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TRANS TO *CIS* PROTON CONCENTRATION GRADIENTS ACCELERATE IONOPHORE A23187-MEDIATED NET FLUXES OF Ca^{2+} ACROSS THE HUMAN RED CELL MEMBRANE

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Ionophore A23187-mediated net influx of Ca^{2+} in ATP-depleted human red cells was studied as a function of the pH and the proton concentration gradient across the membranes. Utilizing the Ca^{2+}-induced increase in K^+ conductance of the cell membranes, various CCCP-mediated proton gradients were raised across the membranes of cells suspended in unbuffered salt solutions with different K^+ concentrations. In ionophore-mediated equilibrium the concentration ratios of ionized Ca between ATP-depleted, DIDS-treated cells and their suspension medium were equal to the concentration ratios of protons raised to the second power. With no proton concentration gradient across the membranes the net influxes of Ca^{2+} as a function of pH resembled a titration curve of a weak acid, with half maximal net influx at pH 7.3, at 100 μM extracellular Ca^{2+}. With cellular pH fixed at various values, the net influx of Ca^{2+} was determined as a function of the proton concentration gradient. A linear relationship between the logarithm of net influx and the difference between extracellular and cellular pH was found at all cellular pH values tested, but the proton concentration gradient acceleration was a function of the cellular pH. Accelerations between 10- and 40- times per unit ΔpH were found and net effluxes were correspondingly decreased. The results are discussed in relation to present models of the mechanism of ionophore A23187-mediated Ca^{2+} transport. The importance of the proton concentration gradient dependency is discussed in relation to the induced oscillations in K^+-conductance of human red cell membranes previously reported (Vestergaard-Bogind and Bennekou (1982) Biochim. Biophys. Acta 688, 37–44).

Introduction

Induction of oscillations in the conductance of the Ca^{2+}-sensitive K^+ channels of human red cell membranes have previously been reported [1]. The oscillations were induced by addition of ionophore A23187 to cells suspended in an unbuffered salt solution containing 15–50 μM Ca^{2+}. In presence of the ionophore CCCP, which maintain protons in electrochemical equilibrium across the red cell membranes [2], any shift in membrane potential was reflected by a shift in the extracellular pH, pH_{ex}, the pH of the intracellular phase, pH_c, remaining constant, since all buffering capacity was confined to this phase. Synchronous oscillations in intracellular Ca concentration, K^+-conductance and membrane potential were shown to take place [3]. Variation in the ionophore-mediated Ca^{2+} influx as a result of the constantly chang-

Abbreviations: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid disodium salt trihydrate; Hepps, *N*-2-hydroxyethylpiperazine-*N'*-3 propanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Trizma, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

ing pH_{ex}, combined with delayed activation of the Ca^{2+} pump [4] were suggested to be important elements of the oscillations [1,3].

If the pH dependence of the ionophore A23187 mediated Ca^{2+} net flux was ascribed simply to the changes in the degree of dissociation of the protonized ionophore [1,5], then the changes in extracellular pH during the oscillations only allowed for a variation in the ionophore-mediated influx by a factor of 2 to 3. The magnitude of the Ca^{2+} net in- and effluxes during a wave of hyperpolarization indicated, that a substantially larger variation took place.

It is generally assumed that the ionophore A23187 mediates exchanges of Ca^{2+} for Mg^{2+} or protons [6,7], and accordingly, that the ionophore-mediated equilibrium distribution of Ca^{2+} across the membranes should be equal to the proton distribution ratio raised to the second power [8]. However, no direct experimental test of the Ca^{2+} distribution as a function of proton distribution across the cell membranes have been reported up till now.

If the ionophore in cases of Ca^{2+} net influx mediates an exchange of extracellular Ca^{2+} for intracellular H^+ and Mg^{2+}, a decrease in extracellular proton concentration at a given fixed cellular pH, that is an increase in the *trans* to *cis* concentration gradient of protons, might result in a significant increase in Ca^{2+} net influx.

In the present paper the ionophore-mediated equilibrium distribution of Ca^{2+} between human red cells depleted in ATP and 2,3-diphosphoglycerate and their medium is shown to be a function of the magnitude of the proton concentration gradient across the membranes over a wide range. The ionophore-mediated net influx of Ca^{2+} into depleted cells has been determined as a function of pH. It is demonstrated, that a *trans* to *cis* concentration gradient of protons accelerate ionophore A23187-mediated net fluxes of Ca^{2+} strongly, the net flux increasing exponentially with an increase in the proton concentration distribution ratio. Finally, it is shown, that there is no direct interference from CCCP on the ionophore A23187-mediated Ca^{2+} netflux.

Materials and Methods

All inorganic salts (pro analysis) and glycolic acid (for synthesis) were purchased from Merck; Hepps (EPPS), Mops, Trizma base and Trizma, iodoacetamide, EGTA and CCCP were from Sigma. CCCP was administered as a concentrated (20 mM) solution in ethanol. Ionophore A23187 was from Calbiochem. The stock solution was a 2.0 mM solution of the ionophore in absolute ethanol for spectroscopy from BDH. From this stock solution, stored at −20°C, diluted solutions of ionophore were currently prepared by further dilution with ethanol. DIDS was from Pierce Chemical Co.

Freshly drawn blood from healthy human donors was heparinized and centrifuged, plasma and the buffy coat were aspirated, and the cells were washed twice in 5 vol. high-K salt solution (90 mM KCl/66 mM NaCl/50 μM EGTA, pH 7.4). The cells were then depleted for ATP by incubation for 2–3 h in depletion Ringer's solution (75 mM NaCl/75 mM KCl/0.1 mM EGTA/10 mM inosine/6 mM iodoacetamide/10 mM Tris, pH 7.7) at 37°C and a hematocrit of 10% [9]. Glycolate (15 mM) was included in the depletion Ringer's solution in order to ensure the breakdown of most of the large 2,3-diphosphoglycerate pool present in red cells [10]. The ATP content of cells depleted in this way was found to be less than 1 μmol/l cells.

After this incubation the cells were washed three times in 5 volumes high-K salt solution and either stored packed on ice or incubated for 30 min at 37°C in high-K salt solution with DIDS ($2 \cdot 10^{-4}$ M). These cells were then washed as above and stored on ice.

Concentration of ionized Ca in the various salt solutions was determined with a Ca^{2+} selective electrode (Selectrode F2112 Ca, Radiometer), standardized with Ca^{2+} buffers [11].

The cellular content of ^{45}Ca was determined by the method of Lew and Brown [12], slightly modified. 100 μl samples of the cell suspension were transferred to a centrifuge tube containing 850 μl of a solution (Trizma acetate 155 mM, EGTA 2 mM, bovine albumin 50 μM, pH 7.75 at 0°C) layered on top of 400 μl di-*n*-butylphthalate (density 1.042–1.045 at 20°C, BDH Lab reagent). The

centrifuge tubes were stored on ice and, immediately after the addition of a 100 μl sample of suspension, the tube with its content was centrifuged at 15000 × *g* for 50 s. The procedure was standardized so that the centrifugation started approx. 5 s after the transference of the 100 μl sample to the phthalate containing tube. The resulting cell pellets were processed for scintillation counting according to Ref. 12.

The initial intracellular pool of exchangeable calcium is less than 1 μmol/l cells [13,14], the extracellular concentration of ionized Ca were 50 or 100 μM and the cytocrit 3.5%. Therefore, the initial intracellular Ca^{2+} compartment is negligible and the ^{45}Ca in- and effluxes can be taken as valid markers of the net in- and effluxes of calcium ions. Since the cells were completely depleted of ATP, there was no Ca^{2+}-pump efflux, and the ^{45}Ca influx thus marked the ionophore-mediated net flux of Ca^{2+}.

Extracellular concentrations and cellular contents of magnesium were determined by atomic absorption spectrophotometry. Hemoglobin was determined with a Test-combination from Boehringer.

Experimental procedure. 200 μl packed cells, depleted of ATP and 2,3-diphosphoglycerate was added to 5000 μl unbuffered salt solution (156 mM (NaCl + KCl)) containing ^{45}Ca-labelled ionized Ca in a total concentration of 52–55 μM. The cell suspension was incubated at 37°C and under vigorous stirring the suspension was slowly titrated to the desired pH value with either 100 mM NaOH/56 mM NaCl or 100 mM HCl/56 mM NaCl. CCCP was then added to a final concentration of 20 μM.

During the following 5–10 min, samples of suspension were taken for determination of the cellular content of ^{45}Ca. Since no ionophore A23187 had been added yet, the ^{45}Ca content of the cell pellets was found to be very low and of an almost constant value. This value was taken to represent trapped extracellular and cell coat bound ^{45}Ca, consistent with the assumption that the initial cellular uptake of ^{45}Ca was negligible.

At zero-time ionophore A23187 was added to a final concentration of 5 μmol/l cells. Within a few seconds the increase in intracellular Ca^{2+} concentration induced an increase in conductance of the Ca^{2+}-sensitive K^+-channels of about three orders of magnitude (see Fig. 1 and Ref. 3). The cell membranes hyperpolarized or depolarized dependent on the extracellular K^+ concentration chosen, and within 10–15 s protons, mediated by CCCP, were distributed across the cell membranes according to the new electrochemical equilibrium. Since all H^+-buffering capacity in the system was confined to the intracellular phase, this new electrochemical equilibrium caused a shift in the extracellular pH only. For a period of several minutes the proton gradient thus established remained almost constant, and during the first one to two minutes samples of suspension were taken for determination of the cellular content of ^{45}Ca. The initial influx of ^{45}Ca was calculated from the slope of the line through the first five to six points (compare Fig. 1).

After about 2 min the experiment was stopped by the addition of 100 μl of saponin solution. As seen from Fig. 1 this addition, which lead to immediate hemolysis of all cells, resulted in an abrupt shift in pH. The new value attained within approx. 30 s represented the intracellular pH which, as mentioned, remained unchanged during the experiment and now impressed the hemolysate this value. In this way the magnitude of the proton concentration gradient during the experiment was determined.

In series of experiments the hemoglobin concentration in each cell suspension was determined, to ensure identical hemotocrit values.

Results

The results presented in Figs. 1 to 10 have all been obtained in experiments performed on cells depleted of ATP and 2,3-diphosphoglycerate in order to avoid interference from the fluxes of the ATP-driven Ca^{2+} pump on the ionophore A23187-mediated net in- and effluxes of Ca^{2+}. The concentration of free Mg^{2+} in these cells was found to be about 1500 μM. No attempt to deplete the cells of Mg^{2+} by the aid of A23187 was made and no Mg^{2+} was added to the salt solutions employed. Thus, steep, but in each experimental series identical, outward concentration gradients of Mg^{2+} existed.

In a series of experiments the increase in ex-

tracellular Mg concentration, during the period used for determination of initial ^{45}Ca influx (approx. 1 min, compare Fig. 1), was determined by atomic absorption spectrophotometry. We found that the net efflux of Mg^{2+} during this period constituted less than 0.5% of the Ca^{2+} net influx taking place simultaneously. That is, more than 99% of the ionophore reflux took place in the form of protonized molecules. In these experiments the concentration gradients of protons corresponded to ($pH_{ex} - pH_c$) values of about 1 unit and these gradients were established at cellular pH values of 6.2, 7.2 and 7.5.

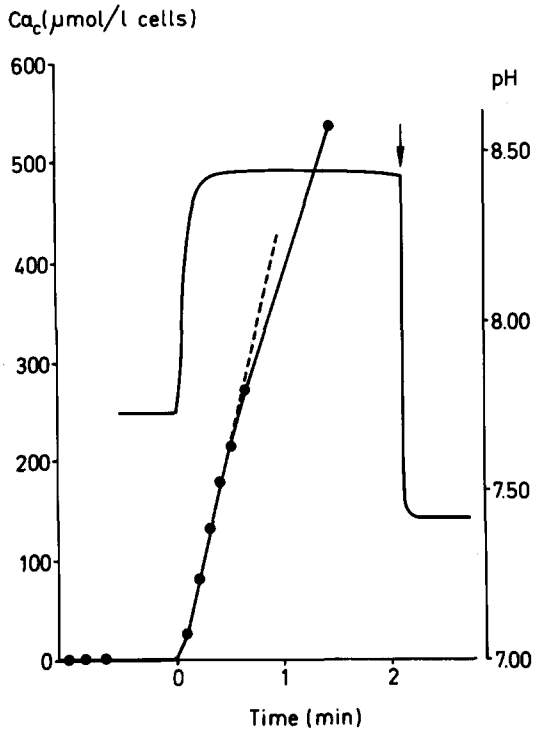

Fig. 1. Results of a typical experiment, in which the ionophore A23187-mediated net influx of Ca^{2+} into depleted cells, in the presence of a proton concentration gradient across the cell membranes, was determined. The pH value of the hemolysate (after addition of saponin, at the end of the experiment, indicated by an arrow) gives the cellular pH during the experiment and the proton concentration gradient corresponds to the difference between the peak value in extracellular pH and the cellular pH. Initial extracellular concentration of ionized Ca, 52 μM and of K^+, 10 mM. CCCP added to a total concentration of 20 μM. At zero time ionophore A23187 was added to a final concentration of 5 μmol/l cells. The increase in extracellular K^+ concentration is not shown. ———, pH; ●———●, cellular Ca content in μmol/l cells.

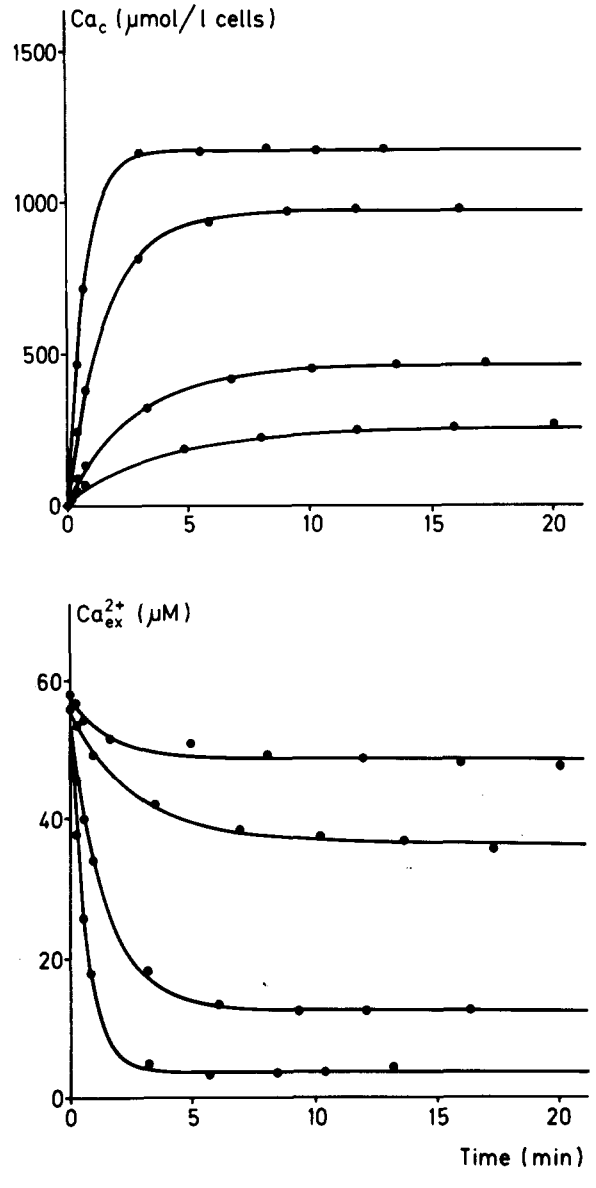

Fig. 2. Ionophore A23187-mediated changes in intra- and extracellular Ca concentrations versus time, with different proton concentration gradients across the membranes of depleted cells pretreated with DIDS. The initial extracellular Ca^{2+} concentration was about 55 μM in all experiments, whereas the initial extracellular concentration of K^+ varied, obtaining in this way proton concentration gradients of different magnitudes (see Experimental procedure). A23187 was added at zero time to a concentration of 10 μmol/l cells. Ordinates are cellular concentration of total Ca in μmol/l cells and extracellular concentration of Ca^{2+} in μM, respectively. Abscissa is time in minutes. ($pH_{ex} - pH_c$) in the experiments were: 0.0, 0.20, 0.64 and 1.05 units, respectively. Compare with Fig. 3. pH_c was 7.1 in all experiments.

Fig. 2 shows A23187-mediated changes in extra- and intracellular Ca^{2+} with time in suspensions of depleted, DIDS-treated cells with different quasi-stationary proton concentration gradients across the membranes. In all experiments the ionophore A23187 concentration was 10 μmol/l cells and the initial extracellular concentration of ionized calcium was about 55 μM. Initial extracellular K^+ concentration was varied in order to obtain the different concentration gradients of protons. The ordinates are total cellular content of calcium in μmol/l cells and extracellular concentration of ionized Ca in μM, respectively. The curves are fitted to single-exponential functions.

DIDS-treated cells were used in these experiments to minimize the decrease with time of the proton concentration gradients. DIDS decreases chloride netflux permeability of the cell membranes substantially [15], resulting in a strongly decreased net efflux of KCl from the cells. In turn, this resulted in slower changes with time in the Nernst equilibrium potentials of potassium ions (E_K) and chloride ions (E_{Cl}) and, therefore, membrane potential (V_m). An initial concentration gradient of protons corresponding to a ($pH_{ex} - pH_c$) value of 1.00 unit only decreased to 0.95 units of pH during 20 min.

The ratio between cellular Ca content and extracellular concentration of ionized Ca at equilibrium as a function of the proton concentration gradient is shown in Fig. 3. Here, the values from the experiments shown in Fig. 2 have been plotted on a log-log scale. As discussed later, the intercept on the ordinate axis gives the negative logarithm to the constant α, which is the ratio between ionized Ca per litre cell water and total Ca per litre cells.

In a series of experiments with different donors α was determined more directly, in the sense that the equilibrium distribution of Ca was determined in suspensions of cells with a membrane potential $\simeq 0$ mV, that is $pH_{ex} \simeq pH_c$. In these experiments α was determined to be 0.19 ± 0.03 (S.D., $n = 7$).

In Fig. 4 the net influx of Ca^{2+} into depleted, DIDS-treated cells at fixed concentrations of ionophore (10 μmol/l cells) and initial extracellular Ca^{2+} (100 μM) is shown as a function of the cellular pH. The suspensions of cells were titrated to the desired pH value before addition of the

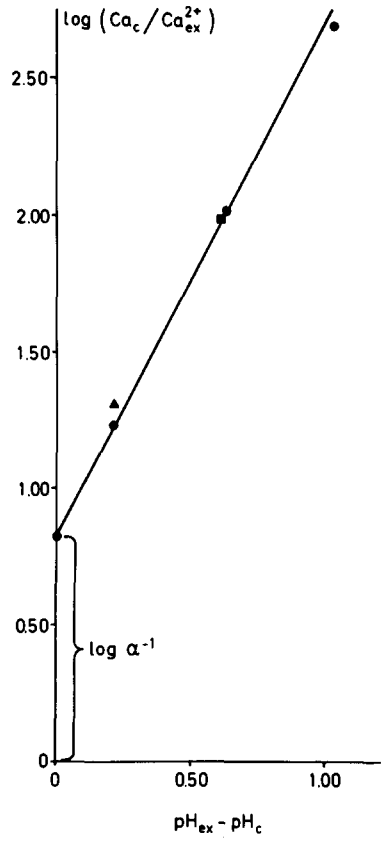

Fig. 3. The logarithm of the ionophore A23187-mediated equilibrium distribution of Ca between cells and medium as a function of ($pH_{ex} - pH_c$). The values indicated with ● are those from the experiments represented in Fig. 2. Thus, Ca_c is the concentration of total Ca per litre cells, whereas Ca_{ex} is the concentration of ionized calcium in the extracellular phase. At ($pH_{ex} - pH_c$) = 0 (that is at a membrane potential of 0 mV) the equilibrium distribution of ionized calcium between the cellular and extracellular phases should be 1.0, and the intercept on the ordinate axis should be equal to the logarithm to α^{-1}, where α is the ratio between ionized calcium per litre cell water and total calcium/l cells. Determined in this way α was 0.19. pH_c was 7.1 in all experiments. ▲, Experiment without DIDS pretreatment; ■, experiment where the initial extracellular concentration of ionized Ca was doubled (102 μM).

ionophore. Using DIDS-treated cells (which have a decreased chloride conductance of the membranes) suspended in 156 mM KCl solution, we obtained maximal depolarizations of the membranes as the ionophore-mediated increase in intracellular Ca^{2+} opened the K^+ channels. In this way, it was possible to determine the net influx of Ca^{2+} as a function of pH_c within the range of 5.8

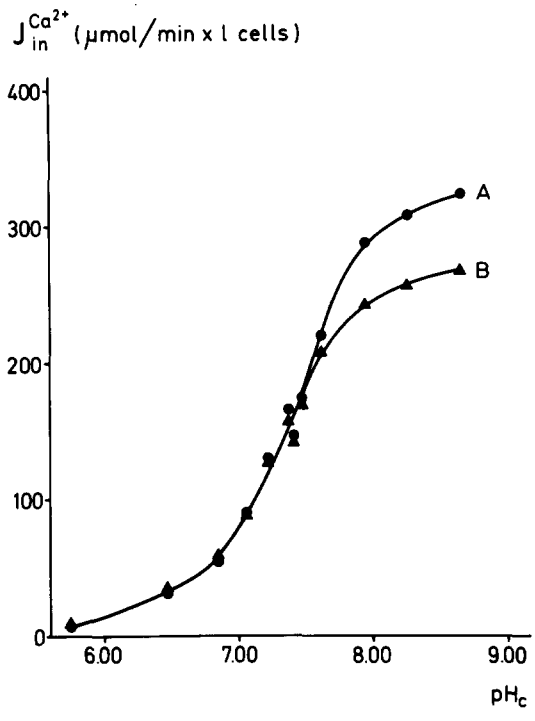

Fig. 4. Ionophore A23187-mediated net influx of Ca^{2+} into depleted, DIDS-treated cells as a function of cellular pH. Initial extracellular concentration of Ca^{2+}, 102 μM and of K^+, 156 mM. Ionophore concentration, 10 μmol/l cells. The ordinate is initial net influx of Ca^{2+} in μmol/l cells per min. The abscissa is cellular pH. The points along curve A shows the flux values directly determined. From $pH_c = 7.2$ and upwards an increasing difference between pH_{ex} and pH_c was found. Curve B shows the calculated initial net influx of Ca^{2+} as a function of pH, with $(pH_{ex} - pH_c) = 0$ at all pH values (for details regarding this correction see text).

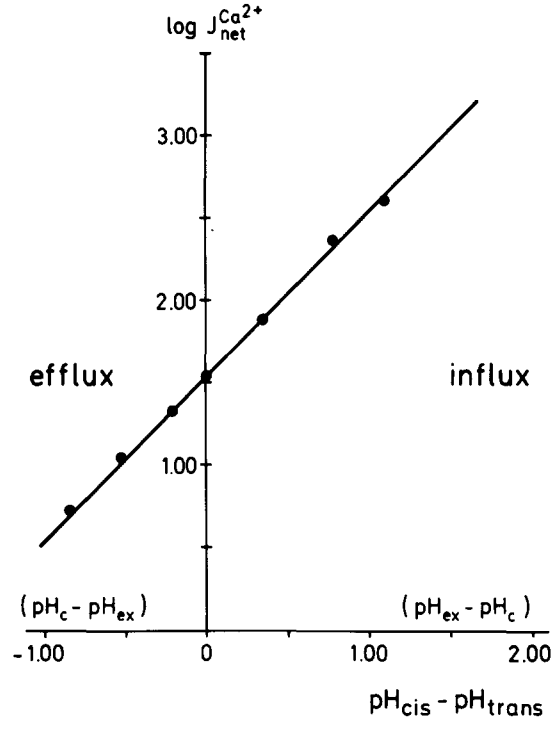

Fig. 5. Ionophore A23187-mediated initial Ca^{2+} net flux as a function of the proton concentration gradient across the membranes. On the ordinate the logarithm to the net flux. To the right of the ordinate axis the fluxes determined are net influxes and to the left the points represent net effluxes. The abscissa is $(pH_{cis} - pH_{trans})$, that is $(pH_{ex} - pH_c)$ to the right and $(pH_c - pH_{ex})$ to the left of the ordinate. The flux determined at $(pH_{cis} - pH_{trans}) = 0$ is an influx, but could in principle equally well have been an efflux. In all experiments pH_c was 7.10 and the concentration of A23187 was 5 μmol/l cells. Initial extracellular Ca^{2+} concentration was 52 μM in all influx experiments, whereas the initial extracellular K^+ concentration varied (see Experimental procedure). For correction of the determined efflux values with respect to the concentration of ionized Ca on the *cis* side, see text.

to 7.2 without a proton concentration gradient present across the membranes.

However, the depolarization was not sufficiently large to counterbalance the high Donnan membrane potentials of cells titrated to higher cellular pH values and thus diminished concentration ratios of chloride ions across the membranes. Therefore, the Ca^{2+} net influx values found at the higher pH_c values (curve A) reflected both dependence of pH_c as such and of the presence of a *trans* to *cis* concentration gradient of protons. Based on the results shown in Figs. 5–7, these flux values have been corrected (curve B) for the acceleration resulting from the determined proton concentration gradients. Curve B thus represents the ionophore-mediated net influx of Ca^{2+} as a function of pH, the intracellular pH being equal to the extracellular pH within the whole range.

In Fig. 5 the ionophore-mediated netflux of Ca^{2+} across the membranes of depleted, DIDS-treated cells is shown as a function of the proton concentration gradient across the membranes. The intracellular pH was kept at a fixed value of 7.10. The initial extracellular concentration of ionized Ca was 52 μM and ionophore A23187 was added to a final concentration of 5 μmol/l cells.

Each point to the right of the ordinate axis represents the logarithm of an initial net influx of

Ca^{2+} in the presence of the proton concentration gradient given on the abscissa as $(pH_{cis} - pH_{trans})$, which in the case of net influxes is the same as $(pH_{ex} - pH_c)$. Each net influx was determined as shown in Fig. 1, and the various proton concentration gradients were raised across the cell membranes by suspending the cells at different initial extracellular K^+ concentrations.

The fluxes, the logarithm of which are plotted on the left side of the ordinate axis, are initial net effluxes of Ca^{2+} and $(pH_{cis} - pH_{trans})$ is now equal to $(pH_c - pH_{ex})$. In these experiments the cells were loaded with Ca^{2+} for about 15 min (compare Fig. 2) and then a concentrated solution of Na-EGTA, with a pH identical to the pH_{ex} of the experiment in question, was added to the extracellular phase to a final concentration of 1 mM. With extracellular concentrations of ionized Ca^{2+} between 3 and 48 μM this concentration of EGTA was sufficient to obtain an extracellular concentration of ionized Ca^{2+} of $\simeq 10^{-8}$ M; and since the net effluxes were very small, the extracellular concentration of ionized Ca was kept almost constant at that value during the experiment. An example of such a net efflux experiment is shown in Fig. 6. Within a couple of minutes after the addition of EGTA the extracellular pH was back to the original value and during the period of efflux determination the change in extracellular pH, and thus in proton concentration gradient, was only about -0.02 units. As discussed above, this constancy in gradient resulted from the DIDS treatment of the cells.

In these net efflux experiments the intracellular concentration of ionized Ca was calculated from the total cellular content of Ca using the factor α. From the fluxes, thus determined, the flux values corresponding to a *cis* concentration of 50 μM Ca^{2+} was calculated, assuming for the narrow range of concentration in question, direct proportionality between flux and Ca^{2+} concentration at the fixed concentration of ionophore A23187.

As seen from Fig. 5, net influx of Ca^{2+}, in the presence of a proton concentration gradient corresponding to $(pH_{ex} - pH_c) = 1.0$, was 10-times larger than when no proton concentration gradient was present. It should be noted, that only the extracellular pH changes as the proton gradient is raised, and that according to Fig. 4, curve A, an

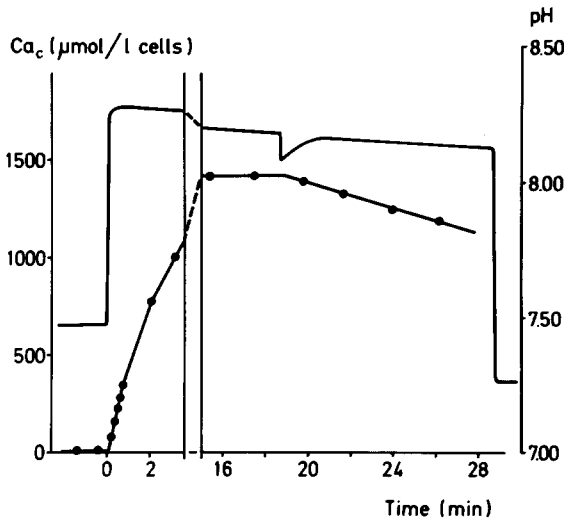

Fig. 6. Ionophore A23187-mediated net efflux of Ca^{2+} from depleted, DIDS-treated cells at $(pH_{ex} - pH_c) = 0.90$. Ordinates are cellular content of Ca in μmol/l cells and pH. Abscissa is time in min. The initial part of the net influx (loading of the cells) is shown. At the transient drop in pH_{ex} (19th minute) a concentrated solution of EGTA was added to a final extracellular concentration of 1 mM. Saponin was added at the end of the experiment, in order to determine the cellular pH (see Experimental procedure). Initial extracellular concentration of ionized Ca, 52 μM and of K^+, 10 mM. The concentration of A23187 was 5 μmol/l cells. ———, pH; ●———●, cellular Ca content in μmol/l cells.

about 3-times lower increase in net influx results from a simple increase in pH from 7.1 to 8.1. Further, it is important to notice that the carrier mediated netflux is directly proportional to the ratio (H^+-*trans*/H^+-*cis*) over a range of 0.1 to 10.

Ca^{2+} net influxes determined in the presence of various proton concentration gradients raised across the membrane of cells with fixed values of intracellular pH of 6.6 and 7.9, respectively, is shown in Fig. 7. At $pH_c = 6.6$ the carrier mediated net influx of Ca^{2+} was found to be a linear function of the proton concentration gradient up to a ratio of 10, but the dependence was steeper than at $pH_c = 7.1$, namely a 27-times increase in influx per ΔpH. At $pH_c = 7.9$ the linear relationship between the logarithm of the net influx and ΔpH was limited to a span of 0.4 units, where the net influx increased by a factor of 32 per ΔpH unit.

The degree of proton concentration gradient acceleration of Ca^{2+} net influx as a function of the

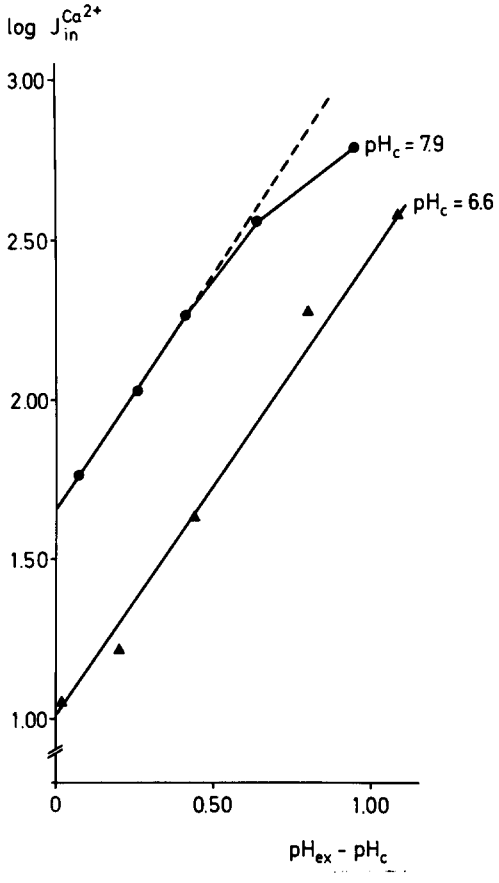

Fig. 7. Logarithm of ionophore A23187-mediated initial net influx of Ca^{2+} versus ($pH_{ex} - pH_c$) at two different cellular pH values. In all experiments the initial extracellular concentration of ionized Ca was 52 μM and the concentration of ionophore A23187 was 5 μmol/l cells. The initial extracellular K^+ concentration was varied (see Experimental procedure). The basis cellular pH value was 6.6 and 7.9, respectively.

fixed cellular pH value at which the gradient was raised is shown in Fig. 8. As seen from this figure a pronounced minimum in proton concentration gradient acceleration of the Ca^{2+} net influx was found at a cellular pH of about 7.1.

In all experiments with proton concentration gradients, so far shown, these were established by CCCP-mediated transfer of protons until electrochemical equilibrium. It was of interest, therefore, to see whether CCCP had any direct effect upon the ionophore A23187-mediated net fluxes of Ca^{2+}. Varying the concentration of CCCP between 7 and 90 μM resulted in identical fluxes and equal degrees of proton concentration gradient acceleration.

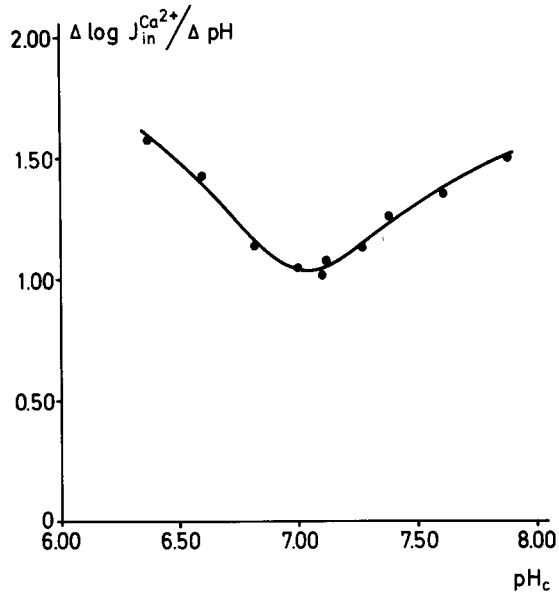

Fig. 8. Proton concentration gradient acceleration of ionophore A23187-mediated Ca^{2+} net influx as a function of cellular pH. Ordinate is the increase in the logarithm to the net Ca^{2+} influx per unit ΔpH. (Compare Figs. 5, 7 and 9.) Abscissa is cellular pH.

Acceleration and deceleration of Ca^{2+} net influxes in the absence of CCCP were studied in a series of experiments in which various concentration gradients of protons were obtained by suspensions of DIDS-treated, depleted cells ($pH_c = 7.28$) in salt solutions buffered with 10 mM Mops or Hepps (Fig. 9). The extracellular concentration of K^+ was 73 mM and the original V_m of about -15 mV should therefore change insignificantly as the Ca^{2+}-sensitive K^+ channels were activated. The cells were treated with DIDS in order to block the anion-exchange mechanism [16], thus minimizing the breakdown with time of the established proton concentration gradients. Since the extracellular pH changed less than 0.01, the maximal change in pH_c, the relative buffering capacities taken into account, was less than 0.02 in the opposite direction.

As seen from Fig. 9 the usual linear relationship was obtained between the logarithm to the net influx and the difference ($pH_{ex} - pH_c$). Comparing Fig. 5 and Fig. 9 it should be noted, that in Fig. 9 the fluxes are all net influxes. Those to the left of the ordinate are net fluxes of Ca^{2+} into cells

with a pH higher than the values of the extracellular phase ($(pH_{ex} - pH_c)$ is negative). The slope of the curve in Fig. 9 was 1.1, a value which inserted in Fig. 8 fitted well. Thus, the same degree of acceleration was found whether the proton concentration gradient was established by extracellular buffering or by the aid of CCCP, mediating an electrochemical equilibration of protons at various membrane potentials.

In Fig. 10 the proton concentration gradient acceleration of Ca^{2+} net influx at $pH_c = 7.16$ is shown at two different degrees of saturation of the ionophore with Ca^{2+}. The concentrations of ionophore and extracellular Ca^{2+} were adjusted so that with different ratios between these concentrations

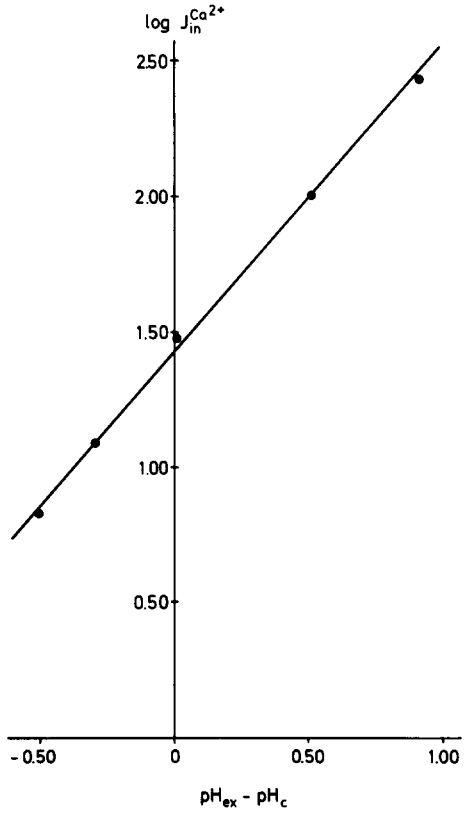

Fig. 9. Logarithm of ionophore A23187-mediated net influx of Ca^{2+} as a function of $(pH_{ex} - pH_c)$ at a fixed value of pH_c of 7.28. The various concentration gradients of protons across the membranes were established by suspending depleted, DIDS-treated cells in salt solutions containing 73 mM KCl, 73 mM NaCl and 10 mM Mops or Hepps, titrated to given values of pH. No CCCP was present in these experiments, and the change in cellular pH with time was insignificant (see text).

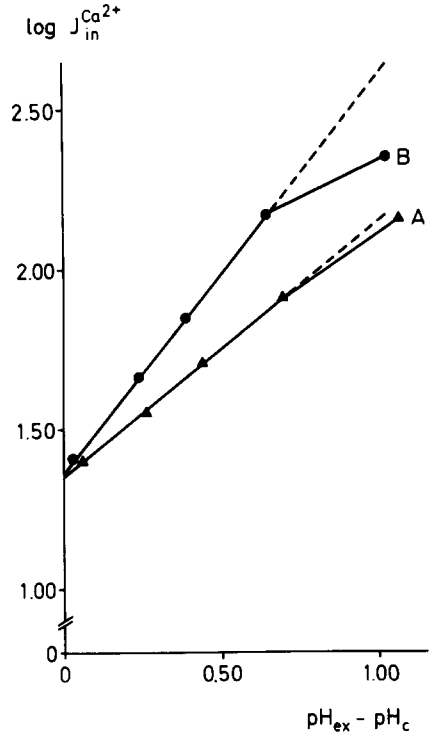

Fig. 10. Logarithm of ionophore A23187-mediated net influx of Ca^{2+} versus $(pH_{ex} - pH_c)$ at two different ratios between initial extracellular Ca and cellular ionophore concentrations. The proton concentration gradients were obtained (as described in Experimental procedure) with a fixed cellular pH of 7.16. Curve A: 1.25 μmoles ionophore per litre cells and an extracellular concentration of Ca^{2+} of 700 μM. Curve B: 10 μmoles ionophore per litre cells and an extracellular concentration of Ca^{2+} of 12 μM.

identical net influxes were obtained without a proton gradient present. In curve A the concentrations were: ionophore 1.25 μmol/l cells, extracellular Ca^{2+} 700 μM; and in curve B the concentrations were: ionophore 10 μmol/l cells, extracellular Ca^{2+} 12 μM. The proton concentration gradients were established in the usual way (compare Fig. 1). At the relatively high saturation of the ionophore with Ca^{2+} the acceleration of net influx was only 6-times per ΔpH unit (curve A), whereas at the relatively lower degree of saturation the acceleration was 18-times per ΔpH unit (curve B).

Discussion

Equilibrium distribution of ionized Ca

As seen from Fig. 3 the equilibrium distribution

of Ca between the intra- and extracellular phases on a log-log scale is a linear function of the ratio of proton concentrations in the phases. The linear relationship indicates that the ratio between the concentrations of ionized Ca per litre cell water and total Ca per litre cells, α, is constant within the range of experimental values (200–1800 μmoles of Ca per litre cells), the logarithm to the magnitude of the reciprocal constant being given as the intercept on the ordinate axis. In this way, α was found to be 0.19.

Ferreira and Lew [17] have previously reported that α was 0.3–0.5 among cells from different donors. They also reported a constant buffering capacity within the range of 1 to 3000 μmoles of total Ca per litre cells. Simonsen et al. [18] later used an α value of 0.16, a value in closer agreement with the value reported here.

The slope of the line in Fig. 3 is almost two, indicating that the equilibrium concentration distribution ratio of ionized Ca equals the proton concentration distribution ratio raised to the second power. This relationship between proton and Ca^{2+} equilibrium distributions in the presence of ionophore A23187 was originally suggested by Pressman [8] as a consequence of the proton-calcium exchange mediated by the ionophore, an observation first reported by Reed and Lardy [19]. In their determination of α, Ferreira and Lew [17] and Lew and Brown [12] based their calculation on the same assumption. In neither of these cases, however, the distribution of Ca^{2+} across the membranes were determined under systematically varied proton concentration distribution ratios, whereas in Fig. 3 the range from 1 to 10 is covered.

pH-dependence of the ionophore-mediated Ca^{2+} net influx

The true pH-dependence of the ionophore mediated net influx of Ca^{2+} should be represented by curve B in Fig. 4, since here pH_{ex} is equal to pH_c within the whole range of pH. The shape of curve B is close to that of a simple titration curve of a monocarboxylic acid, and the curve shows a half maximal net influx at pH 7.3 (100 μM extracellular Ca^{2+}, 10 μmoles ionophore per litre cells). Curve B thus represents the variation with pH in the amount of ionophore anion present in the interfaces of the membrane, available for Ca^{2+} transport. It should be noted, however, that parameters such as binding of ionophore to intracellular proteins [20], binding of ionophore to the lipid membrane [7], and surface charge density are all included in curve B Fig. 4 as functions of pH.

Proton concentration gradient acceleration of Ca^{2+} net influx

The curves in Figs. 5–9 clearly demonstrate that a concentration gradient of protons across the membrane increases the ionophore mediated net flux of Ca^{2+} in the opposite direction. As seen from Fig. 5 a linear relationship between the logarithm of the net flux and $(pH_{cis} - pH_{trans})$ was found within a range of up to two units of ΔpH.

That it is the variation in concentration gradient which is decisive and not simply the simultaneous variation in extracellular pH is underlined by two facts. Firstly, net effluxes of Ca^{2+} were decreased by exactly the same factor by which the net influxes were increased (Fig. 5). In net efflux experiments the variation in pH were on the *trans* side, whereas in net influx experiments the pH variation took place on the *cis* side. Thus, the system behaves symmetrically with respect to the effect of the proton concentration gradient. Secondly, with a fixed intracellular pH of 7.1 an acceleration of Ca^{2+} net influx of 10-times per unit of ΔpH was found (Fig. 5). Raising different proton concentration gradients across the membranes, with a cellular pH of 7.1 as basis, results in concurrent changes in extracellular pH within the steep range of curve A, Fig. 4. Raising comparable gradients, with a cellular pH of 7.9 as basis, results in concurrent changes in extracellular pH from 8.1 and upwards to 9.1. According to curve A, the increase in net influx of Ca^{2+} should be only about 15%, within this range of pH. With a cellular pH of 7.9 the dependence on the proton concentration gradient was found, however, to be even larger than with pH 7.1 as the cellular basis, namely about 30-times per unit of ΔpH (see Fig. 7).

Acceleration of ionophore A23187-mediated Ca^{2+} net fluxes across lipid bilayer membranes by a positive *trans* to *cis* concentration gradient of protons has been reported by Wulff and Pohl [5].

Since they found that the ionophore did not transport protons across a lipid bilayer membrane, they mentioned the acceleration as a peculiar experimental fact but did not discuss it.

Direct ionophore A23187-mediated Ca^{2+}-H^+ exchange across the membranes of rat erythrocytes was demonstrated by Reed and Lardy [19]. Addition of A23187 (about 50 μmol/ cells) to erythrocytes suspended in 150 mM choline chloride, 2 mM $CaCl_2$, 0.1 mM KCl, buffered with 5 mM Tris-HCl (pH 7.4) resulted in a Ca^{2+}-H^+ exchange lasting about 90 s and with a maximum ratio of 0.8–1.3 between protons released and calcium taken up by the cells. Addition of ionophore in 10-times lower concentration produced no measurable proton efflux.

Comparing the degrees of proton concentration gradient acceleration of the net influx of Ca^{2+} as presented in Fig. 5 and Fig. 9, the absence of any influence of the membrane potential is convincing. In the experiments depicted in Fig. 5 the membrane potential varies from about 0 mV to about -80 mV concurrently with the variation in proton concentration gradient, whereas in the experiments represented in Fig. 9 the membrane potential was about -15 mV at all values of proton concentration gradient. Corrected for the difference in pH_c between the two types of experiments, identical values of acceleration was found. In accordance with Reed and Lardy [19], Pfeiffer et al. [6] and Kolber and Haynes [7] we therefore consider a consistent transport mechanism to be an electroneutral exchange of Ca^{2+} for Mg^{2+} or H^+ via the neutral species A_2M and AH.

Since the concentration of ionized Mg in the ATP and 2,3-diphosphoglycerate depleted cells was as high as approx. 1500 μM and the affinity of the ionophore for Mg^{2+} is only about 2.5-times lower than that for Ca^{2+} [7], we determined the netflux of Mg^{2+} from the cells into the Mg^{2+}-free salt solutions. During the period of initial net influx of Ca^{2+} (approx. 1 min), less than 0.5% of the ionophore efflux was in the form of the Mg-ionophore complex. This is a surprisingly low value, but allows for the assumption, that the carrier recruitment for the Ca^{2+} net influx takes place as a reflux of undissociated ionophore, AH.

Kolber and Haynes [7] have shown that the association and dissociation processes of the various complexes take place instantaneously in the region of polar heads and glycerol backbones at the interface of the lipid membrane and the waterphase. Furthermore, they have determined the rate constants for translocations across a phospholipid bilayer of the calcium and proton complexes to be 0.1–0.3 s^{-1} and 28 s^{-1}, respectively [7]. While the existence of the proton concentration gradient acceleration of ionophore-mediated Ca^{2+} net flux is therefore understandable, it is not easy to see why the acceleration as a function of the cellular pH passes through a minimum at a value of 7.1.

In their experiments and kinetic equations Kolber and Haynes [7] only deal with transports between phases with identical pH, and Ca^{2+} is equilibrated across the vesicular membranes within a second or less. They assume that undissociated ionophore A23187, AH, is in instantaneous equilibrium across the lipid membranes. However, the ratio between ionophore A23187 present and Ca^{2+} transported was very high and the average turnover of the ionophore molecules in an experiment was $\leqslant 1$.

Assuming, that in our experiments 50% of the ionophore added was bound to intracellular proteins, the turnover of the ionophore molecules in the membranes can be calculated. During an initial period of about 1 min we found absolutely constant net influxes of Ca^{2+} within the range of 10 to 500 μmol/l cells per min. With an assumed concentration of transporting ionophore A23187 of 2.5 μmol/l cells the resulting turnover numbers are between 0.1 and 7 s^{-1}. During proton concentration gradient accelerated net influxes the turnover number may therefore be 5- to 10-times as high as in the experiments reported by Kolber and Haynes.

We find that the minimum in acceleration at $pH_c = 7.1$ (Fig. 7) most probably is explained by a shift in the rate determining factor from one process to another, as $pH_c \simeq 7.1$ is passed. Thus at low pH_c, instantaneous equilibrium across the membrane of the species AH might prevail, as suggested by Kolber and Haynes [7]. The proton concentration gradient acceleration should then be explained mainly by the increase in the concentration of A^- in the cis side region with increasing pH_{ex}. This effect would be expected to decrease

with an increase in overall pH, and therefore in the A^-/AH ratio. With increasing pH_c (and therefore decreasing concentration of AH in the membrane) equilibration of AH across the membrane might not be instantaneous and carrier-recruitment, that is *trans* to *cis* flux of AH, might now be the rate limiting step. If this is the case, the proton concentration gradient acceleration should, in the range of pH_c of 7.1 and upwards, be explained by an increase in the net efflux of AH, resulting from an increase in the concentration gradient driving this flux.

Besides the basis pH_c, the proton concentration gradient acceleration, as seen from Fig. 10 was found to be a function of the degree of saturation of the ionophore. Increasing the ratio between extracellular Ca^{2+} and cellular ionophore from the usual value of 10 (cf. Fig. 5) to 560 resulted in a decrease in acceleration from 12- to 6-times per ΔpH unit. A decrease of the Ca^{2+}/ionophore ratio to 1.2 resulted in an increase in acceleration from 12- to 18-times per ΔpH unit. It should be noted that in the case with low ionophore and high Ca^{2+} concentration the ionophore is still far from saturation with Ca.

The important parameter might here be the concentration ratio $[AH]:[A_2Ca]$ in the membrane. In parallel with the suggestion of a shift in rate limiting factor as pH_c increases, the rate-limiting and proton concentration gradient-dependent factor at high ionophore concentration should be the concentration of A^- in the *cis* side of the membrane, AH being in equilibrium across the membrane. At low ionophore and high Ca^{2+} concentration AH might not be in equilibrium across the membranes and the rate-limiting step would be shifted to the carrier recruiting *trans* to *cis* flux of AH, a flux that increases as the driving gradient across the membrane is increased. If this is the case, both absolute magnitudes of acceleration and the position of the minimum in Fig. 8 should be functions of the ratio between extracellular Ca^{2+} and cellular ionophore concentrations.

Proton concentration gradient acceleration and oscillations

In relation to the induced oscillations in conductance of the Ca^{2+}-sensitive K^+ channels of human red cells previously reported [1,3], the proton concentration gradient acceleration and deceleration of ionophore A23187-mediated net fluxes of Ca^{2+} across red cell membranes support some of the proposed explanations of this phenomenon.

In the oscillation experiments variation in membrane potential were monitored as CCCP mediated changes in pH of an unbuffered extracellular phase (Ref. 1, see also Experimental procedure) and large changes in the concentration gradient of protons across the membranes took place. During the first wave of an oscillation a hyperpolarization of about 60 mV occurred [1], resulting in ($pH_{ex} - pH_c$) values of about 1.2 units. The intracellular pH was about 7.15 and the variation in ionophore-mediated influx of Ca^{2+} was therefore close to the linear relationship shown in Fig. 5.

The range of ionophore-mediated Ca^{2+} influx within which oscillation was induced, was found to be very narrow [1]. This can now be explained as follows: Below a certain threshold value the induced net influx is so small, that activation of the Ca^{2+} pump by the increase in cellular concentration of ionized Ca takes place sufficiently fast, and an increased pump efflux therefore balances ionophore mediated influx in a new steady state with an only slightly elevated cellular Ca^{2+} and an insignificant increase in the K^+ conductance. Above this threshold intracellular Ca^{2+} increases so rapidly, that the activation of the Ca^{2+} pump, which takes place with a significant characteristic delay [4], can not counterbalance the ionophore-mediated Ca^{2+} influx sufficiently fast. The intracellular concentration of ionized Ca therefore increases above the threshold at which the K^+ channels are activated, and the cell membranes hyperpolarize with about 60 mV, corresponding to an increase in the ($pH_{ex} - pH_c$) value of about 1. As a result of this new, high value of ($pH_{ex} - pH_c$) the ionophore-mediated Ca^{2+} influx becomes about 10-times higher than the initially induced value and the pump is further activated. A peak in cellular Ca^{2+}-concentration occurs only if the delayed pump flux can achieve a magnitude sufficiently high to turn this strongly increased net influx into a net efflux of Ca^{2+}.

During the phase of depolarization the maximally activated Ca^{2+} pump, on the other hand, maintain a high efflux of Ca^{2+} while a sharp decrease in ionophore-mediated influx takes place,

and the delayed inactivation of the pump [4] in combination with this decrease result in a tendency of the pump to overshoot.

Single transient increases in intracellular Ca induced by addition of ionophore A23187 to human red cells suspended in buffered media without CCCP present have been studied by Scharff et al. [21]. They found that the course of a single transient in cellular calcium concentration could be fully explained in quantitative terms by the ionophore-induced increase in Ca^{2+} influx and the delayed activation of the Ca^{2+} pump. It was at the same time clear, that oscillation in the cellular Ca level would occur only if the influx of Ca^{2+} varied with time. Since the cells were suspended in buffered, high-K salt solution in the absence of CCCP, the ionophore-mediated Ca^{2+} influx did not change in consequence of a varying extracellular pH in their experiments.

The importance of the Ca^{2+} pump in the oscillating system was originally supported by the finding, that no oscillations could be induced in ATP-depleted cells [1]. Addition of the ionophore to a suspension of these cells resulted in a constant hyperpolarization. Surprisingly enough, addition of EGTA in surplus to the extracellular phase did not result in a closure of the K^+ channels, even if the ionophore concentration was doubled [1]. Based on the assumption that the cells were depleted of Ca, it was suggested, that if ATP was totally absent, the K^+ channels could not be inactivated, even though the cellular Ca^{2+} concentration decreased below the threshold value. Now this phenomenon can easily be explained by the strong deceleration of ionophore mediated net efflux of Ca^{2+} from cells hyperpolarized about 60 mV.

Since the variation in proton concentration gradient, and hence in ionophore A23187-mediated influx, is mediated by CCCP, it is understandable, that no oscillations could be induced in the absence of CCCP or if the extracellular phase was heavily buffered.

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References

1 Vestergaard-Bogind, B. and Bennekou, P. (1982) Biochim. Biophys. Acta 688, 37–44
2 Macey, R.I., Adorante, J.S. and Orme, F.W. (1978) Biochim. Biophys. Acta 512, 284–295
3 Vestergaard-Bogind, B. (1983) Biochim. Biophys. Acta 730, 285–294
4 Scharff, O. (1980) in Membrane Transport in Erythrocytes: Alfred Benzon Symp. 14 (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), pp. 236–250, Munksgaard, Copenhagen
5 Wulf, J. and Pohl, W.G. (1977) Biochim. Biophys. Acta 465, 471–485
6 Pfeiffer, D.R., Taylor, R.W. and Lardy, H.A. (1978) Ann. N.Y. Acad. Sci. 307, 402–423
7 Kolber, M.A. and Haynes, D.H. (1981) Biophys. J. 36, 369–391
8 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530
9 Lew, V.L. (1971) Biochim. Biophys. Acta 233, 827–830
10 Rose, Z.B. (1976) Biochem. Biophys. Res. Commun. 73, 1011–1018
11 Tsien, R.Y. and Rink, T.J. (1981) J. Neurosci. Methods 4, 73–86
12 Lew, V.L. and Brown, A.M. (1979) in Detection and Measurement of Free Ca^{2+} in Cells (Ashley, C.C. and Campbell, A.K., eds.), pp. 423–432, Elsevier/North-Holland Biomedical Press, Amsterdam
13 Bookchin, R.M. and Lew, V.L. (1980) Nature 284, 561–563
14 Lew, V.L., Tsien, R.Y. and Miner, C. (1982) Nature 298, 478–481
15 Knauf, P.A., Fuhrmann, G.F., Rothstein, S. and Rothstein, A. (1977) J. Gen. Physiol. 69, 363–386
16 Cabantchick, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207–226
17 Ferreira, H.G. and Lew, V.L. (1976) Nature 259, 47–49
18 Simonsen, L.O., Gomme, J. and Lew, V.L. (1982) Biochim. Biophys. Acta 692, 431–440
19 Reed, P.W. and Lardy, H.A. (1972) in The Role of Membranes in Metabolic Regulation (Mehlman, M.A. and Hanson, R.W., eds.), pp. 111–131, Academic Press, New York
20 Lew, V.L. and Simonsen, L.O. (1980) J. Physiol. 308, 60P
21 Scharff, O., Foder, B. and Skibsted, U. (1982) Biochim. Biophys. Acta 730, 295–305