of dye (3.5 µM final concentration). The percentage change in fluorescence, % ΔF, is defined to be (final-initial)/initial where the initial value refers to the steady level of fluorescence attained after addition of dye to a cell suspension and the final value refers to the fluorescence 30 sec after addition of A 23187 or valinomycin. Small changes in fluorescence (1 to 10% ΔF) could be conveniently amplified and displayed with signal noise envelopes of 0.5% ΔF. Each point on the figures represents the average of at least two determinations.

The DW-2 spectrophotometer was also used in the split-beam mode for recording absorption spectra.

Uncorrected excitation and emission spectra were obtained in the analog mode of the SLM fluorimeter at 800 to 1100 volts and at 100 sensitivity using a 16-nm excitation bandpass and an 8-nm emission bandpass. Data was acquired for 1 sec at successive 1-nm wavelength increments, stored in the volatile memory, and replotted onto an x-y recorder. Use of the SLM fluorimeter showed that the signal noise levels of cell suspensions (0.4 to 1.2% HCT) containing dye greatly exceeded the noise level of dye in the absence of cells at comparable photon count rates. With cells in the absence of dye, the double excitation and single emission monochromators effectively eliminated all scattered excitation light from reaching the detector, implying that the signal noise level of cells in the presence of dye is due to scattering of emitted fluorescent light and to variations in the number and orientation of cells in the beam. For kinetic runs with the SLM fluorimeter, optimal S/N was achieved by using the RG 645 cut-off filter and a frosted quartz screen in place of the emission monochromator.

Ion Fluxes

The effect of WW 781 on Ca-induced K efflux was tested by using ^{86}Rb as a tracer for K according to a procedure similar to that described by Reichstein and Rothstein (1981). Fresh blood was drawn and washed as in previous experiments in chilled media containing 5 mM KCl, 145 mM NaCl and 5 mM HEPES (pH 7.6 at 25 °C). The red cells were loaded with tracer during overnight incubation in a 37 °C shaking water-bath at 20% hematocrit in media containing 5 mM KCl, 138 mM NaCl, 1 mM EGTA, 5 mM glucose, 10 µ Ci/ml ^{86}Rb and 20 mM HEPES (pH 7.4 at 37 °C). Penicillin G potassium and streptomycin sulfate were added and the time into and out of the incubation was recorded. After incubation, the cells were diluted, centrifuged, and then resuspended 5 times in ice-cold tracer-free media containing 150 mM NaCl or choline Cl and 5 mM HEPES (pH 7.3 at 25 °C). The washed packed cells were kept on ice for use the same day. The flux media, contained in 25-ml Erlenmyer flasks, were equilibrated on a 37 °C shaking water bath and at desired times packed red cells were added with a tuberculin syringe to 1% hematocrit. Within 30 sec, 3.5 µM WW 781 was added and the flux was initiated by addition of 2.4 µM A 23187. At desired time intervals, 1.3-ml samples were taken via syringe and transferred to 1.5-ml capacity microcentrifuge tubes. The centrifuge (Eppendorf 5412) was run for 5 sec at 23 °C and a 1-ml sample of the supernatant was pipetted immediately for subsequent counting (Gamma 8000, Beckman Instruments, Inc.). The remaining portions of each suspension were lysed with 15 µl of 5% Triton X-100 and 1 ml was counted (cpm lysate) along with supernatants from the timed samples (cpm sample). The fraction F of ^{86}Rb remaining in the cells at each time point was computed from $F = 1 -$ (cpm sample/cpm lysate). Rate constants, $^{o}k_{Rb}$ (hr^{-1}), were obtained from the slopes of linear regressions of log F vs. time, in which zero time was at the addition of A 23187 and the sample time was at the start of centrifugation. With immediate pipetting of the supernatants, all slopes were semi-logarithmic for at least 5 min and all intercepts were within 5% of unity. WW 781 was found to be nonhemolytic for at least 30 min at 37 °C.

Reagents

Dye, ionophores and drugs were added from the following stock solutions in EtOH: 1 mg/ml WW 781 (triethanolamine salt, gift of Dr. A.S. Waggoner, Department of Biological Science, Carnegie Mellon University, Pittsburgh, Pa.), 3 mM valinomycin (Calbiochem), 2.4 mM A 23187 (Calbiochem), 3 mM gramicidin D (Sigma), 10 mg/ml oligomycin (65% A, 20% B, 15% C, Sigma), 3.7 mg/ml quinine sulfate (Sigma), and 3 mM FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, gift of Dr. P.G. Heytler, E.I. Dupont De Nemours and Co.). Trifluoperazine hydrochloride (10 mg/ml) was obtained as SK and F Stelazine concentrate. A stock solution of DIDS (4,4′-diisothiocyano-2,2′-disulfonic acid stilbene from Sigma) was prepared daily at 0.5 mg/ml in 150 mM NaCl. All other chemicals were reagent grade.

Results

The first section of results describes basic spectral properties of WW 781 in suspensions of human red blood cells and in solvents of varying dielectric constant. The next section describes experiments using ionophores to evaluate the correlation between the fluorescence of WW 781 and E_m of red cells and to calibrate fluorescence vs. E_m. Then various experimental conditions are examined in order to compare changes in dye fluorescence with known properties of K permeability when the Gardos effect is initiated with A 23187 in the presence of Ca_o (Lew & Ferreira, 1976; Reed, 1976; Gardos et al., 1977; Yingst & Hoffman, 1978). The final section of Results defines conditions under which WW 781 may serve as a continuous monitor of time-dependent changes in E_m.

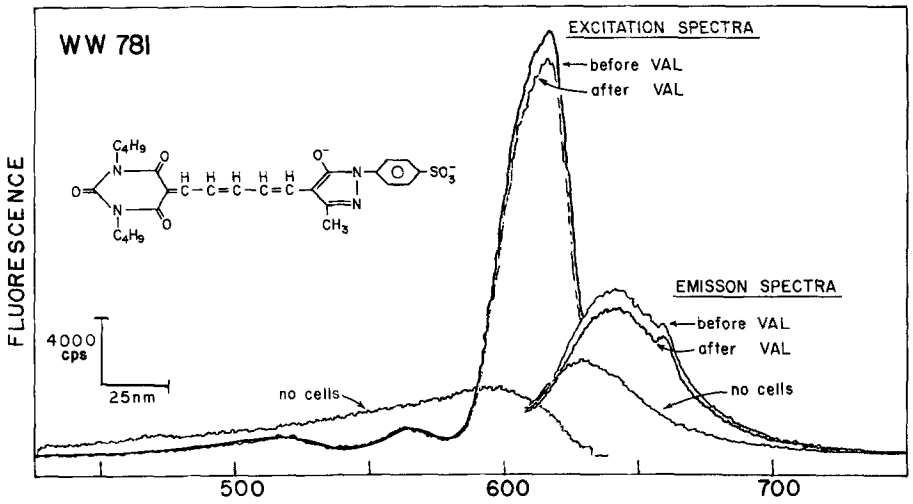

Fig. 1. Excitation and emission spectra (uncorrected) of the oxonol dye, WW 781 (bis-[3-methyl-1-*p*-sulfophenyl-5-pyrazolone-(4)]-pentamethine oxonol). The lower traces refer to dye in buffer. The upper traces refer to dye in the presence of a suspension of human red blood cells before and after hyperpolarization by addition of 1 μM valinomycin (*VAL*). Excitation spectra ($\lambda em =$ 643 nm) are on the left and emission spectra ($\lambda ex = 593$ nm) are on the right. The buffer (2.5 ml) consisted of 5 mM KCl, 145 mM NaCl, and 5 mM HEPES (pH 7.4 at 37 °C), to which was added 62.5 μl of 50% hematocrit washed red cells (upper traces only) followed by 3.5 μM WW 781. *See* Materials and Methods for additional details

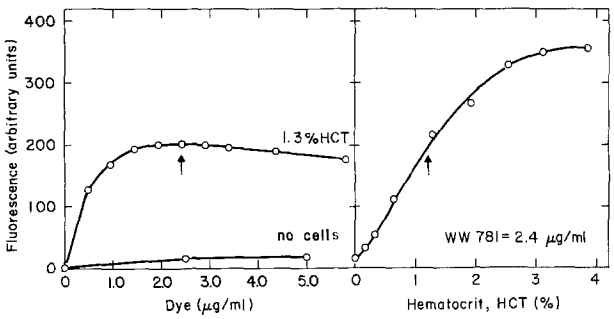

Fig. 2. Effect of dye and cell concentrations on the fluorescence of WW 781. As described in Materials and Methods, a 50% HCT suspension of human red blood cells was prepared in media containing 5 mM KCl, 145 mM NaCl, and 5 mM HEPES buffer, pH 7.4 at 22 °C. The left panel shows the steady levels of fluorescence attained 1 min after successive additions of dye, 1 to 3 μl each, to a cuvette containing 3 ml buffer alone (lower curve) or to one containing buffer plus 80 μl of the cell suspension (upper curve). The right panel shows the steady levels of fluorescence 1 min after successive additions of cell suspension, 10 to 50 μl each, to a cuvette containing buffer plus 10 μl dye. The final concentration of dye and cells are as indicated. The arrows show the dye and cell concentrations chosen for subsequent experiments

Table 1. Spectral properties of WW 781 in solvents of varying dielectric constant[a]

Solvent	Dielectric constant	Maximum absorption (nm)	Molar extinction coefficient ($\times 10^{-5}$ M^{-1} cm^{-1})	Relative fluorescence
Water	78.5	603	1.0	–
Buffer	–	603	1.1	1.0
Methanol	32.6	609	1.2	1.8
Ethanol	24.3	613	1.1	2.8
Octanol	10.3	619	1.1	15.4
Chloroform	4.8	621	1.1	3.4
Benzene	2.3	615	0.64	6.0
Dioxane	2.2	468	0.34	–

[a] Split-beam absorption spectra of dye in each solvent were recorded versus solvent. Molar extinction coefficients were determined for λmax in the range of 0 to 15 μM [dye], using a formula weight of WW 781 equal to 759 g/mole. The dielectric constants (25 °C) of the solvents are taken from the Handbook of Chemistry and Physics (1981/82). The values for relative fluorescence are for 7.0 μM dye solutions, a concentration at which fluorescence as a function of [dye] has reached a maximal value

Spectral Properties of WW 781 and Calibration of Fluorescence vs. E_m

The chemical structure of WW 781 is shown in Fig. 1, along with uncorrected excitation and emission spectra of the dye in a cuvette containing buffer without cells and of dye in a suspension of human red blood cells before and after hyperpolarization by addition of the K-ionophore, valinomycin. With cells, the excitation maximum was recorded at 614 nm with maximum emission at 640 nm. Two pertinent features of these spectra are that the fluorescence intensity of WW 781 is red-shifted by 10 nm and enhanced in the presence of cells, and that hyperpolarization of the cells by valinomycin causes a decrease in the fluorescence

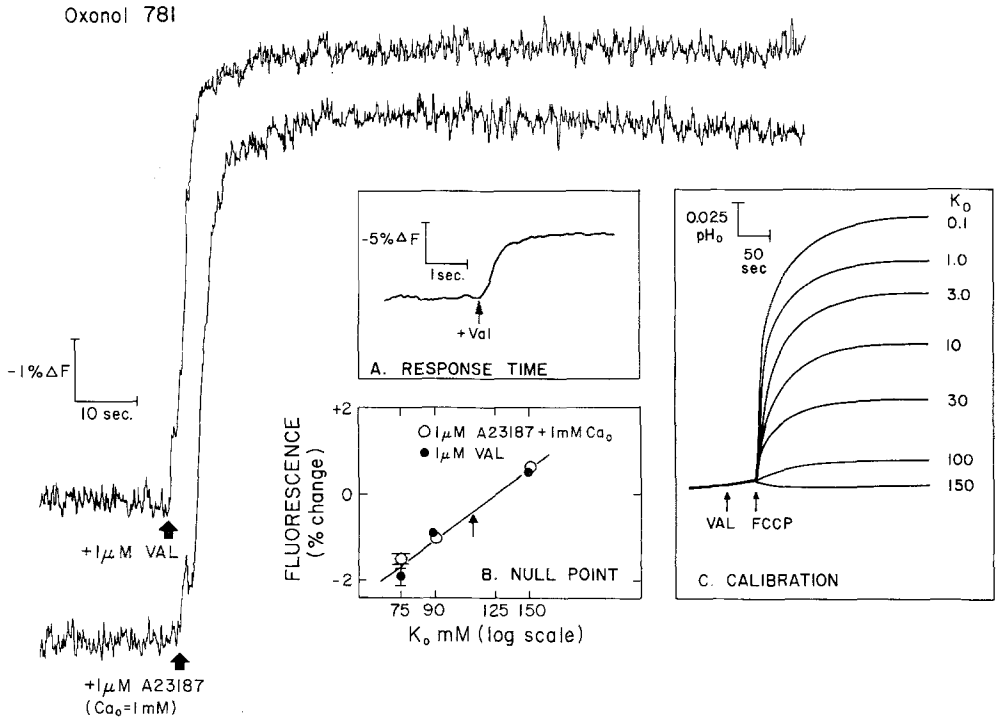

Fig. 3. Changes in the fluorescence of WW 781 after inducing KCl diffusion potentials by adding valinomycin (upper trace) or A 23187 (lower trace) to red cells. To cuvettes containing 3 ml of the same medium used in Fig. 2 were added 75 μl of 50% HCT red cells followed by 3.2 μM WW 781, and the fluorescence was recorded at 25 °C. For the upper trace, the diffusion potential was induced by adding 1 μM valinomycin and for the lower trace, 3 μl of 1 M $CaCl_2$ was added (1mM final Ca_T) followed by 1 μM A 23187. *A*. Response time. Inset *A* shows that when the fluorescence is recorded at reduced amplifier gain and with the chart speed increased by a factor of 10 to 1 sec/inch, the response of dye fluorescence to the diffusion potential induced by valinomycin is complete within 1 sec. *B*. Null point. Inset *B* shows identical null points obtained with valinomycin and with Ca plus A 23187 (*see* text). Points represent average of duplicate determinations. The arrow designating the null point is displaced to −0.4% ΔF to correct for the effect of EtOH. *C*. Calibration. Inset *C* shows the changes in pH_o which occur after addition of 1 μM valinomycin and 10 μM FCCP to a series of cuvettes containing unbuffered isotonic suspensions of DIDS-treated cells at 1.2% HCT in medium consisting of *x* mM KCl and (150-*x*) mM NaCl. After the initial washing three times in 150 mM NaCl, the cells were treated at 25% HCT with 10 μM freshly prepared DIDS for 10 min at 23 °C, and then returned to ice until needed. Simultaneously determined changes in pH_o and fluorescence allow calibration of fluorescence vs. E_m, as described in Fig. 4

intensity without a detectable spectral shift. The two dips at 540 and 576 nm in the excitation spectra with cells correspond to the absorption peaks of hemoglobin. The absorption spectrum of WW 781 in the presence of cells (*not shown*) resembled the excitation spectrum seen in Fig. 1, but with a single major peak at 603 nm. Addition of valinomycin resulted in no detectable change (<0.005 OD) in the wavelength or magnitude of light absorption by the dye.

The results in Fig. 2 (left panel) show that addition of varied amounts of WW 781 to a 1.3% hematocrit suspension of red blood cells (upper curve) results in an enhancement of dye fluorescence by 15- to 20-fold over that exhibited by the same amount of dye added to cell-free buffer (lower curve). Similarly, the increased fluorescence resulting from the addition of successive amounts of cells to a cuvette containing a constant amount of dye is shown in Fig. 2 (right panel). When dye absorption spectra, molar extinction coefficients, and relative intensities of fluorescence were determined in seven solvents of varying dielectric constant (Table 1), it was found that as the polarity of a series of alcohols decreased, WW 781 exhibited a red-shifted major absorption peak and an enhanced fluorescence. In 2-octanol, the dye fluorescence was 15-fold higher than in aqueous buffer. These results suggest that the enhanced fluorescence of WW 781 in red cell suspensions may be due to association of dye with hydrophobic regions of the red cell membrane.

Addition of 1 μM valinomycin to cells in low K medium in the presence of dye at systematically varied cell and dye concentrations led to the choice of 1.2% hematocrit and 2.4 μg/ml dye as conditions (indicated by arrows on Fig. 2) which gave a maximal change in fluorescence in response to a given change in E_m. The upper trace in Fig. 3 shows a typical kinetic record of a change in fluo-

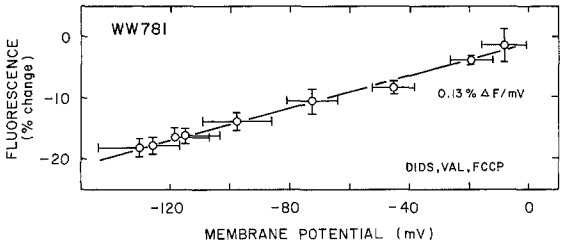

Fig. 4. Calibration of fluorescence vs. E_m for WW 781. In unbuffered DIDS-treated cell suspensions as described in Fig. 3, Inset *C*, the changes in the fluorescence of WW 781, as induced by 1 μM valinomycin at varied K_o, were recorded simultaneously with the changes in pH_o, as induced by subsequent addition of 10 μM FCCP. At each K_o between 0.1 and 150 mM, the membrane potential was computed from $\Delta E_m = -(RT/F)\Delta pH_o$, where $RT/F = 58.7$ mV at 23 °C. The error bars represent ± 1 SD for data from 5 independent experiments. Linear regression yields a slope of 0.13 ± 0.03 (SD, $n=5$) % ΔF/mV

rescence in response to hyperpolarization induced by valinomycin. In these and subsequent traces, an upward pen deflection represents a decrease in fluorescence corresponding to hyperpolarization of the membrane dominated by the outwardly directed K gradient. By speeding up the chart recorder and decreasing the amplifier gain, and by using an optimal speed of stirring the magnetic spin bar in the cuvette, an upper limit for the response time of WW 781 to the potential change was determined to be 1 sec, a value limited by the time required to mix the ionophore into the suspension (Fig. 3, inset *A*). This response time is about an order of magnitude faster than obtained with permeant cyanine dyes, such as diS-C_3 (5) (Sims et al., 1974). Attempts were made to measure the change in binding of dye to red cells possibly associated with a change in E_m, but the change in the fluorescence of the medium as determined after centrifuging the cuvette both before and after addition of valinomycin was too small to give precise results. The magnitude of the change in dye fluorescence induced by valinomycin decreased between 0.1 mM $[K]_o$ and the null point, as $[K]_o$ was increased by isotonic substitution with $[Na]_o$. Between the null point, and 150 mM K_o, the dye fluorescence increased (partially shown in Fig. 3*B*, solid circles). A similar pattern was obtained with gramicidin in a series of suspensions in which choline was substituted for $[Na]_o$, and in which $[K]_o$ was varied from 1 to 150 mM by isotonic substitution for $[choline]_o$. Choline was used because of the poor discrimination between Na and K by gramicidin, and because choline is larger than tetramethylammonium, which was shown to be almost totally excluded from the gramicidin channel (Myers & Haydon, 1972). Representative traces obtained with gramicidin at varied $[K]_o$ may be found in a review article (Freedman & Laris, 1981). With both ionophores, a decrease in fluorescence is associated with hyperpolarization, while an increase in fluorescence corresponds to depolarization.

The changes in fluorescence obtained with WW 781 are about an order of magnitude smaller than displayed by the permeant cyanine dyes with the same ionophores and K gradients. We also note that WW 781, like diS-C_3 (5), works as well with valinomycin-induced potentials in resealed ghosts, and in right-side-out and inside-out vesicles as it does with intact cells. In each of these systems the changes of fluorescence of WW 781 are too small to be accurately calibrated by measurements of dye binding (Hladky & Rink, 1976) or by equilibrium fluorescence with Gibbs-Donnan potentials (Freedman & Hoffman, 1979*b*). A new method of calibrating changes in dye fluorescence (% ΔF) versus ΔE_m is based on the proposal of Macey, Adorante and Orme (1978) that addition of a proton ionophore to red cells in unbuffered media results in changes in pH_o which are related to ΔE_m as follows:

$$\Delta E_m = -(2.3\ RT/F)\ \Delta pH_o \qquad (1)$$

where $2.3\ RT/F = 58.7$ mV at 23 °C. As pointed out by Wieth, Brahm and Funder (1980), Eq. (1) is valid only if the Cl^-/HCO_3^- exchanger, which can hold protons in a steady state away from electrochemical equilibrium, is inhibited – a condition satisfied by treatment of red cells with DIDS. The data in Fig. 3, inset *C*, show that addition of valinomycin to an unbuffered suspension of DIDS-treated red cells results in a negligible ΔpH_o due to the low proton or OH^- permeability of the cells. Subsequent addition of 10 μM FCCP, a proton ionophore (Heytler & Prichard, 1962), allows protons to equilibrate with E_m, and pH_o then rises or falls in accordance with ΔE_m, as set by K_o. Simultaneous measurements of fluorescence and pH_o and use of Eq. (1) yields a calibration of 0.13 ± 0.03 (SD, $n=5$) % ΔF/mV for the conditions studied, with a linear response between +5 and −120 mV (Fig. 4). As a control, application of the ΔpH_o calibration method to diS-C_3 (5) gave results (*not shown*) in agreement with those previously determined by other methods (Hladky & Rink, 1976; Freedman & Hoffman, 1979*b*). In cells untreated with DIDS, but treated with valinomycin and FCCP, the K_o at the null point, or $K_o^{n.p.}$, at which $\Delta pH_o = 0$, is another way of estimating the resting potential. With blood from one donor (pH 7.4,

23 °C), $E_m = -(RT/\mathbf{F}) \ln (K_c/K_o^{n.p.}) = -10$ mV, in satisfactory agreement with the values of -12 and -9 mV, which we obtained for the same donor at 37 °C using the fluorescence null point method (Hoffman & Laris, 1974) with WW 781 and diS-C_3 (5), respectively.

Ca-Induced Changes in E_m

Ca-induced K Loss in the Absence and Presence of WW 781. In view of previous reports that cyanine dyes selectively inhibit the Gardos effect (Simons, 1976*a*, 1979), the occurrence of Ca-induced loss of K was examined in the presence and absence of WW 781. Cells suspended in media containing 5 mM KCl, 145 mM NaCl, and 5 mM HEPES (pH 7.4 at 37 °C) had ratios $[Cl]_c/[Cl]_o$ of 0.67 ± 0.05 (SD, $n=3$) in the absence of WW 781 and 0.66 ± 0.01 (SD, $n=3$) in the presence of 3.5 μM WW 781, indicating that the dye does not perturb the normal resting potential, which in this experiment was -11 mV from E_{Cl}. Isotonic 5% HCT suspensions containing 1 mM $CaCl_2$ with and without WW 781 were prepared and the Gardos effect was initiated by adding 1 μM A 23187. After incubation for 15 min at 37 °C, cell K and Na contents were analyzed. Control suspensions containing WW 781, but without A 23187, maintained normal K and Na contents, as shown in the left-most bargraph of Fig. 5. In the absence of WW 781, addition of 1 μM A 23187 caused a pronounced net efflux of K without much gain of Na (Fig. 5, second from left). In another experiment with $[K]_o$ at zero (nominally), 0.1, 2 and 5 mM (Fig. 5), the presence of 3.5 μM WW 781 did not prevent the Ca-induced selective K loss. Additional data (*not shown*) at varied [dye] and $[K]_o$ from both experiments indicated that the amounts of K remaining in the cells 15 min after addition of A 23187 in the presence of dye was 30 to 50% of that in paired samples without dye. However, the major conclusion to be drawn from these results is that the presence of WW 781 is compatible with the occurrence of Ca-induced selective loss of K. A closer examination of unidirectional K efflux in the presence and absence of WW 781 will be described later.

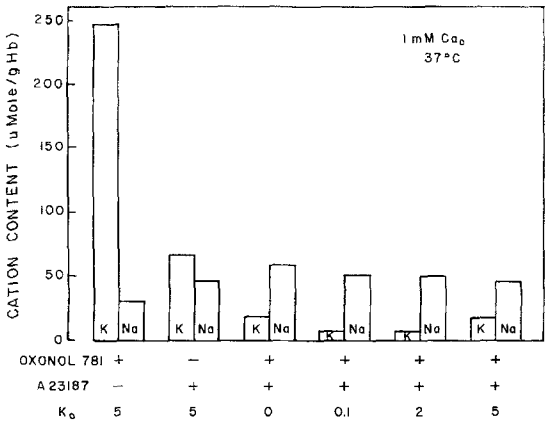

Fig. 5. Lack of inhibition by WW 781 of the Ca-dependent selective loss of K from human red blood cells. The results shown above are from two experiments. For the experiment summarized in the left two bar graphs, the cells were washed as described in Materials and Methods, and resuspended to 1.2% HCT. For the experiment summarized in the right four bar graphs, the cells were washed in media containing 150 mM NaCl and 5 mM HEPES (pH 7.4 at 22 °C), then resuspended to 60% HCT and divided into portions. Each portion was then washed twice more in cold media containing 0 to 5 mM KCl as indicated, 150 to 145 mM NaCl, and 5 mM HEPES (pH 7.4 at 22 °C), and finally diluted to 1.2% HCT. For both experiments, $CaCl_2$ (1 M stock) was then added to 1 mM and the suspensions were equilibrated in a shaking water bath at 37 °C. To each suspension 3.5 μM WW 781 and 1 μM A 23187 were then added as indicated. Fifteen minutes after addition of the Ca ionophore, six 10-ml aliquots of each suspension were centrifuged, three for triplicate analysis of cell K and Na, and three for parallel analyses of water content as described in Materials and Methods

Effects of $[Ca]_o$ and [A 23187]. When 1 μM A 23187 was added to a red cell suspension containing 1 mM $[Ca]_o$, an immediate decrease in fluorescence was observed which was comparable to that induced by valinomycin (Fig. 3, lower trace). The fluorescence change induced by A 23187 in the presence of Ca_o resembled those obtained with valinomycin and with gramicidin, and by analogy could be attributed to a change in E_m were it not for the possible complication that entry of Ca might alter the dye fluorescence independently of the effect of E_m. A test for such a possibility is shown in Fig. 3, inset *B*. In this experiment, the K_o at which no change in fluorescence occurs upon addition of ionophore (designated the "null point"), was determined by interpolation. At the null point, the K equilibrium potential, E_K, after addition of ionophore is equal to the initial resting potential; therefore, E_m does not change upon addition of the ionophore. If there were a direct effect of Ca_c on dye fluorescence, independent of the effect of E_m on fluorescence, it would be seen as an offset of the null point in contrast to the results shown in Fig. 3, inset *B*. At the null point, Ca may enter the cells freely but the fluorescence does not change. The fact that the same null point is obtained either with valinomycin or with A 23187 plus Ca establishes that the amount of Ca_c which is sufficient to induce the change in K conductance in red cells is low enough to avoid complications from direct effects of Ca on dye fluorescence.

Table 2. Comparison of null points with valinomycin and with Ca+A 23187[a]

K_o (mM)	Fluorescence (% change)	
	Valinomycin	Ca+A 23187
75	−1.0	−1.1
90	−0.7	−0.8
142.5	+0.4	+0.2

[a] Cells were washed and prepared as described in Materials and Methods. The sample cuvette initially contained 3 ml of medium consisting of x mM KCl, where $x = 75$, 90 or 142.5, (142.5-x) mM NaCl, 5.0 mM $CaCl_2$, 0.5 mM EGTA, and 5 mM HEPES buffer (pH 7.2 at 37 °C). Cells (75 µl of 50% HCT suspensions) and 3.5 µM dye were added, and after equilibration at 37 °C either 1 µM valinomycin or 1 µM A 23187 was added. Values of % ΔF are averages of duplicate determinations and are corrected for the −0.4% ΔF due to addition of EtOH

Injection of 1 to 10 mM $[Ca]_o$ to cuvettes containing suspensions of intact red cells or leaky white ghosts in the presence of dye but in the absence of A 23187 does increase the initial fluorescence substantially (+2% ΔF/mM $[Ca]_o$ for intact cells and +8% ΔF/mM $[Ca]_o$ for ghosts) even though ΔE_m is negligible. The results in Table 2 show that the same null point is obtained with valinomycin and with A 23187 even at 5 mM $[Ca]_o$. At this concentration, when Ca is able to enter the cells at the null point, an offset of the null point would be expected if dye had permeated and had bound to the inner membrane surface. The observation that the null point is invariant at 5 mM $[Ca]_o$ is consistent with, but does not prove the hypothesis that WW 781 is impermeant.

In inducing the fluorescence change of WW 781, A 23187 is half-maximally effective at 0.04 µM in 1.3% HCT suspensions (Fig. 6*A*), and the 1 µM concentration chosen for subsequent studies is maximally effective. In comparison, ionomycin, which is also a Ca ionophore (Liu & Hermann, 1976), is half maximally effective at 0.25 µM (dose-response curve not shown). The effect of $[Ca]_o$ on the rapid fluorescence changes of WW 781 is shown in Fig. 6*B*. In these experiments $[Ca]_o$ was buffered with 0.5 mM EGTA. At a half-maximally effective total Ca of 0.24 mM, it may be estimated (Raaflaub, 1956; Simons, 1976*b*) that the ionized external $[Ca^{++}]_o$ would be approximately 0.07 µM, in agreement with tracer efflux studies employing A 23187 (Reichstein & Rothstein, 1981). After hyperpolarization of the cells with A 23187 in the presence of Ca_o, subsequent addition of excess EGTA promptly restores the fluorescence of WW 781 back to the original level (*not shown*).

Effect of pH_o. The effect of pH_o on the changes in fluorescence of WW 781 induced by addition of 1 µM A 23187 at 0.5 mM Ca_o and 0.5 mM EGTA is shown in Fig. 7. A complicated relationship between the effects of pH and K_o on Ca-induced

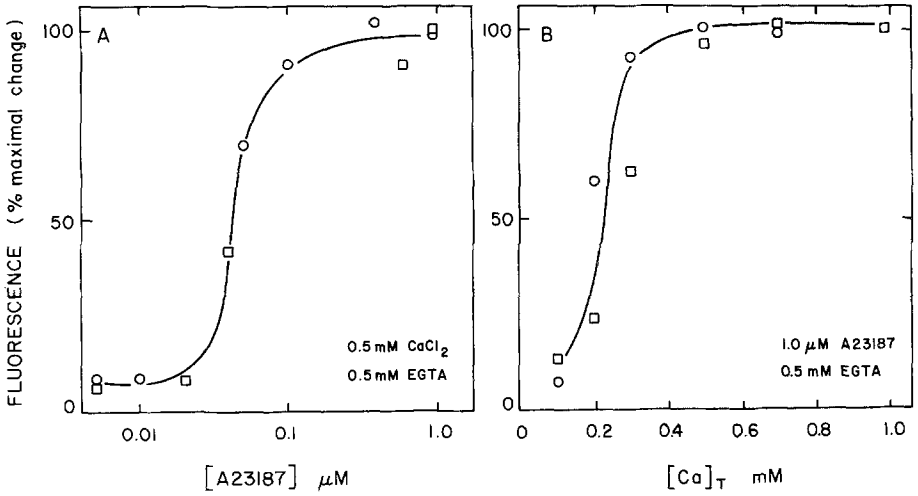

Fig. 6. Percentage change of fluorescence of WW 781 induced by Ca ionophore at varied [A 23187] and $[Ca]_T$. In *A*, the sample cuvettes contained 3 ml of 5 mM KCl, 145 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM EGTA, and 5 mM HEPES buffer, pH 7.2 at 37 °C. After adding 75 µl of 50% HCT red cell suspension and 3.5 µM WW 781, the diffusion potential was induced by adding 1 µl of A 23187 (the 3 mM stock ionophore solution had been diluted to produce final concentrations ranging from 0.005 to 1.0 µM). In *B*, the sample cuvettes initially contained 1 mM KCl, 149 mM NaCl, 0.5 mM EGTA, 5 mM HEPES buffer (pH 7.2 at 37 °C) and 0 to 1 mM $CaCl_2$. Cells and dye were added as in *A*, and the diffusion potential was induced at 37 °C with 1 µM A 23187. For *A* and *B*, the percentage change in fluorescence was noted 1 min after addition of A 23187. The circles and squares represent data from two experiments, normalized by dividing each % ΔF by the maximal % ΔF attained on a given day at 1 µM A 23187 and at 1 mM $(Ca)_T$

^{42}K efflux has previously been briefly described (Hoffman et al., 1980). In the present experiments, the fluorescence changes were independent of pH_o in the range 6.5 to 7.5. Outside of this range, the voltage response was dependent on K_o, being more sensitive to pH_o at 0.01 mM K_o (Fig. 7, dotted line) than at 1.0 mM K_o (Fig. 7, solid line). These results would predict that under the conditions employed, the change in K conductance responsible for the voltage change should be activated by K_o at pH 6 but not at neutral pH.

Effects of K_0 and Na_0. The aforementioned expectation was confirmed by the results shown in Fig. 8, in which the fluorescence changes of WW 781 induced by A 23187 were determined in suspensions in which K_o was systematically varied from 0.1 to 142.5 mM at pH 6.0 and at pH 7.2 in media containing sufficient Na or choline to maintain isotonicity. In Na-containing media at pH 7.2 (Fig. 8*A*), maximal hyperpolarization was observed at 0.1 mM K_o, and as K_o was increased

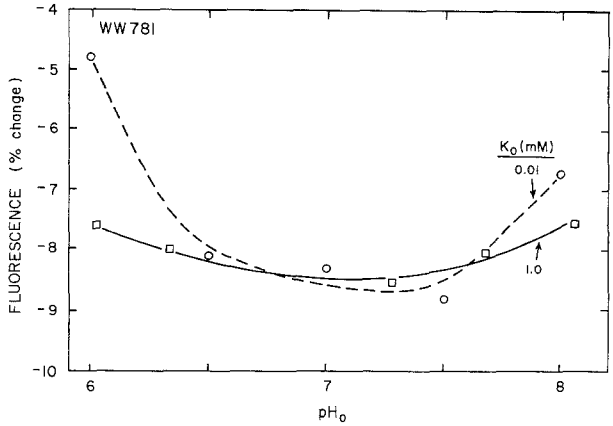

Fig. 7. Effect of pH_o on the percentage change of fluorescence of WW 781 induced by A 23187 at two external K concentrations. Cells were washed and prepared as described in Materials and Methods. The sample cuvettes contained 3 ml of media consisting of 0.01 mM KCl (circles, dotted line) or 1.0 mM KCl (squares, solid line), 150 or 149 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM EGTA, 5 mM HEPES buffer (pH 6.0 to 8.0 at 37 °C), and cells at 1.2% HCT. After adding 3.5 μM dye and equilibrating at 37 °C, the diffusion potential was induced with 1 μM A 23187 and the percentage change in fluorescence was noted when the signal reached a new plateau

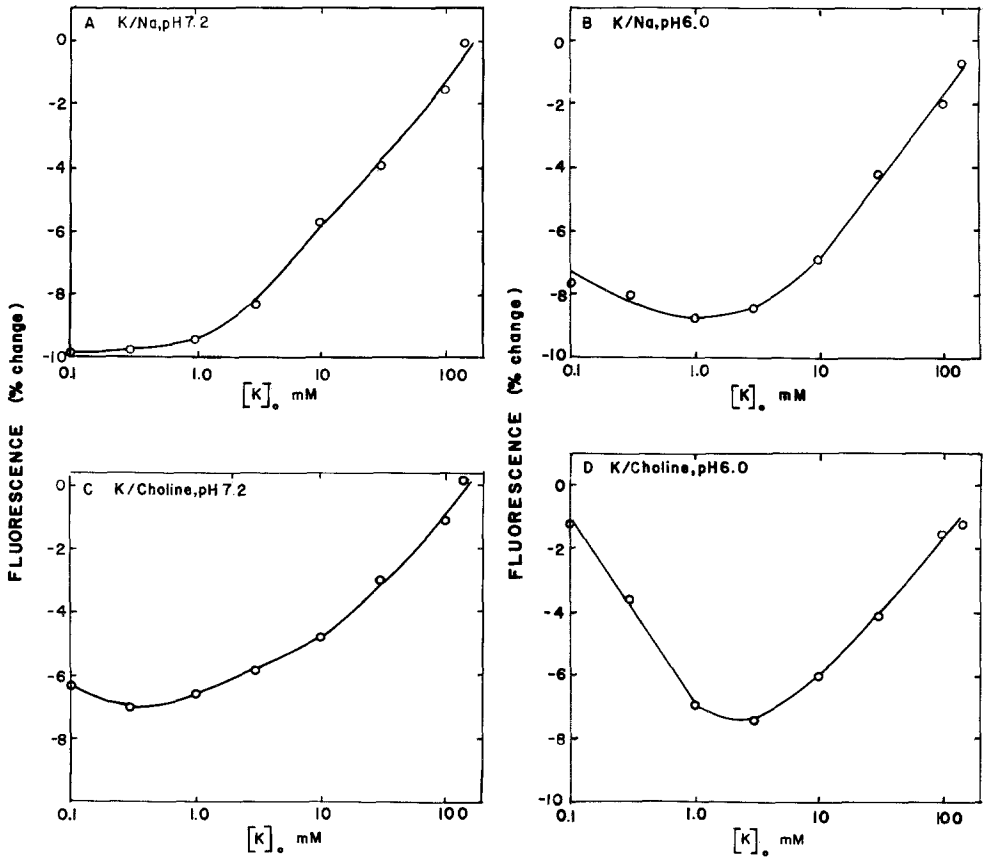

Fig. 8. Effect of K_o on the percentage change of fluorescence of WW 781 induced by A 23187 at two pH's and in the presence and absence of Na_o. Cells were washed and prepared as described in Materials and Methods. The cuvettes contained 3 ml of media consisting of x mM KCl, where x=0.1, 0.3, 1.0, 3.0, 10, 30, 100 and 142.5 mM, (142.5-x) mM NaCl (in *A* and *B* above) or (142.5-x) mM choline Cl (in *C* and *D* above), 0.5 mM $CaCl_2$, 0.5 mM EGTA, and 20 mM HEPES buffer (pH 6.0 in *B* and *D* above, or pH 7.2 in *A* and *C* above, all adjusted at 37 °C). Cells, dye and 1 μM A 23187 were added as described in Fig. 7, and the percentage change in fluorescence was determined in duplicate and averaged for each solution

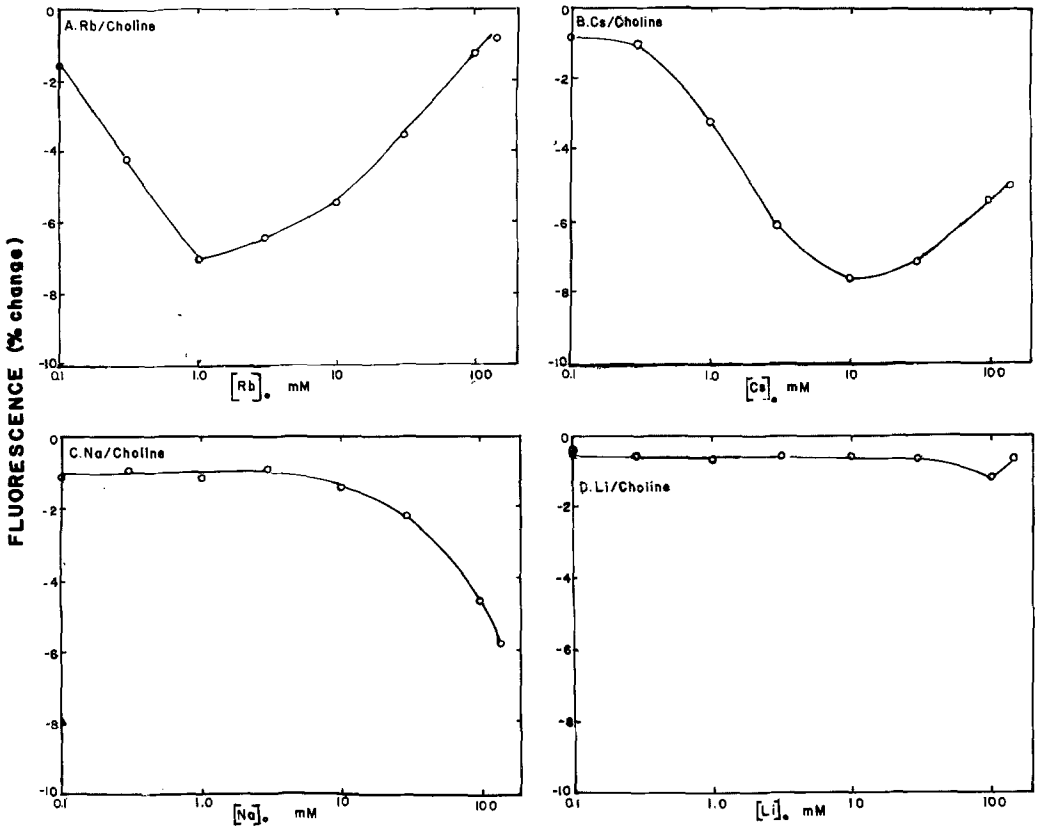

Fig. 9. Ionic selectivity of the Ca-dependent fluorescence changes of WW 781 related to activation and to conductance. Cells were washed and prepared as described in Materials and Methods. The cuvettes contained 3 ml of media consisting of x mM RbCl (panel *A* above), CsCl (panel *B*), NaCl (panel *C*) or LiCl (panel *D*), where $x = 0.1$, 0.3, 1.0, 3.0, 10, 30, 100 and 142.5 mM, (142.5-x) mM choline Cl, 0.5 mM $CaCl_2$, 0.5 mM EGTA, and 20 mM HEPES buffer (pH 6.0 at 37 °C). Cells, dye and 1 μM A 23187 were added as described in Fig. 7, and the percentage change in fluorescence was noted in duplicate for each solution

the extent of hyperpolarization decreased monotonically in accordance with the decreased outward K gradient. At pH 6.0 (Fig. 8*B*), activation of the increased K conductance by K_o was evidenced by the slightly increasing extent of hyperpolarization between 0.1 and 1.0 mM K_o, followed by decreased hyperpolarization as K_o was increased further. In Na-free choline-containing media at pH 7.2 (Fig. 8*C*), activation by K_o was again only slightly apparent with a maximal hyperpolarization occurring at 0.3 mM. However, at pH 6.0 in choline-containing media (Fig. 8*D*), there was a barely detectable fluorescence change at 0.1 mM K_o, and as K_o was raised a maximal hyperpolarization was evident at 3 mM K_o followed by decreasing responses at higher K_o. The fact that the biphasic response to K_o was more apparent in the absence of Na than in its presence (*compare* Fig. 8*B* and 8*D*) is consistent with competition between Na_o and K_o at the external activating site, i.e., that Na_o is a competitive activator. As a control, under the conditions of Fig. 8*D*, namely choline media at pH 6.0, valinomycin-induced changes in dye fluorescence showed a monophasic response to varied K_o, similar to the pattern of results seen in Fig. 8*A*. The interactions between Na_o and K_o at the external activating site seen in Fig. 8 are consistent with previous descriptions of Na activation of K efflux (Knauf et al. 1975; Hoffman & Blum, 1977), and activation of Ca-dependent K efflux by K_o has been studied in ATP-depleted cells (Kregenow & Hoffman, 1972; Hoffman & Blum, 1977) and in red cell ghosts (Knauf et al., 1975; Simons, 1979; Heinz & Passow, 1980).

The dependence on K_o illustrated by the results in Fig. 8*A* was also found in additional experiments in Na-containing media under other conditions which included raising $[CaCl_2]_o$ to 10 mM, lowering $[CaCl_2]_o$ to a submaximally effective level of 0.2 mM (with 0.5 mM EGTA), raising $[MgCl_2]_o$ to 10 mM, using cells which had been depleted of ATP and other metabolites by incubation for 35 hr at 37 °C in the absence of glucose, and also by inducing the Gardos effect with ionomycin instead of with A 23187. Activation of the Ca-dependent fluorescence changes by K_o at pH 6 (Fig. 8*D*), but

not at pH 7 (Fig. 8*C*), is also in accordance with the known properties of the Gardos effect. At 1% HCT, below which the dye was not usable, and with an initial K_o between 0.01 and 10 mM, addition of A 23187 caused a rise in K_o amounting to 0.4 ± 0.1 mM (SD, $n = 10$) in 1 to 2 min, as determined by flame photometric analyses of supernatants of cell suspensions which were taken from the cuvettes and centrifuged before and immediately after addition of the Ca ionophore. Simons (1979) reported that activation by K_o at pH 7.1 is maximal at 0.15 mM in Ca-loaded ghosts, and Hoffman et al. (1980) reported that reducing pH_o raises the apparent $K_{1/2}$ for K activation, in agreement with the results in Fig. 8*C* and *D*.

Ionic Selectivity. In order to begin to characterize the ionic selectivity of the fluorescence changes associated with both activation and conductance, each of the five alkali cations was tested to determine the concentration which permits Ca plus A 23187 to induce the voltage change. Suspensions were prepared at pH 6 with each alkali cation ranging from 0.1 to 142.5 mM, and with isotonicity maintained by choline Cl. The results (Fig. 9) show that in addition to K, activation occurs with Rb, Cs and Na, but is not detectable with Li. The order of effectiveness in activating the fluorescence change was Rb, $K > Cs > Na$.

From the curve shown in Fig. 9*C*, in which $[Na]_o$ was increased while maintaining isotonicity by decreasing $[choline]_o$, it is not possible to conclude whether the increasing hyperpolarization is due to activation by $[Na]_o$, or, alternatively, to a possible decrease of inhibition by choline as its concentration is decreased. The experiments with choline (Figs. 8 and 9) were designed under the assumption that choline does not interact with the system that mediates Ca-induced K conductance. To test this assumption, the Gardos effect was induced by adding A 23187 to suspensions in which $[choline]_o$ was raised from 0.1 to 139.5 mM while maintaining isotonicity by decreasing either NaCl in one series or LiCl in another series. The media were adjusted to pH 6.0 at 37 °C and also contained 0.5 mM $CaCl_2$, 0.5 mM EGTA, 20 mM HEPES, and 3 mM KCl, sufficient K_o to induce the full hyperpolarization. The fluorescence changes were maximal in all of these media. Since there was no indication of inhibition by choline, the curve in Fig. 9*C* must result from activation by Na_o. A lack of inhibition by choline was also demonstrated by Kregenow and Hoffman (1972) by a similar experiment with tracer fluxes.

The same series of experiments which define the selectivity of activation also begins to describe, at least semiquantitatively, the selectivity of the conductance pathway itself. The greater the conductance of an external cation, the lower the concentration needed to reach the null point. One interpretation of the increasing portions of the curves in Figs. 8 and 9 is that while the conductances of Na and Li are undetectable, K and Rb have comparable ionic conductances both of which are greater than that of Cs. While this pattern of selectivity agrees with that determined by measuring ionic fluxes from ATP-depleted intact cells (Kregenow & Hoffman, 1972) and from Ca-loaded ghosts (Simons, 1976*c*), a quantitative analysis of the selectivity ratios for ionic conductances is not possible until further experiments define the possible specific inhibitory effects of the various cations as their concentrations are changed.

Effects of Inhibitors. In the presence of an outward KCl gradient, hyperpolarization at low K_o (Figs. 3 and 6–9) implies that $P_K > P_{Cl}$ and thus the rate of net loss of KCl is limited by the Cl conductance. By reducing the Cl conductance with the specific inhibitor DIDS (Knauf et al., 1977), the Ca-induced hyperpolarization is expected to be larger than in the absence of the anion inhibitor (*see* Hoffman & Knauf, 1973). The results in Table 3 show that the decrease in fluorescence of WW 781 is doubled in cells treated with DIDS as compared to untreated control suspensions.

In order to test further the hypothesis that the changes in fluorescence of WW 781 are indicative of voltage changes associated with Ca-induced K conductance, several known inhibitors of the Gardos effect were examined for their ability to

Table 3. Effect of DIDS on Ca-induced hyperpolarization[a]

K_o	DIDS	% ΔF	
0	−	−5.5,	−5.8
	+	−12.8,	−11.8
3	−	−5.2,	−5.4
	+	−10.0,	−10.5

[a] Cells were washed and prepared in choline solution as described in Materials and Methods. The cuvettes contained 3 ml of medium consisting of 142.5 mM choline Cl, 0.5 mM $CaCl_2$, 0.5 mM EGTA, and 20 mM HEPES buffer (pH 7 at 37 °C). The samples with 3 mM KCl were made by injecting 9 µl of 1 M KCl into the cuvettes. For samples without DIDS, 75 µl of an ice-cold 50% HCT suspension was added to the cuvette. For DIDS-treated samples, 10 µM freshly prepared DIDS were added to a 25% HCT suspension at 25 °C and incubated for 10 min before replacing the suspension on ice. Therefore, 150 µl of the 25% HCT suspension was added to the cuvette for the DIDS-treated samples. Dye (3.5 µM) was added and after equilibration to 37 °C, 1 µM A 23187 was added and the % ΔF recorded

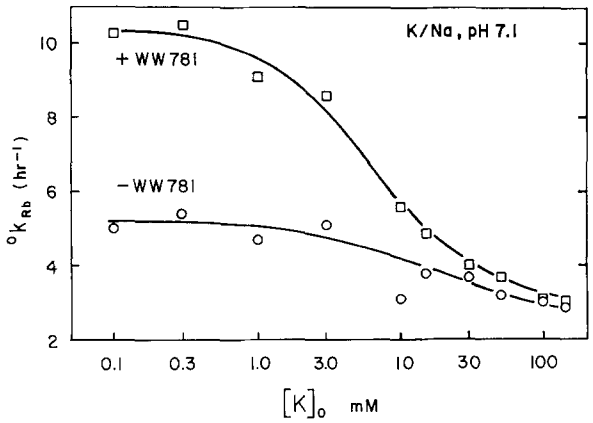

Fig. 10. Effect of K_o on Ca-induced Rb efflux in the presence and absence of WW 781. Cells were washed and loaded overnight with ^{86}Rb, used as tracer for K as described in Materials and Methods. The media for fluxes consisted of x mM KCl, where $x=0$, 0.1, 0.3, 1.0, 3.0, 10, 30, 100 and 142.5 mM, (142.5-x) mM NaCl, 1 mM $CaCl_2$, 3 mM HEDTA, and 5 mM HEPES (pH 7.1 at 37 °C). Cells, 3.5 μM dye (upper curve only), and 2.4 μM A 23187 were added, samples were taken at four time points, and rate constants, $^0k_{Rb}$, were estimated as described in Materials and Methods

inhibit the Ca-induced change in dye fluorescence. The compounds studied included quinine (Armando-Hardy et al., 1975; Reichstein & Rothstein, 1981), oligomycin (Blum & Hoffman, 1971; Riordan & Passow, 1971; Hoffman & Blum, 1977), and trifluoperazine (Hoffman et al., 1980). From dose response curves, half-maximal inhibition of the fluorescence changes induced by 1 μM A 23187 at 0.5 mM Ca, 0.5 mM EGTA, and 5 mM K_o were obtained with 160 μM quinine, 2 μg/ml oligomycin, and at 1 mM K_o with 5 μM trifluoperazine. While quinine and oligomycin inhibited both at pH 7 and at pH 6 in media containing either Na or choline (as in Fig. 8), trifluoperazine inhibited the voltage changes only at pH 6 in choline media, and was ineffective in the other three media described in Fig. 8.

Effect of WW 781 on Ca-Induced Rb Efflux

The study of K activation kinetics with tracer fluxes involves complications including irreversible inactivation at low K_o, time-dependent changes in rate constants, nonlinearities due to altered internal ionic concentrations, changes in cell volume, and possible cellular heterogeneity (Knauf et al., 1975; Heinz & Passow, 1980). Nevertheless, we measured initial unidirectional rates of Ca-induced Rb efflux at varied $[K]_o$, using ^{86}Rb as an economical tracer for K, in order to ascertain the extent to which WW 781 might itself influence the transport kinetics. The results in Fig. 10 show that in the presence of dye, the Ca-induced Rb efflux is greater than in the absence of dye, and the difference is greater at low K_o with greater extents of hyperpolarization. At 0.3 mM K_o, stimulation of $^0k_{Rb}$ by WW 781 is evident at 0.03 μM dye, reaches twofold at 2 μM dye, and continues to increase at 10 μM dye. This stimulation of efflux by WW 781 is problematical for a quantitative analysis correlating fluxes and voltages. The inhibitory effect of K_o on $^0k_{Rb}$ contrasts with the stimulatory effect of K_o on valinomycin-mediated K efflux (Hunter, 1977; Wieth & Tosteson, 1979), and agrees with previous studies of the Gardos effect in ATP-depleted cells (Blum & Hoffman, 1971; Kregenow & Hoffman, 1972; Hoffman & Blum, 1977) as well as in ghosts (Knauf et al., 1975; Heinz & Passow, 1980).

Time-Dependent Changes in Fluorescence and E_m

Based on previous microelectrode measurements of E_m of the large red cells of *Amphiuma* (Lassen, Pape & Vestergaard-Bogind, 1976, 1980), it was concluded that the Ca-induced hyperpolarization is transient, returning to the level of the resting potential in 10 min even though the level of $[Ca]_c$ continues to increase in this time period. Consequently, we attempted to use WW 781 to monitor E_m of human red blood cells over a period of 10 min after inducing the Gardos effect. From the results described so far, it is apparent that the initial change in the fluorescence of WW 781 after addition of A 23187 in the presence of Ca_o correlates well with the expected membrane potentials associated with Ca-induced K conductance. However, under certain conditions the fluorescence change exhibits biphasic kinetics, such that at a maximally effective Ca_o the immediate initial rapid decline of about 8% associated with hyperpolarization remains stable for a certain time period, but then there is a subsequent kinetically distinct slow decline of fluorescence which reaches an additional 35% over 10 min (Fig. 11). The higher the Ca, the sooner is the onset of the slow decline (Figs. 11 and 12). The magnitude of the slow secondary decrease in dye fluorescence reaches 4 to 5 times the maximal change obtained with valinomycin at low K_o, a change which seems too large to be associated with a change in E_m. The secondary decrease does not occur with valinomycin, but only with Ca plus A 23187, and is inhibited by Mg such that at 3 mM $[Mg]_o$ the dye fluorescence decreases to a peak value and then levels off and

remains stable. Between 5 and 20 mM $[Mg]_o$ the dye fluorescence decreases to a peak value and then spontaneously increases back to the initial baseline at a rate which increases with increasing $[Mg]_o$ (*not shown*). These effects of Mg also occur with resealed ghosts. Addition of membrane-free hemolysate to a red cell suspension containing WW 781 causes an increase, rather than a decrease, in dye fluorescence, an observation which argues against the possibility that the slow decrease in fluorescence is due to dye permeation with quenching by intracellular components, as occurs with diS-C_3 (5). With intact cells in the absence of Mg_o, the slow secondary decline of WW 781 fluorescence after addition of A 23187 occured at the same rate irrespective of K_o between 1 and 150 mM, further indicating that this particular fluorescence change is unrelated to ΔE_m. When A 23187 was added near the null point, and with either Ca_o or Mg_o present at concentrations ranging from 0 to 20 mM, the slow change occurred in the presumptive absence of ΔE_m, and was activated specifically by Ca and not by Mg.

At 1.1 mM $CaCl_2$ and 1 mM EGTA, raising Mg_o from 0 to 20 mM systematically inhibited the extent of the slow decline. Adding excess EGTA immediately stopped the slow decline, and the fluorescence remained at a steady level according to its value at the time of EGTA addition. Moreover, the slow decline was not inhibited by quinine, oligomycin, or by trifluoperazine when tested at the null point. All of these observations together served to differentiate the slow change from the potential-dependent rapid change of the fluorescence of WW 781. The only similarity found between the immediate fast change and the subsequent slow change was that both changes involved a quenching of fluorescence without a detectable shift in the location of the excitation and emission peaks. One possible explanation is that a slow decrease in binding of dye to the membrane results either directly or indirectly as a consequence of the gain of Ca or the loss of Mg by the cells as mediated by A 23187.

Because the slow changes of dye fluorescence interfered with continuous monitoring of red cell E_m, experiments were conducted to compare the Ca-activation thresholds for the rapid change and for the slow change. For this purpose the [EGTA] was raised to 7 mM for greater Ca buffering, and the results (Figs. 11 and 12) showed that the rapid potential-dependent dye response is activated at a calculated ionized external Ca concentration of approximately 0.01 μM, which is ten times lower than that which activates the subsequent slow decline. With this information, a $[Ca]_o$ was defined which gave maximal stimulation of K conductance but which was insufficient to activate the interfering slow change for 9 min. Thus, the results at 7 mM EGTA and 4 mM $[Ca]_o$ showed that after inducing the Gardos effect with 1 μM A 23187, the rapid change in the fluorescence of WW 781 is stable for at least 9 min (Fig. 11). At submaximally effective levels of Ca_o, the dye fluorescence appears

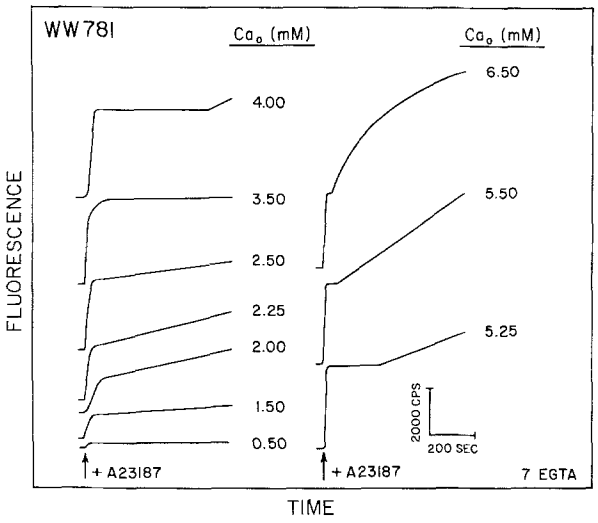

Fig. 11. Time-dependent changes in the fluorescence of WW 781 at varied Ca_o. Cells were washed and prepared as described in Materials and Methods. The cuvettes contained media consisting of x mM $CaCl_2$, where $x=0$ to 7 mM in 0.5-mM increments, with additional points at 2.25, 5.25, 8, 9 and 10 mM, $(145.5-1.5x)$ mM NaCl, 1 mM KCl, 7 mM EGTA, and 5 mM HEPES (pH 7.2 at 37 °C). Cells, dye and 1 μM A 23187 were added as described in Fig. 7, and the fluorescence was recorded for 10 min

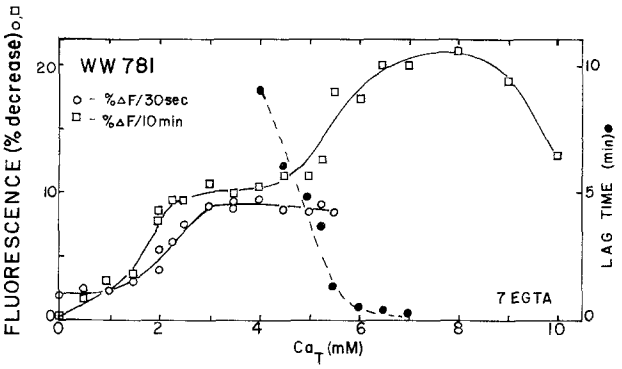

Fig. 12. Fast and slow components of the change in fluorescence of WW 781. From each of the traces shown in Fig. 11, the percentage change in fluorescence was measured 30 sec after addition of A 23187 for the fast change (empty circles) and at 10 min for the slow change (squares). Also shown (filled circles) is the effect of Ca_o in decreasing the lag time (right oridnate) between the addition of A 23187 and the start of the slow change

to drift toward the maximally stimulated level. Under the conditions employed in Fig. 11, transient changes in E_m were not apparent at any level of Ca_o. Moreover, the semilogarithmic plots of Ca-dependent ^{86}Rb efflux had constant slopes for at least 10 min in the presence and absence of WW 781.

The effects of high concentrations of Ca in initiating the slow change would complicate the study of E_m when the Gardos effect is induced by adding 10 mM Ca to cells or ghosts depleted of ATP (Kregenow & Hoffman, 1972; Knauf et al., 1975). Propranolol, which has also been used to induce the Gardos effect (Ekman, Manninen & Salminen, 1969), is incompatible with the use of WW 781 because the drug increases the dye fluorescence in the absence of cells. For these reasons, the most convenient way found to study the effect of Ca on E_m of human red cells was to increase $[Ca]_c$ by adding the ionophore A 23187 in the presence of controlled amounts of $[Ca]_o$.

Discussion

This paper summarizes the results of experiments using the fluorescent oxonol dye, WW 781, to monitor the changes in E_m associated with the Ca-induced increase in the K permeability of human red blood cells. That the observed changes in fluorescence correlate with ΔE_m is demonstrated by the responses to gramicidin (*see* Freedman & Hoffman, 1979*c*; Freedman & Laris, 1981) and to valinomycin (Fig. 3), and by the correspondence with ΔpH_o in unbuffered suspensions of cells treated with DIDS and the proton ionophore FCCP (Figs. 3*C* and 4). The change in fluorescence in response to varied concentrations of A 23187 and Ca_o (Fig. 6), and of K_o (Fig. 8), the reversibility with excess EGTA, the inhibition by oligomycin, quinine, and trifluoperazine, the activation by external K (Fig. 8*D*) and the ionic selectivity of activation and conductance (Fig. 9) all correlate with the known properties of the Gardos effect and thus indicate that the optical signals are associated with the system which mediates the Ca-dependent increase in K conductance. Moreover, control experiments (Fig. 3, inset *B*, and Table 2) established that the amount of Ca_c which maximally stimulates the fluorescence change is insufficient to affect dye fluorescence independently of the effect of E_m on fluorescence.

The magnitude of the maximal change in fluorescence in response to hyperpolarization with valinomycin at low K_o (Fig. 3) is -8% with a signal-to-noise ratio of 16. WW 781 has a calibration of 0.13% ΔF/mV (Figs. 3*C* and 4), a response time of <1 sec (Fig. 3*A*), and a detection limit for ΔE_m of 10 mV. This voltage-dependent optical signal is the largest that we have observed in red cell suspensions with a presumably impermeant fast dye. Based on correlations between molecular structure and the permeability of red cells to a large number of organic anions (Motais, 1977), the two separated negative charges of WW 781 would suggest impermeance, although this point is difficult to establish at micromolar dye concentrations. On the basis of the results with red cells, it would seem worthwhile to test WW 781 and related fast dyes with other nonexcitable cells, such as leukocytes, macrophages, platelets and other systems where calcium and E_m appear to be involved in stimulus-response coupling, but where the interpretation of optical potentiometric signals from permeant dyes may be mixed with contributions from the membranes of internal organelles (*see* Freedman & Laris, 1981, for review).

Adaptation of the method of Macey et al. (1978) using a glass electrode to monitor ΔpH_o in an unbuffered red cell suspension (Fig. 3, inset *C*) represents a new way of calibrating optical potentiometric indicators. This method is most accurate for cells treated with DIDS (Wieth et al., 1980), since it is necessary to prevent the Cl^-/HCO_3^- exchanger from holding pH in a steady state away from thermodynamic equilibrium. Whether DIDS specifically affects the relationship between % ΔF and ΔE_m (Fig. 4) could be tested by using a variety of inhibitors of anion exchange. If DIDS does not affect the dye calibration, then the value of 0.13 ± 0.03 (SD, $n=5$) % ΔF/mV could be used to measure E_m in cells which have not been treated with DIDS. It is noteworthy that in the absence of DIDS, the pH electrode tends to underestimate ΔE_m while the calibrated dye gives a more accurate measurement. However, the stimulation of Ca-dependent Rb efflux by WW 781 (Fig. 10) complicates quantitative correlations between voltages and fluxes with this particular dye. Preliminary studies with related dyes indicate that the extent of stimulation depends on which specific dye is utilized.

Another object of our studies with WW 781 was to determine the time-dependence of ΔE_m after inducing the Gardos effect with A 23187. This question was prompted by reports of a transient hyperpolarization of *Amphiuma* red blood cells after exposure to Ca (Lassen et al., 1976, 1980). The occurrence in human red cells of a slow Ca-dependent change in dye fluorescence probably un-

related to ΔE_m interfered with our ability to monitor voltages continuously. Under conditions in which Ca is buffered at a level below that which activates the slow change (Figs. 11 and 12), our results suggest that in human red blood cells, the Ca-induced ΔE_m is stable at maximally effective Ca_o and tends to increase toward the maximal ΔE_m at submaximally effective Ca_o (Fig. 11), as if partial activation of the Gardos effect leads in time to a maximally activated state. This aspect of the results is consistent with the fact, which we confirmed (*data not shown*), that Ca uptake continues to proceed for 10 to 20 min after induction of the Gardos effect with A 23187 (Sarkadi, Szasz & Gardos, 1976).

All of our results indicate that the rapid changes in the fluorescence of the oxonol dye, WW 781, as induced by A 23187 in the presence of Ca_o, correlate well with the known properties of the Gardos effect, that the changes in fluorescence can be assigned mV values based on the changes in pH_o in unbuffered suspensions of DIDS-treated human red cells, and that the information thus obtained with optical potentiometric indicators will aid in increasing our understanding of how Ca affects and possibly regulates cellular membrane potentials.

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