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# SPONTANEOUS INACTIVATION OF THE $Ca^{2+}$-SENSITIVE $K^+$ CHANNELS OF HUMAN RED CELLS AT HIGH INTRACELLULAR $Ca^{2+}$ ACTIVITY

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**Ionophore A23187-mediated $Ca^{2+}$-induced oscillations in the conductance of the $Ca^{2+}$-sensitive $K^+$ channels of human red cells were monitored with ion specific electrodes. The membrane potential was continuously reflected in CCCP-mediated pH changes in the buffer-free medium, changes in extracellular $K^+$ activity were followed with a $K^+$-selective electrode, and changes in the intracellular concentration of ionized calcium were calculated on the basis of cellular $^{45}Ca$ content. An increased cellular $^{45}Ca$ content at the successive minima of the oscillations where the $K^+$ channels are closed indicates that the activation of the channels might be a ($dCa^{2+}/dt$)-sensitive process and that accommodation to enhanced levels of intracellular free calcium may occur. An incipient inactivation of the $K^+$ channels at intracellular ionized calcium levels of about 10 μM and a concurrent membrane potential of about −65 mV was observed. At a membrane potential of about −70 mV and an intracellular concentration of about $2 \cdot 10^{-4}$ M no inactivation of $K^+$ channels took place. Inactivation of the $K^+$ channels is suggested to be a compound function of the intracellular level of free calcium and the membrane potential. The observed sharp peak values in cellular $^{45}Ca$ content support the notion that a necessary component of the oscillatory system is a $Ca^{2+}$ pump operating with a significant delay in the activation/inactivation process in response to changes in cellular concentration of ionized calcium.**

## Introduction

$Ca^{2+}$-sensitive $K^+$-specific ion channels have been demonstrated in the membranes of different cell types from different species [1]. Physiologically, this channel is of obvious importance as a component in the second messenger system which, in addition to the $K^+$ channel, includes the transmitter- and voltage-sensitive $Ca^{2+}$ channel, the adenylate cyclase and the $Ca^{2+}$ pump [1,2].

Whereas the physiological importance of the $Ca^{2+}$-sensitive $K^+$ channel in the membrane of the human erythrocyte is doubtful, these cells lend themselves as an experimental unit in the study of the opening and closing processes of this channel.

In a recent paper [3] ionophore A23187-mediated $Ca^{2+}$-induced oscillations in the $K^+$ conductance of the human erythrocyte membrane were described. The oscillations in the conductance of $Ca^{2+}$-sensitive $K^+$ channels were suggested to be partly based on the delayed response of the activation/inactivation of the $Ca^{2+}$ pump to rapid changes in the concentration of ionized calcium reported by Scharff and Foder [4]. Another physiologically important parameter suggested to be decisive for the oscillatory $K^+$ conductance was a spontaneous closure of the $K^+$ channels taking place while the intracellular concentration of free calcium was still at a maximum. It was thus proposed that the closure of the $K^+$ channels was not

Abbreviations: Mops, 4-morpholinepropanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

a consequence of a decreasing level of intracellular $Ca^{2+}$ concentration resulting from a sharply increased $Ca^{2+}$-pump activity. On the contrary, the indirectly observed decrease in intracellular $Ca^{2+}$ concentration after the peak-value of hyperpolarization was proposed to be the concerted effect of two factors: a decreasing ionophore-mediated $Ca^{2+}$ influx caused by the decreasing gradient of protons across the cell membranes resulting from the incipient inactivation of the $K^+$ channels, and a delayed increase in the $Ca^{2+}$-pump rate.

CCCP used to monitor the membrane potential unfortunately strongly influenced the response of the $Ca^{2+}$ electrode, and the explanation of the oscillations outlined above therefore had to be tentative [3]. In the present work, the variations in intracellular $Ca^{2+}$ concentration during oscillations were monitored with $^{45}Ca$. The results obtained clearly demonstrate that the inactivation of the $Ca^{2+}$-sensitive $K^+$ channels begins while the intracellular $Ca^{2+}$ concentration still is maximal and that the activation of the $Ca^{2+}$ pump is delayed.

## Materials and Methods

All inorganic salts (pro analysis) and glucose were purchased from Merck. Mops, Trizma base and Trizma, and CCCP were from Sigma. CCCP was administered as a concentrated (20 mM) solution in ethanol. The ionophore A23187 was from Calbiochem. A 2 mM stock solution of the compound in absolute ethanol for spectroscopy (BDH) was stored at −20°C. Dilute solutions of ionophore were prepared by adding 1 vol. of stock solution to 9 vol. of ethanol.

The experiments were performed using cells from freshly drawn blood from healthy human donors. The cells were washed twice in low-calcium Ringer's solution (155 mM NaCl/1 mM KCl/0.15 mM $MgCl_2$/5 mM glucose) and then either preincubated at 37°C in Mops/Ringer (144 mM NaCl/1 mM Mops, pH 7.40) at a cytocrit of 20%, or stored up to 3 h as packed cells in an ice-bath. The cells preincubated in Mops/Ringer were washed twice in 5 vol. low-calcium Ringer just before a sample of packed cells was taken for an experiment.

In all experiments the cells were suspended in the unbuffered low-calcium Ringer at 37°C. $Ca^{2+}$ contamination of the inorganic salts resulted in a measured $Ca^{2+}$ activity of approx. $5 \cdot 10^{-6}$ M in this solution. The addition of $^{45}Ca$ (in 5 mM $CaCl_2$) resulted in a total initial activity of $Ca^{2+}$ in the extracellular phase of about $10^{-5}$ M. Extracellular $Ca^{2+}$ activities were determined with a $Ca^{2+}$-selective electrode standardized with $Ca^{2+}$ buffers of comparable activities [5].

For a discussion of the estimation of changes in membrane potential of red cells suspended in unbuffered salt solutions and using CCCP as a proton equilibrator, see Macey et al. [6]. Wieth et al. [7,8] have shown that the very efficient anion-exchange system of the human red cell membrane together with the high carbonate dehydratase activity of the cellular phase is able to degrade the proton gradient established by CCCP (e.g., in case of hyperpolarization) in the presence of sufficient bicarbonate. In the absence of CCCP the effect of this system, the so-called Jacobs-Stewart cycle [9] is dependent on the concentration of bicarbonate present and is based on the electrosilent exchange of $Cl^-$ and $HCO_3^-$, which, combined with the carbonate dehydratase-catalyzed reaction, will proceed until identical hydroxyl and chloride ion gradients are established. Thus, in the presence of CCCP and sufficient bicarbonate the proton gradient across the membrane will be somewhere between the chemical and the electrochemical equilibrium depending on the relative efficiency of the Jacobs-Stewart cycle and the CCCP system.

In experiments in which either argon was blown over the vigorously stirred incubated cells for 5 min before the addition of the ionophore A23187, or the anion-exchange system was inhibited by prior addition of 0.2 mM of the potent inhibitor picrate, or the cells had been preincubated for more than one hour (pH 7.5, 37°C) with the carbonate dehydratase inhibitor acetazolamide (approx. 2.5 mM) the magnitude of the changes in extracellular pH was compared to that of those obtained under the usual standard conditions. The results indicated that peak values of hyperpolarizations of about −75 mV (absolute scale) as evaluated by the peak values in extracellular pH, were underestimated by maximally 5 mV under usual experimental conditions. With respect to the problems addressed in this paper, this deviation

was considered to be of minor importance.

Since the intracellular buffering capacity is more than 200 times that of the extracellular phase, the intracellular pH remains constant during an experiment. The membrane potential before the addition of the ionophore A23187, that is the Nernst potential of $Cl^-$, and thus the membrane potentials on the absolute scale were obtained by determining the change in pH on total hemolysis of the cells at the end of the experiment [3,6]. Preparation of the $K^+$- and $Ca^{2+}$-selective electrodes as well as the CCCP interference with the response of the latter have been reported previously [3].

The cellular content of $^{45}Ca$ was determined by the method of Lew and Brown [10], slightly modified. 100 $\mu$l samples of the cell suspension were transferred to a centrifuge tube containing 900 $\mu$l salt solution (75 mM KCl/75 mM NaCl/10 mM Tris-HCl (pH 7.0, 0°C)/2 mM EGTA and 150 $\mu$M bovine albumin) layered on top of 400 $\mu$l 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 the 100-$\mu$l sample of suspension, the tube with its content was centrifuged at $13000 \times g$ for 15 s. The procedure was standardized so that the centrifugation started 5 s after the transference of the 100-$\mu$l sample of suspension to the phthalate tube. The resulting cell pellets were processed for scintillation counting according to Ref. 10.

*Experimental procedure*

A sample of packed cells was suspended in the appropriate volume of low-calcium Ringer at 37°C and vigorous stirring was begun. 20 $\mu$l $^{45}Ca$ (as a 1 mM $CaCl_2$ solution, 7 Ci/g Ca) was added, and after a couple of minutes a sample of extracellular phase was taken for subsequent measurement of $Ca^{2+}$ activity. CCCP was then added to the suspension and for the next 5 to 10 min three or four samples for determination of the cellular $^{45}Ca$ content were taken as well as samples for determination of the total concentration of $^{45}Ca$ in the suspension and initial extracellular $K^+$ concentration (flame photometry). Ionophore A23187 was then added and samples for determination of cellular $^{45}Ca$ were taken during the ensuing oscillations. At the end of the experiment a concentrated saponin solution was added and the pH value of the resulting hemolysate was recorded, the absolute scale of membrane potentials being established in this way.

In the experiments where a quasi steady state occurred after one wave of hyperpolarization (Fig. 2) samples for determination of cytocrit and cellular ion concentrations were taken initially and during this period in a parallel experiment.

## Results

In the experiments presented in Figs. 1–3 initial extracellular $Ca^{2+}$-activities were determined on samples taken after the addition of $^{45}Ca$ but before the addition of CCCP and ionophore A23187 to the cell suspension. Since the initial intracellular pool of exchangeable calcium is less than 1 $\mu$mol per litre cells [11] and the measured extracellular $Ca^{2+}$ activities were over $10^{-5}$ M, the intracellular $Ca^{2+}$-compartment, even at a cytocrit of 10 to 15%, is negligible and the $^{45}Ca$ in- and effluxes can be taken as valid markers of the net in- and effluxes of calcium ions.

The intracellular $Ca^{2+}$ concentration at various times during the oscillations was calculated from the measured cellular $^{45}Ca$ content assuming a negligible initial intracellular $Ca^{2+}$ activity. The cellular water content in which the $^{45}Ca$ was distributed was calculated from changes in either cytocrit value or extracellular $K^+$ activity. In the latter case, the loss in cellular $K^+$ was considered to be equal to a loss in isotonic KCl.

According to Ferreira and Lew [12], within the range of cellular calcium of $10^{-6}$ to $10^{-4}$ M, 50 to 70% of the intracellular calcium is bound (although exchangeable). A factor of 0.35 between concentrations of ionized and total calcium was used in the calculation of ionized calcium (cf. Table I).

In Fig. 1 the recordings of the extracellular activities of $H^+$, $K^+$ and $Ca^{2+}$ and the cellular concentration of $^{45}Ca$ versus time are shown. Before the addition of ionophore A23187 all extracellular activities remained constant with time and 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 $^{45}Ca$, consistent with the assumption

that the initial cellular content of $^{45}Ca$ was zero.

The addition of ionophore A23187 (15 $\mu$mol per litre cells) resulted in a rapid uptake of $^{45}Ca$ into the cells, an almost linear increase in extracellular $K^+$ activity and a steep increase in extracellular pH with time. The increase in pH in the buffer-free extracellular phase indicates a strong hyperpolarization of the cell membranes, an increase of one pH unit in principle corresponding to a change in membrane potential of the cells of $-61$ mV.

In the experiments shown in Fig. 1 the hyperpolarization culminates at a value of $-65$ mV (absolute scale) and a depolarizing phase follows. During this phase the net efflux of $K^+$ decreases and eventually ceases and the membrane potential adjusts to a value a few mV more negative than

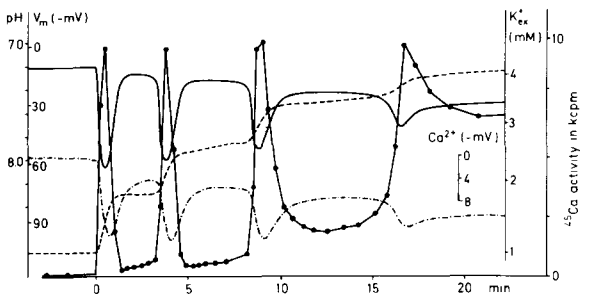


Fig. 1. Cellular $^{45}Ca^{2+}$ content (●———●), extracellular pH ($V_m$) (———), $K^+$ activity (------) and $Ca^{2+}$-electrode potential (·-·-·-·) in a suspension of glucose-fed cells (cytocrit 3.7%) as functions of time. Extracellular $Ca^{2+}$ activity before addition of the ionophore was $1.8 \cdot 10^{-5}$ M. CCCP was added to a final concentration of 20 $\mu$M and at zero-time ionophore A23187 was added to a final concentration of 15 $\mu$mol per litre cells. pH values and calculated corresponding membrane potentials are shown on the double-ordinate axis to the left. The absolute scale of membrane potential was established by adding concentrated saponin solution to the cell suspension at the end of the experiment and recording the pH of the resulting hemolyzate. Extracellular $K^+$ activity (in mM) and $Ca^{2+}$-electrode potentials (in mV) are indicated on the inserted ordinate axis. Total drift in the $K^+$ electrode during the experimental period was under +0.25 mM. Note that a large part of the changes in $Ca^{2+}$-electrode potentials reflects a varying CCCP interference with the electrode (see text). The cellular contents of $^{45}Ca$ are given in kcpm. Note that a scale of the cellular concentrations of calcium calculated on the basis of the $^{45}Ca$ contents cannot be inserted because of the substantial decrease in cell volume taking place during each wave of hyperpolarization (see text). For these corresponding intracellular concentrations of calcium see Table I. Hemolysis after 25 min was under 0.5%. The abscissa is time in minutes after addtion of ionophore A23187.

the initial value of $-13$ mV (absolute scale).

Almost simultaneously with the initiated depolarization cellular $^{45}Ca$ reaches a maximum value corresponding to an intracellular level of free calcium of about $3 \cdot 10^{-5}$ mol per litre original cell water (see also Table I). Obviously, the incipient decrease in $K^+$ conductance occurs while the cellular $Ca^{2+}$ concentration is close to maximum, but the exact displacement in time of the $^{45}Ca$ maximum relative to that of the hyperpolarization is impossible to determine with the technique used.

The succeeding decrease in cellular $^{45}Ca$ is very sharp and results, within less than a minute, in a minimum corresponding to a cellular concentration of ionized calcium of about $6 \cdot 10^{-7}$ mol per litre original cell water. During the following waves, the maximum values of cellular $^{45}Ca$ are approximately the same as those found during the first wave, whereas the minium values during the depolarized phase increases by about one order of magnitude (see Table I).

As can be seen in Fig. 1 the degree of hyperpolarization decreases from wave to wave, reflecting the fact that the Nernst potential of $K^+$ decreases, especially because of the increase in the extracellular $K^+$ activity, while the Nernst potential of $Cl^-$ increases, in particular during the second and subsequent waves, with decreasing intracellular concentration of chloride.

It should be noted that the $K^+$ efflux is zero only during the first depolarized phase (first minimum), that is all the $K^+$ channels are closed. This was true in all experiments but in some experiments this was also the case during the second minimum. The observed increase in extracellular $K^+$ during subsequent oscillations was not due to experimental error or cell hemolysis. After 25 min only about 0.7% of the cells were hemolyzed (but more than half of these cells hemolyzed instantaneously upon the addition of the ionophore A23187) and the drift in the $K^+$ electrode was found by flame-photometric control to be less than +0.24 mM. Therefore the slight increase in extracellular $K^+$ activity seen during the second and following phases of depolarization represents a few open $K^+$ channels.

As discussed in detail previously [3] $CCCP^-$ exerts a strong influence on the $Ca^{2+}$ electrode, and since the CCCP anions redistribute constantly

TABLE I

CALCULATED CONCENTRATIONS OF CALCIUM AT THE VARIOUS MAXIMA AND MINIMA IN CELLULAR $^{45}Ca$ CONTENT SHOWN IN FIG. 1

The concentration of ionized calcium in μmol per litre calculated cell water (row 3) is calculated from the corresponding concentrations per litre original cell water (row 2) assuming that the observed increase in extracellular $K^+$ activity can be regarded as the result of an efflux of a 155 mM KCl solution from the cells. As discussed in the text this correction can be applied for the first oscillation but becomes increasingly inapplicable for subsequent oscillations.

| | Peak | | | | Minimum | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 |
| Concentrations of total Ca in μmol per litre original cell water | 85 | 85 | 88 | 87 | 1.6 | 3.2 | 16 |
| Concentration of ionized Ca in μmol per litre original cell water | 30 | 30 | 31 | 30 | 0.55 | 1.1 | 5.6 |
| Concentrations of ionized Ca in μmol per litre calculated cell water | 32 | 36 | ~ 40 | - | 0.65 | 1.4 | ~ 8 |

during a wave of hyperpolarization, the $Ca^{2+}$-electrode response could be taken only as an indication of the direction of the actual changes in extracellular $Ca^{2+}$ activity taking place. It is remarkable, therefore, that the $Ca^{2+}$-electrode recordings, included in the figures for comparison, and the cellular changes in $Ca^{2+}$ concentrations as evaluated by the measured cellular $^{45}Ca$ correspond so well to each other as a function of time.

In oscillations induced in cells suspended at a cytocrit of 3.7% (original standard conditions [3]) the intervals between the waves of hyperpolarization generally were about 5 min. The same type of oscillations could be induced at higher cytocrit values, but here another type of oscillation in which a quasi-steady state of up to 20 min between the first and the second wave could also be induced. In order to analyze further the changes in cellular $^{45}Ca$ and especially the changes in cytocrit and cellular ion concentrations under a more favourable compartment ratio, oscillations were induced in cells suspended at a cytocrit of about 10%. In Fig. 2 are shown the changes in the various parameters during the first wave of hyperpolarization from such an oscillation.

Since the increase in extracellular $K^+$ activity very rapidly becomes almost linear with time, the cellular concentration of free $Ca^{2+}$ corresponding to the half-maximum value of $K^+$ efflux cannot be exactly determined. Based on the data presented in Fig. 2, a reasonable estimate is that the $K_{\frac{1}{2}}$ value is lower than the cellular concentration of free calcium about 10 s after the addition of ionophore A23187, which is under $0.5 \cdot 10^{-6}$ M.

At the peak value of membrane potential, that is at the maximum of $K^+$ conductance when the closing of $K^+$ channels starts, the intracellular $^{45}Ca$ reaches a value corresponding to an intracellular Ca concentration of $6.5 \cdot 10^{-5}$ mol per litre original cell water, or $2.5 \cdot 10^{-5}$ mol ionized calcium per litre actual cell water.

While the extracellular concentration of $Ca^{2+}$ in the experiment represented in Fig. 1 decreased about 10% the extracellular concentration of $Ca^{2+}$ in the experiment at 11% cytocrit was decreased about 40% at the peak of intracellular $^{45}Ca$ content.

As indicated in Fig. 2 a quasi-steady state was established after the wave of hyperpolarization. The intracellular Ca concentration reached a minimum of 1.1 μM after about 90 s, increasing during the following 5 min to 1.9 μM (original cell water concentrations), corresponding to 0.5 and 0.8 μmol of ionized calcium per litre actual cell water, respectively. The membrane potential, which initially was −11 mV, was −14 mV (absolute scale), and the net efflux of $K^+$ was zero. The cytocrit had changed from 11 to 9%, corresponding to a loss of about 175 ml water per litre cells assuming that no change in the degree of packing of the cells took place.

The cellular concentrations of $K^+$ and $Cl^-$ at the peak of hyperpolarization were calculated on the basis of the recorded change in extracellular

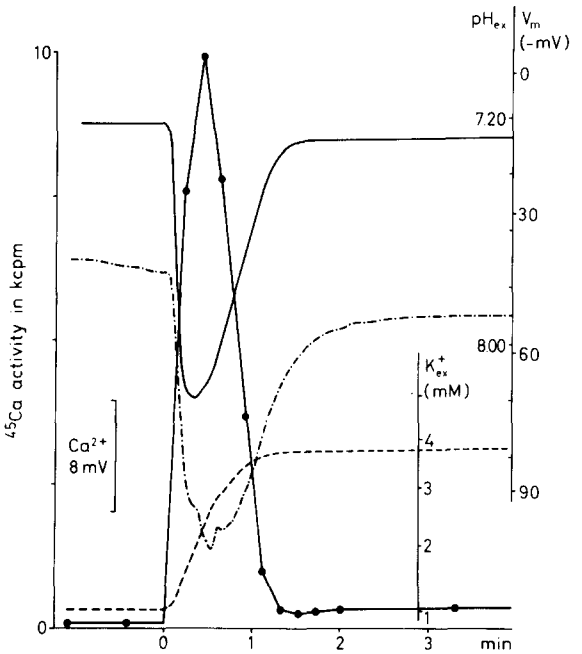


Fig. 2. Cellular $^{45}Ca$ content (●———●), extracellular pH ($V_m$) (———), $K^+$ activity (- - - - -) and $Ca^{2+}$-electrode potential (·–·–·) in a suspension of glucose-fed cells (cytocrit 11%) as functions of time. The extracellular $Ca^{2+}$ activity before addition of the ionophores was $10^{-5}$ M. CCCP was added to a final concentration of 50 $\mu$M and at zero-time ionophore A23187 was added to a final concentration of 6.5 $\mu$mol per litre cells. Coordinate axes are as in Fig. 1. The peak value of cellular $^{45}Ca$ corresponds to a cellular ionized Ca concentration of $2.5 \cdot 10^{-5}$ mol per litre actual cell water. Cellular $^{45}Ca$ contents after the wave of hyperpolarization correspond to concentrations of ionized calcium per litre actual cell water which increased from 0.5 to 0.8 $\mu$M. At the peak of cellular $^{45}Ca$ content, the decrease in the extracellular $Ca^{2+}$ concentration was about 60%. The cytocrit after the wave of hyperpolarization was 9%. After the wave of hyperpolarization the membrane potential of the 'resting' cells was −14 mV. Based on a cellular loss of isotonic KCl the membrane potential (that is $E_{Cl}$) calculated from the change in extracellular $K^+$ activity was −14.5 mV. $K^+$ net efflux at the peak of hyperpolarization was 1.75 mol per litre cells per hour. Hemolysis was 0.5% and drift in $K^+$ electrode was zero.

$K^+$ activity, assuming that during the initial short phase of hyperpolarization the net-efflux of $K^+$ took place as an efflux of isotonic KCl solution. Since the $P_K : P_{Na}$ selectivity ratio of the $Ca^{2+}$-sensitive $K^+$ channel is probably higher than $10^4$ [13], the net flux of $Na^+$ can be neglected. At the peak of hyperpolarization the trans-membrane current is zero and the transference number, conductance and permeability of $K^+$ can be calculated using the constant field equations. Inserting the net efflux of $K^+$ at the peak of the wave (0.5 mmol per litre cells per s) $T_{K^+}$ was calculated to be 0.6 and $P_{K^+}$ was $2 \cdot 10^{-7}$ cm/s. Lew and Ferreira [14] have reported a $P_{K^+}$ of $1.6 \cdot 10^{-7}$ cm/s for cells suspended in a $K^+$-equilibrium Ringer and ionophore A23187 loaded with about $3 \cdot 10^{-4}$ M $Ca^{2+}$. The permeability was here calculated on the basis of $^{42}K$-equilibration rates.

In an experiment parallel to that of Fig. 2, measured cellular contents of $K^+$, $Na^+$, $Cl^-$ and water were in reasonable agreement with the values calculated on the basis of a cellular loss of isotonic KCl-solution. While the changes during the first wave of hyperpolarization in an oscillation thus can be reasonably accounted for, the situation after four waves is more complicated (Fig. 1). Thus, the total loss of cellular chloride calculated under the above-mentioned assumptions surpasses the original cellular content by about 30%. A detailed analysis of the changes in cellular ion concentrations during an oscillation is in progress.

An upper threshold of ionophore-mediated $Ca^{2+}$ influx, above which oscillations in the $K^+$ conductance could not be induced, has previously been demonstrated [3]. At and above this threshold value a substantial but sustained net efflux of $K^+$ was induced, the $K^+$ conductance remaining almost constant or decreasing only slightly with time (see also Fig. 3).

The cellular uptake of $^{45}Ca$ in three parallel experiments, in which the initial $Ca^{2+}$ influx ranged from the normal to above the upper threshold value, is shown in Fig. 3. Since the initial extracellular $Ca^{2+}$ activity in these experiments was as high as $7 \cdot 10^{-5}$ M a sufficiently high influx could be induced with as little as 2.5–6 $\mu$mol ionophore A23187 per litre cells. In the experiment of Fig. 3B, the cellular $^{45}Ca$ content reached a peak value within about 30 s, but the decrease in $^{45}Ca$ just after the peak was much slower than those seen in Figs. 1, 2 and 3A. Moreover, within about 3 min, while the cellular content was still very high, the net efflux of $^{45}Ca$ was reduced to almost zero. The peak value of cellular $^{45}Ca$ corresponded to an intracellular concentration of ionized calcium of about $10^{-4}$ mol per litre and the net efflux levelled

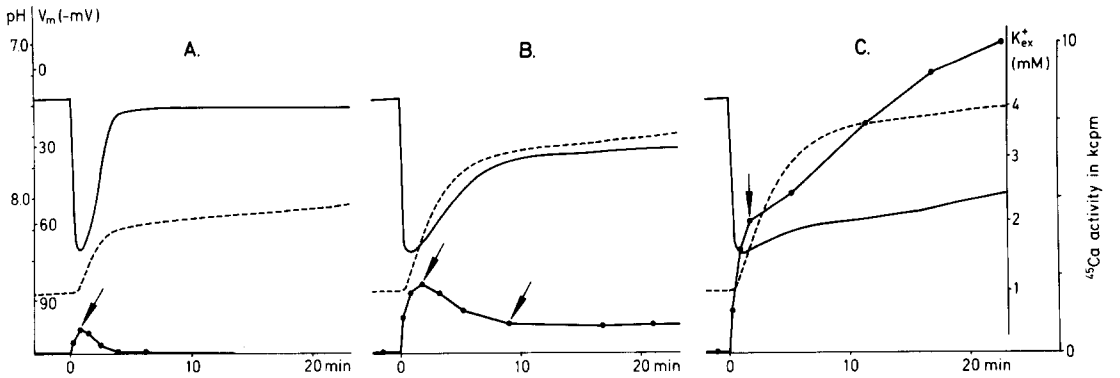


Fig. 3. Cellular $^{45}Ca$ content (●———●), extracellular pH ($V_m$) (———) and $K^+$ activity (- - - - -) in suspensions of glucose-fed cells (cytocrit 3.7%) as functions of time. The initial extracellular $Ca^{2+}$ activity was $7 \cdot 10^{-5}$ M in all three experiments. CCCP was added to a final concentration of 20 μM and at zero-time ionophore A23187 was added to final concentrations of (A) 2.5, (B) 4, and (C) 6 μmol per litre cells. The $K^+$ net efflux about 30 s after the addition of ionophore A23187 was in (A) about 1400 and in (B) and (C) about 1550 mmol per litre cells per h. The arrows indicate the $^{45}Ca$ content to which the following calculated cellular concentrations of ionized calcium per litre original cell water correspond: (A) $3 \cdot 10^{-5}$ M, (B) $10^{-4}$ M and $4 \cdot 10^{-5}$ M and (C) $1.8 \cdot 10^{-4}$ M. Coordinate axes as in Fig. 1.

off at about $4 \cdot 10^{-5}$ mol per litre original cell water. The substantial decrease in cell volume with time which takes place almost constantly in the experiments presented in Figs. 3B and 3C (see above) results in an appreciably higher $Ca^{2+}$ concentration when calculated per litre actual cell water, especially after 120 s.

The $Ca^{2+}$ influx in the experiment of Fig. 3B is close to the upper threshold, whereas in the experiment represented in Fig. 3C the net influx of $Ca^{2+}$ by far surpasses this threshold value. Comparing Figs. 3A and 3C it can be seen from both the $K^+$ and from the pH ($V_m$) records that at a membrane potential of −70 mV, absolute scale, and an intracellular ionized calcium concentration of about $10^{-4}$ M, only a partial inactivation of the $K^+$ channels takes place during the first minutes after the peak of hyperpolarization. Above $(1.5–2) \cdot 10^{-4}$ mol $Ca^{2+}$ per litre original cell water inactivation of $K^+$ channels does not take place in strongly hyperpolarized cell membranes.

## Discussion

### *Activation of the $K^+$ channels*

In the first analysis of the oscillations [3] it was assumed that an overshoot of the $Ca^{2+}$ pump was an essential event and that the intracellular concentration of calcium after each wave of hyperpolarization was brought back to the initial value or lower. With a certain delay this should result in a sharp decrease in the fraction of active $Ca^{2+}$-pump units which in turn should lead to a new wave of ionophore-mediated net influx of $Ca^{2+}$. As seen from Fig. 1, this assumption was not quite correct. During the minima of cellular $Ca^{2+}$ concentration (Fig. 1 and Table I) the $K^+$ channels remained closed for minutes at calculated cellular ionized calcium concentrations of 0.6 to 8 μM, and only opened again upon a new burst in $Ca^{2+}$ influx. In experiments at higher cytocrit (Fig. 2) the channels remained closed for up to 20 min while the calculated intracellular concentration of ionized calcium slowly increased from about 0.5 to 1 μM. It should be noted that these minima concentrations of intracellular free calcium are very high compared to the experimental initial values which should be close to that of physiological steady state. Recently, Lew et al. [15] by an elegant non-disruptive loading of intact red cells with a Ca-chelator, have shown that the physiological steady-state concentration of free calcium is as low as 10–30 nM.

As mentioned in the Results, the increases in intracellular $^{45}Ca$ and $K^+$ efflux (Fig. 2), just after the addition of ionophore A23187, take place so fast that the cellular concentration of ionized Ca at which half-maximal $K^+$ efflux was reached can

be only roughly estimated to be below 0.5 $\mu$M.

The relation between opening of closed $K^+$ channels and intracellular $Ca^{2+}$ concentration seen during the oscillations in Fig. 1, indicates that the channels during the second and subsequent waves might be opened by a sufficiently rapid increase in the existing level of cellular $Ca^{2+}$ activity, whereas the channels might accommodate, within certain limits, to a slow increase in ionized calcium. This indicates that the gating mechanism may involve a $(dCa^{2+}/dt)$-sensitive process.

Since the membrane potential decreased a few millivolts from minimum to minimum a possible voltage dependence of the gating processes (discussed in the next section) might explain the finding that, although the levels of intracellular calcium increased from minimum to minimum, the majority of the $K^+$ channels were still found to be inactivated. To explain this phenomenon only in term of a voltage-dependent gating of the $K^+$ channel requires that the voltage dependence in the range of $-10$ mV to $-25$ mV is very strong.

Studying the characteristics of the $Ca^{2+}$-sensitive $K^+$ channel in vesicles (inside-out and right-side-out types) prepared from human red cell membranes Lew et al. [16] and Garćia-Sancho et al. [17] recently demonstrated that the individual $Ca^{2+}$-sensitive $K^+$ channels responded in an all-or-nothing fashion to $Ca^{2+}$ activation. However, the threshold sensitivity of the channels of the various vesicles varied from 2 to 10 $\mu$M [16] and $10^{-7}$ to $10^{-4}$ M $Ca^{2+}$ ($Mg^{2+}$ concentration 0.5 mM) [17]. Among possible kinetic characteristics which could explain the graded vesicular responses, Lew et al. [16] mention the possibility of a $(dCa^{2+}/dt)$-sensitive gating mechanism.

*Inactivation of the $K^+$ channels*

The variations in cellular $^{45}Ca$ during oscillations demonstrate unambiguously that inactivation of the $K^+$ channels begins while the intracellular $Ca^{2+}$ concentration is still close to maximum. The closing process, therefore, is not the result of a $Ca^{2+}$-pump-mediated decrease in the intracellular concentration of free calcium to a magnitude below the $K_m$ value of the channels.

In search of a possible time-dependent inactivation of the $K^+$ channels, Brown and Lew [18] added ionophore A23187 in a concentration of 10 $\mu$M to human red cells suspended in $K^+$-equilibrium Ringer medium (75 mM KCl) containing about $3 \cdot 10^{-4}$ M $^{45}Ca$-labelled $Ca^{2+}$ and 10 mM inosine. The induced $K^+$ permeability which was calculated from the rate constants of $^{42}K$ equilibration 15, 30 and 45 min after addition of ionophore showed no significant variance, indicating a lack of spontaneous inactivation of the $K^+$ channels.

The discrepancy between the results of Brown and Lew [18] and those represented in Fig. 1, most probably can be explained by two marked differences in experimental conditions. First, in the present study, the cellular concentration of ionized calcium during the first wave of hyperpolarization (Fig. 1) only exceeded $5 \cdot 10^{-5}$ M for about 30 s, whereas in the experiments of Brown and Lew a cellular $Ca^{2+}$ content of about $3 \cdot 10^{-4}$ M was maintained during the entire experimental period [18]. As seen from Fig. 3, this value is in the range of intracellular $Ca^{2+}$ ($(1-2) \cdot 10^{-4}$ M ionized calcium) found when the $Ca^{2+}$ influx was close to the upper threshold value, above which no inactivation of the $K^+$ channels took place at a membrane potential of about $-65$ mV, absolute scale.

Secondly, in the experiments of Brown and Lew the membrane potential should be constant at about $-15$ mV so that a possible voltage dependence of the conductance of the $K^+$ channels would not be seen. Although voltage-dependent conductance has been reported for the $Ca^{2+}$-dependent $K^+$ channels of the central neurones of *Helix aspersa* [19] and *Aplysia* [20], it is not known whether the conductance of the $Ca^{2+}$-dependent $K^+$ channels of the human red cell membrane is voltage dependent.

The occurrence of the oscillations in $K^+$ conductance is, in addition to the spontaneous closing of the $K^+$ channels and the delayed activity of the $Ca^{2+}$ pump most probably based on a feed-back amplification of the ionophore-mediated $Ca^{2+}$ influx during the phase of hyperpolarization: the ionophore-mediated $Ca^{2+}$ influx results in an increase in $K^+$ conductance which leads to a hyperpolarization of the membrane and a CCCP-mediated alkalinization of the extracellular phase, resulting in an increase in the ionophore A23187-mediated $Ca^{2+}$ influx (see Ref. 3). Therefore, a simple

substitution of extracellular $Na^+$ for $K^+$ in varying degrees would not demonstrate a possible influence of the membrane potential of the $K^+$ conductance as a separate function.

Based on the fact that a transient depolarization could be induced in cells suspended in 156 mM KCl, it was previously concluded that it was unlikely that the closing process was voltage dependent. Because of the lack of feed-back amplification the levels of intracellular $Ca^{2+}$ concentration may, however, have been small compared to those found in transient hyperpolarizations. This problem will be further addressed.

*Role of the $Ca^{2+}$ pump*

Although a $Ca^{2+}$-pump-mediated decrease in the intracellular concentration of ionized calcium does not initiate inactivation of the $K^+$ channels, the active transport of calcium ions is crucial for the induction of oscillations. This was previously demonstrated by the fact that no oscillations could be induced in ATP-depleted cells [3]. Without an active transport the intracellular level of $Ca^{2+}$ would continue to increase and the inactivation of the $K^+$ channels would stop and most probably reverse (compare Fig. 3).

That the net-efflux of $Ca^{2+}$ accompanying the depolarization or the cell membrane has to be mediated by the $Ca^{2+}$ pump can also be seen by the line of arguments illustrated in Fig. 4. Here the changes in pH ($V_m$) and cellular $^{45}Ca$ content with time in the experiment presented in Fig. 2 are redrawn in a slightly simplified form. At, for example, $-45$ mV (absolute scale) on the ascending and descending legs of the curve of membrane potential we can roughly estimate the magnitude of the net in- and effluxes of $Ca^{2+}$, using the calculated cellular $Ca^{2+}$ concentrations. Besides identical proton gradients and CCCP distributions across the membranes [3], the concentration gradients of ionized calcium are not very different in these two situations. Therefore, the ionophore-mediated $Ca^{2+}$ influx should be almost the same at A and B (Fig. 4), even if the above discussed proton gradient facilitation of the ionophore A23187-mediated transmembranous calcium transport occurs. Assuming that the pump efflux at A is negligible the net-influx at this time is identical to the ionophore-mediated influx at A

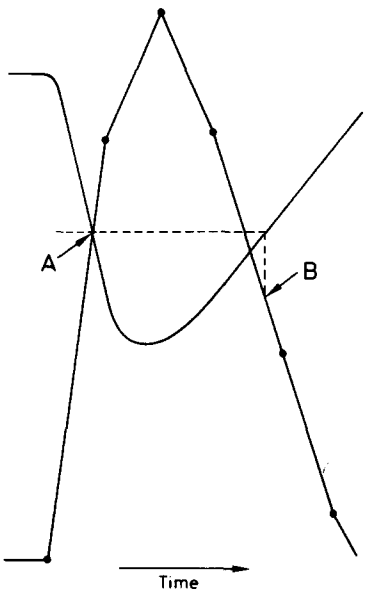


Fig. 4. Changes in $pH_{ex}$ ($V_m$) and cellular $^{45}Ca$ content with time. The figure is based on the experiment represented in Fig. 2 which has been redrawn in a slightly simplified form in order to illustrate the principles used in estimating the $Ca^{2+}$-pump net efflux at B. At the intersections A and B the membrane potential and accordingly the proton- and CCCP-gradients across the membranes are identical. Because of the decrease in cell volume during the wave of hyperpolarization the calculated cellular concentrations of total calcium are almost identical (approx. $3.5 \cdot 10^{-5}$ M). The pump flux is calculated from the equations: $J_C^A - J_p^A = J_{net}^A$ and $J_p^B - J_C^B = J_{net}^B$, where $J_{net}$ is the net flux and $J_C$ and $J_p$ are the ionophore-mediated influx and the pump-mediated efflux, respectively.

which according to the above argument is equal to the ionophore-mediated net influx at B. The pump-mediated efflux then equals the net efflux at B plus the ionophore-mediated net influx at A. Based on this assumption a rough calculation of the pump rate results in a maximum value of 13 mmol per litre cells per h. This rate of pumping is maintained for a period of 10–20 s only so that plentiful ATP should be present. Based on the maximum $Ca^{2+}$-ATPase activity observed in preparations of the enzyme from human red cell membranes the calculated maximum rate of the $Ca^{2+}$ pump of human red cells is about 20 mmol $Ca^{2+}$ per litre cells per h [21].

It should be noted that the assumption of a negligible pump efflux in A (Fig. 4) is permissible only if activation of the $Ca^{2+}$ pump in response to

sudden increases in the concentration of ionized calcium is delayed. The existence of delays in the activation or inactivation of the $Ca^{2+}$ pump by sudden changes in $Ca^{2+}$ activity was demonstrated by Scharff and Foder [4]. The magnitudes of these delays at physiological levels of calmodulin (cf. Table IV in Ref. 22) allow the assumption of a negligible (compared to the maximum value) pump rate in A (Fig. 4). Furthermore, the mere presence of an active $Ca^{2+}$ pump with ample ATP is most probably not sufficient to explain the characteristic peak values in intracellular $Ca^{2+}$ concentration seen in Figs. 1 and 2. If we assumed that the $Ca^{2+}$ pump was activated without a delay by an increase in cellular $Ca^{2+}$ concentration, the net influx and thereby the increase in cellular $Ca^{2+}$ concentration would be reduced almost immediately. This would result in a diminished activation of $K^+$ channels and thus in the degree of hyperpolarization. This, however, would reduce the feed-back amplification of the ionophore-mediated calcium influx. In a kinetic analysis of the time-dependent changes in cellular $Ca^{2+}$ concentration in erythrocytes exposed to ionophore A23187, Scharff et al. [23,24] have shown that no peak occurs in this concentration unless the pump is delayed in its response to increases in $Ca^{2+}$ concentration.

Probably, the oscillations presented in Figs. 1 and 2 are induced by a calcium net influx just large enough to ensure an increase in $K^+$ conductance of magnitude sufficient for a feed-back amplification of the original influx to occur. If this results in a cellular $Ca^{2+}$ concentration of about $5 \cdot 10^{-5}$ M at the maximum of hyperpolarization (about $-75$ mV, absolute scale), then at this point an incipient inactivation of the $K^+$ channels closely followed by a maximum in $Ca^{2+}$ pumping will reverse the sequence of events.

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