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The Ca^{2+}-sensitive K^+-conductance of the human red cell membrane is strongly dependent on cellular pH

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The conductance of the Ca^{2+}-sensitive K^+-channels in human red cell membranes has been determined as a function of the intracellular pH. A sudden increase in the intracellular concentration of ionized calcium was established by addition of ionophore A23187 to a suspension of cells in buffer-free, Ca^{2+}-containing salt solution. At the various cellular pH-values cellular concentrations of ionized Ca, saturating with respect to activation of the Ca^{2+}-sensitive K^+-conductance, were obtained by the use of varied concentrations of extracellular Ca^{2+} and added ionophore A23187. Changes in membrane potential was monitored as CCCP-mediated changes in extracellular pH. Initial net effluxes of K^+, cellular K^+ contents and the K^+ Nernst equilibrium potentials were calculated from flame photometric measurements. Cellular Ca-contents were determined by aid of ^{45}Ca. With cellular Ca^{2+} at the saturating level with respect to activation of the K^+-channel the K^+-conductance calculated from these data was independent of extracellular pH and a steep function of cellular pH with a half maximal conductance of 31 $\mu S/cm^2$ at a cellular pH of 6.1. The K^+-conductance is not a simple function of cellular pH (pH_c). From $pH_c = 6.5$ and down to $pH_c = 6.0$ a Hill-coefficient of 2.5 was found, indicating cooperativity between at least two sites regulating the conductance. Below $pH_c = 6.0$ an extremely high Hill-coefficient of 11 was found, probably indicating that the additional titration of the channel protein leads to an increased cooperativity. The importance, as a physiological regulatory mechanism, of a K^+-conductance increasing from zero to maximal conductance within less than one unit of pH, is discussed.

Introduction

The conductance of the Ca^{2+}-sensitive K^+-channel of the human red cell membranes, first observed by Gárdos [1], has during the recent years been extensively characterized with respect to the sensitivity towards intracellular concentrations of ionized Ca (cf. Refs. 2, 3), extracellular concentrations of K^+ (cf. Refs. 4, 5); intracellular concentration of Na^+ (cf. Ref. 4) etc.

At the same time, a possible dependence of the Ca^{2+}-regulated K^+-conductance on pH, intra- as well as extra-cellularly, has only been briefly commented on in a couple of papers [6,7], in which the combined effects of variations in extracellular pH and concentration of K^+ was the central issue. Since competition between Ca^{2+} and H^+ in the intracellular phase with respect to binding to the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Mes, 2-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Trizma base, tris(hydroxymethyl)-aminoethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Ca^{2+}-sensitive site of the conductance pathway might well be of importance, we address in the present paper the pH-dependence of the Ca^{2+}-sensitive K^+-conductance of the human red cell membrane, and especially the dependence on the intracellular pH. The K^+-conductance was determined at a fixed initial extracellular K^+ concentration of 1 mM and an intracellular concentration of ionized Ca, which was found to be saturating with respect to the activation of the K^+-conductance. Preliminary accounts of this work (the results were obtained with a slightly different technique) have been presented in abstract form [8].

Materials and Methods

All inorganic salts (pro analysis) were purchased from Merck; Hepps (EPPS), Mes, Mops, Trizma base and CCCP were from Sigma. The ionophore A23187 was from Calbiochem; DIDS was from Pierce Chemical Co., di-*n*-butylphthalate and sucrose (Aristar) were from BDH. The test-combination for determination of hemoglobin was from Boehringer.

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/150 μM $MgCl_2$/50 μM EGTA (pH approx. 7.4)). The cells were depleted of ATP and 2,3-diphosphoglycerate as previously described [9], washed three times in high-K salt solution and stored on ice as a suspension (hematocrit approx. 15%).

Cellular contents of ^{45}Ca, K^+, and hemoglobin and extracellular concentrations of K^+ were determined by the phthalate-method of Lew and Brown [10] modified in order to determine cellular and extracellular concentrations of K^+. In centrifuge tubes 875 μl of a solution (155 mM Trizma acetate/2 mM EGTA/3.3 mM $LiNO_3$ (pH 7.7)) was layered on top of 400 μl di-*n*-butylphthalate (density 1.042–1.045 at 20°C) and the tubes were stored on ice.

During an experiment 100 μl samples of the cells suspension (hematocrit $\simeq$ 3.1%) were transferred to the cold phthalate-tubes, and 5 s later the tubes were centrifuged for 30 s at 18000 $\times$ *g*. The 3.1 μl of cells were thus isolated as a pellet under the phthalate layer. Extracellular concentrations of K^+ were determined by flame photometry directly on the diluted top-phases of the phthalate tubes. The rest of the top-phase and the phthalate were removed and the cell-pellets were processed for scintillation counting of the ^{45}Ca content according to Ref. 10. In addition the pellet-contents of hemoglobin and K^+ were determined.

The determination of K^+ was performed on a flame photometer (Radiometer, FLM 3) based on an internal Li-standard. The initial concentrations of ionized Ca in the various extracellular phases were controlled with a Ca^{2+}-selective electrode (Selectrode F1221 Ca, Radiometer).

In each experiment cells were suspended in a buffer-free salt solution at a hematocrit of 3.1% in the presence of 20 μM CCCP. Using the method of Macey et al. [11], the membrane potential of the suspended cells was continuously monitored by recording of the extracellular pH (see also 'Experimental procedure; and Fig. 2).

In each experimental series the cellular content of water was determined on samples of packed cells from the stock suspension of cells stored on ice.

Experimental procedure

From the stock suspension of cells a sample was taken and the cells were spun down and washed once in the incubation-solution (155 mM NaCl/1 mM KCl/150 μM $MgCl_2$/10 μM EGTA). 100 μl of packed cells were transferred to 3000 μl of this solution, thermostatted at 37°C. Applying a 1 ml syringe mounted on a step-motor, the cell suspension was under vigorous stirring titrated slowly to the desired pH-value with 100 mM HCl/56 mM NaCl. Chemical equilibration of protons across the cell membranes took place rapidly, mediated by the anion-exchange system. During the titration 2 M sucrose solution was added at intervals in order to maintain the original cellular volume. The amounts of 2 M sucrose solution which had to be added at given pH-values were calculated from Fig. 1A, which represents the results from a series of control-experiments. In these, cell volume and resting membrane potential were determined as functions of pH_c at conditions otherwise identical to those of the flux-experiments.

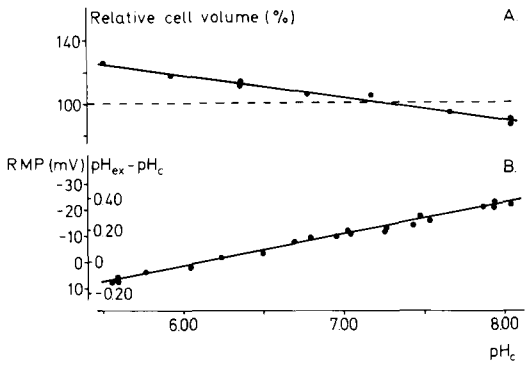

Fig. 1. Curve A shows the relative cell volume as a function of cellular pH (pH_c). The cell volume was calculated from measurement of the concentration of hemoglobin in samples of packed cells. Variations in trapped extracellular volume were corrected for by means of ^{22}Na. Curve B shows the resting membrane potential (RMP) as a function of pH_c. For details regarding measurements of the membrane potentials see text.

$Ca/^{45}Ca$, as $CaCl_2$, was added to the calculated extracellular concentration (see later) and CCCP was added to a final concentration of 20 μM. Samples were taken for determination of total hemoglobin and ^{45}Ca, initial contents of cellular ^{45}Ca and K^+, and concentration of extracellular K^+. The initial cellular content of ^{45}Ca (no ionophore A23187 had been added yet) was taken to represent trapped extracellular and cell-coat bound ^{45}Ca (see Refs. 10 and 12).

At zero time ionophore A23187 was added in the amount calculated (see later), and during the following ~ 90 seconds 100-μl samples of suspension were transferred to cold-stored phthalate tubes (see above) at intervals of about 7 s, determining in this way the net influx of $Ca/^{45}Ca$ into the cells and net-efflux of K^+ from the cells. Extracellular pH was recorded, thus monitoring the hyperpolarization of the cells (see Fig. 2). A sample of suspension was taken for determination of hemolysis, and at the end of the experiment saponin was added and pH decreased within a few seconds to the value of the hemolysate. Since all buffering capacity was confined to the cellular phase the pH of the hemolysate reflected the original cellular pH value, a value which during the experiment remained constant. The absolute value of the membrane potential during the experiment was calculated from the difference between the peak-value of the extracelluar pH, and the cellular pH-value [9,11], assuming that a ΔpH of 1.0 unit was equal to a membrane potential of -61 mV.

In the calculation of the intracellular concentrations of ionized Ca from the determined ^{45}Ca-contents of cell pellets the previously found value of 0.19 for α [13] was used; α being the ratio between the concentration of ionized Ca per liter cell water and total Ca per liter cells [14]. The same value of α was used at all pH-values although, as found by Simons [2] and Foder et al. [15], α will increase with decreasing cellular pH. Thus the cellular concentrations of ionized Ca at the lower pH_c-values were somewhat underestimated, but the crucial point was to achieve sufficiently high values (see later).

Results and Discussion

In Fig. 2 are shown the results of a typical experiment in which the net efflux of K^+ and the corresponding hyperpolarization of the cell membranes were determined at a given cellular pH. As seen from the figure, the addition of the ionophore within a few seconds resulted in a sharp increase in cellular Ca^{2+} and a constant increase in extracellular K^+-concentration. Simultaneously the extracellular pH increased sharply as protons (mediated by CCCP) approached a new electrochemical equilibrium distribution across the membrane. A peak value in pH_{ex} was reached within about 30 seconds, after which pH_{ex}, and accordingly the membrane potential, stayed relatively constant for some minutes. It should be noted, that although the extracellular pH increased about 0.8 units the increase in the extracellular K^+-concentration was constant during the experimental period.

As mentioned, the extracellular concentration of ionized Ca applied and the amount of ionophore A23187 added were calculated for each experiment. We have recently shown [13] that the A23187-mediated net influx of Ca^{2+} into human red cells is strongly accelerated by a *trans* to *cis* proton gradient, that is by a positive value of the difference ($pH_{ex} - pH_c$). Therefore, the sharp increase in extracellular pH seen in Fig. 2 results in an acceleration of the ionophore-mediated Ca^{2+} net influx compared to the initial influx.

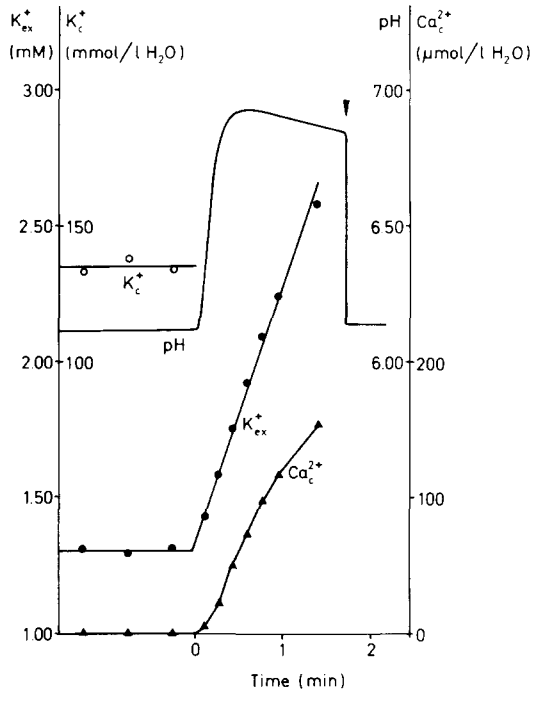

Fig. 2. Results of a typical experiment. Membrane potential (V_m) was calculated from the continuously recorded CCCP-mediated variation in extracellular pH (pH_{ex}) and pH of the hemolysate (see text). The K^+-net efflux from the cells was calculated from the slope of the curve showing concentrations of extracellular potassium (K^+_{ex}) as a function of time. K^+ Nernst equilibrium potential (E_K) was calculated from the measured initial cellular K^+-concentration (K^+_c) and K^+_{ex} at the peak value of V_m. During the ~ 90 s of K^+ net efflux the cells loose isotonic KCl and the intracellular concentration of K^+ changes negligibly (not shown in the figure). ^{45}Ca-measurements allow for calculation of cellular concentration of ionized calcium (Ca^{2+}_c) (the value at the peak of membrane potential in this experiment was 96 μM). The abscissa is time in minutes, the ordinates are concentrations of K^+_c and K^+_{ex} in mmoles per liter cellular and extracellular waterphase, respectively, and cellular concentration of ionized Ca in μmoles per liter cell water and pH. CCCP was added to a total concentration of 20 μM. The ionophore A23187 was added at zero time. At the time indicated by the arrow the cells were hemolysed by addition of saponin, determining in this way the cellular pH.

In agreement with the results of Funder and Wieth [16], we found that at lower pH-values ($pH_c < 6.10$–6.15) the initial difference ($pH_{ex} - pH_c$) was negative (see Fig. 1B). To obtain a sufficiently high ionophore-mediated net influx of Ca under these conditions we had to take into account the decreased efficiency of A23187 at for example pH 5.8 as compared to pH 6.5 (cf. Fig. 4 in Ref. 13), and that at $pH_c = 5.8$ the initial difference ($pH_{ex} - pH_c$) was of the same size as at $pH_c = 6.5$, but negative (see Fig. 1). At $pH_c = 5.8$ no change in membrane potential and accordingly in the difference ($pH_{ex} - pH_c$) took place, while at $pH_c = 6.5$ the cell membranes hyperpolarized about 60 mV, resulting in an increase in ($pH_{ex} - pH_c$) of about one unit. Accordingly, the A23187 mediated net influx at $pH_c = 5.8$ was decreased by a time-invariant proton concentration gradient in the same direction as the calcium concentration gradient, whereas at $pH_c = 6.5$ an opposite directed increasing proton concentration gradient accellerated the ionophore mediated calcium net influx (compare Fig. 5 in Ref. 13). With the strong pH-dependence of the Ca-induced hyperpolarization it was chosen to aim at reasonably equal cellular concentrations of ionized Ca within the saturating level with respect to the Ca^+-activation of the K^+-conductance. Apparent K_m-values of Ca^+-sensitive K^+-conductance of the human red cell membrane of 1 μM [2] and of 2–3 μM [3] of ionized Ca have been reported. Accordingly, we aimed at intracellular concentrations of ionized Ca of about 50 μM at the peak of hyperpolarization. The corresponding extracellular Ca^{2+}-concentration and ionophore concentration per liter of cells appropriate at each pH_c-value were estimated from Figs. 4 and 8 in Ref. 13.

In the experiments represented in Figs. 3–6 the cellular concentration of ionized calcium at the peak of hyperpolarization (at about $t = 30$ s) were within the concentration range of 20–200 μM. It should be noted, however, that during the remaining experimental period these concentrations were still increasing substantially. Control experiments showed that the initial conductances of the K^+-channel were found to be identical whether the peak-value mentioned above was 20 or 200 μM of ionized calcium. Thus, neither a possible inhibition at the higher values nor sub-maximal activation at the lower values could be detected during the 90 s experimental period.

In Figs. 3 and 4 the net effluxes of K^+ and the corresponding membrane potentials are shown as functions of the cellular pH. Both the net effluxes of K^+ and the corresponding membrane potentials varied from donor to donor. But since higher net

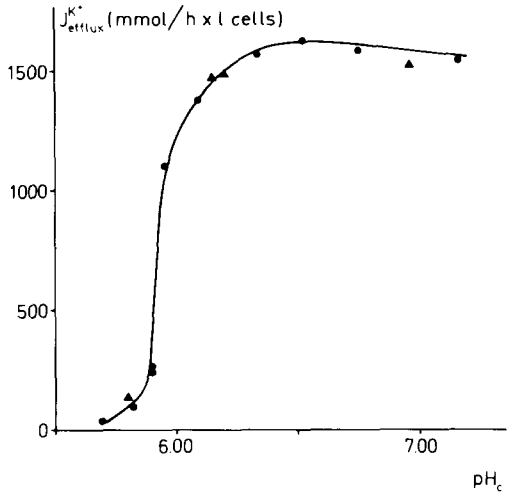

Fig. 3. The K^+ net effluxes, J^K_{efflux} versus cellular pH (pH_c). ●——●, represents fluxes determined as described in 'Experimental procedure' and Fig. 2, ▲, represents fluxes from cells where Cl^- was replaced by NO_3^- and the anion-exchange mechanism was inhibited with DIDS. These fluxes were because of the higher anionconductance ($g_{NO_3} > g_{Cl}$) found to be 1.47-times the corresponding fluxes in chloride cells and has therefore been divided by this factor. The abscissa is pH_c and the ordinate is K^+ net efflux in mmol/l cells per h.

effluxes were always found together with a lower degree of hyperpolarization, that is an increased driving force ($V_m - E_K$), these variations most

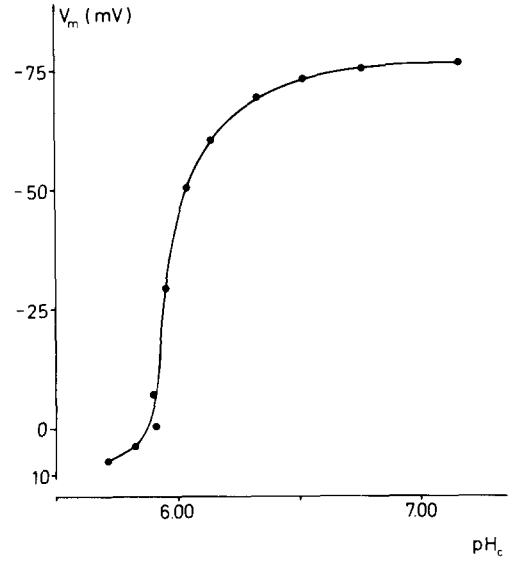

Fig. 4. The peak-membrane potentials (V_m) corresponding to the K^+ net effluxes in Fig. 3, as function of cellular pH (pH_c).

probably originated in variance in Cl^--conductance between 20 and 30 $\mu S/cm^2$ [17], and not in K^+-conductance, among the donors.

Monitoring the hyperpolarization of the cell membranes by a CCCP-mediated increase in the extracellular pH presents an additional problem. The net efflux of K^+ as a function of cellular pH was determined at extracellular pH-values which, as discussed above, at $pH_c > 6.0$ were substantially higher than the cellular pH-values. The fact that the net efflux of K^+ from the cells was found to be constant during the increase in extracellular pH (see Fig. 2 and Ref. 12) indicates that the dependence on extracellular pH is minimal or absent. All the same, the possible influence of variation in the extracellular pH on the net efflux of K^+ from cells at a given fixed value of cellular pH was investigated in a separate series of experiments.

In these experiments all Cl^- in the ATP depleted cells was substituted for by NO_3^-. After titration to the desired cellular pH, DIDS was added to the suspension to a final concentration of 50 μM, and the suspension was left for 10 min at 37°C in order to achieve a sufficiently high degree of inhibition of the anion exchange system (cf. Ref. 18). The suspension of cells was centrifuged and a 100-μl sample of packed cells transferred to 3000 μl salt solution containing 2 mmol/l of Hepps, Mes or Mops adjusted to the value of pH_{ex} chosen. Ca was added to the final concentration calculated, while CCCP was omitted, and at zero time the calculated amount of A23187 was added.

The inhibition of the anion-exchange system with DIDS and the absence of CCCP made it possible to maintain pH-differences across the membranes as high as 2 units for several minutes. On the other hand anion-conductance is also substantially inhibited by DIDS [19], but the substitution of NO_3^- for Cl^- resulted in an anionconductance sufficiently high to ensure a reasonably high net efflux of K^+. Since CCCP was omitted no determination of membrane potentials was possible in these experiments. The cellular pH at the end of these experiments was determined by hemolysis of cells, which had been spearated from the buffered extracellular phase by centrifugation and resuspended in 156 mM NaCl. The changes in cellular pH were less than 0.02.

At $pH_c = 5.80$ identical net effluxes of K^+ were

found at extracellular pH-values as different as 6.3, 6.9 and 7.9 (Fig. 5A). At $pH_c = 5.68$, where in accordance with the experimental results shown in Fig. 3 the net efflux was zero, an increase in the extracellular pH to 6.1 and 6.8, respectively, did not induce any net efflux of K^+ from the cells (Fig. 5B).

We therefore conclude that within the range of pH in question the net efflux of K^+ is the same whether the extracellular pH is identical with or higher than pH_c. Accordingly, the net effluxes in Fig. 3 and the corresponding membrane potentials in Fig. 4 are functions of the cellular pH, as indicated on the abscissa, and there is no interference from the varying CCCP-mediated increases in the extracellular pH during the efflux-period.

A complex effect of variation of pH on K^+ efflux from energy-depleted human red cells suspended at different values of extracellular K^+-concentration has been reported by Hoffman et al. [6] and has later been confirmed by Freedman and Novak [7]. At an extracellular concentration of K^+ of 1 mM Hoffman et al. find that the K^+ efflux is about three times lower at pH 6.15 than at pH 7.15. Since the cells were not treated with DIDS the effect seen probably reflects a corresponding difference in cellular pH. There is therefore no disagreement between their result and those presented in Fig. 3.

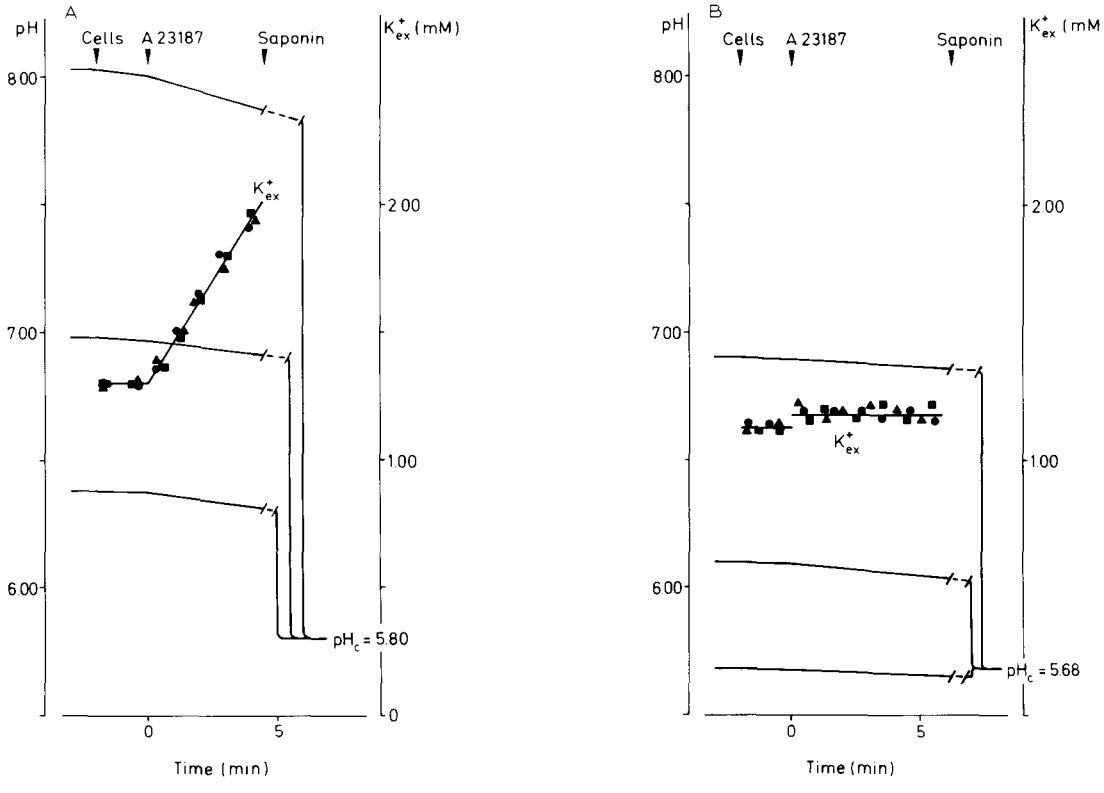

Fig. 5. K^+ net efflux from cells with a fixed cellular pH of 5.80 (A) and 5.68 (B) at different extracellular pH-values. All cellular Cl^- was replaced by NO_3^- and DIDS was added (50 μM) in order to achieve inhibition of the anion-exchange mechanism. No CCCP was present. A23187 was added at zero time. The last arrow indicates addition of saponin to the cells, which were first separated from the buffered extracellular phase by centrifugation and then resuspended in a buffer-free medium in order to measure cellular pH (dotted section of the pH-curves). Panel A shows three experiments with $pH_c = 5.80$ and the extracellular phase buffered at pH = (7.9, 6.9 and 6.3). In these experiments the K^+-net efflux was in average 307 mmol/h per liter cells. Panel B shows three experiments with $pH_c = 5.68$ and the extracellular phase buffered at pH = (5.7, 6.1 and 6.8). In all experiments K^+-net efflux was zero. The abscissa is time in minutes, the ordinates pH and extracellular concentration of K^+ in mM.

In a recent abstract Heinz and Hoffman [20] report that in energy-depleted. Ca-loaded human red cells treated with DIDS, the ^{42}K single fluxes at $pH_c \sim 7.1-7.3$ are about 20-times lower at $pH_{ex} = 5$ than at $pH_{ex} = 7.0$. They conclude that the major effect may be the result of a proton-potassium competition for transport on the Ca-sensitive conductance pathway. In the experiments of Heinz and Hoffman [20] the differences $(pH_{ex} - pH_c)$ are negative, whereas those shown in Figs. 5A and 5B are positive. Their results are therefore not a variance with our conclusion, that in the present context variation in extracellular pH is insignificant.

Since the net efflux of K^+ at a given pH_c is independent of the CCCP-mediated increases in pH_{ex}, we can calculate the conductance of K^+ (g_K), using the equation: $g_K = FJ_K/(V_m - E_K)$ [21]. Here F is Faradays number, and E_K is the Nernst equilibrium potential of K^+ across the membranes. E_k was calculated from the intra- and extracellular concentrations of K^+ determined at the peak of hyperpolarization. Since the original cell volume was maintained by addition of sucrose a surface area of $1.75 \cdot 10^7$ cm^2 per liter packed cells was used throughout. The conductance, in $\mu S/cm^2$, as a function of the cellular pH is shown in Fig. 6.

As seen from the figure the conductance is a very steep function of the cellular pH with a half maximal value at $pH_c \simeq 6.1$. As mentioned this is the value found at a cellular concentration of ionized Ca, which is saturating with respect to Ca^{2+}-activation of the K^+-conductance. Preliminary experiments have indicated, however, that a displacement of the curve to the right takes place at lower intracellular concentrations of ionized Ca. With the present technique a sufficient control of the intracellular Ca at low levels of Ca unfortunately is very difficult.

In Fig. 7 is shown a Hill-plot of the conductance as a function of intracellular pH (same data at Figs. 3, 4 and 6). From $pH_c = 6.5$ and down to $pH_c = 6.0$ a Hill-coefficient of 2.5 was found indicating a cooperative effect between at least two sites regulating the conductance. That the Ca^{2+}-sensitive K^+-channel is activated by reaction with two Ca-ions has been suggested by Grygorczyk and Schwarz [22]. In a patch-clamp study of the

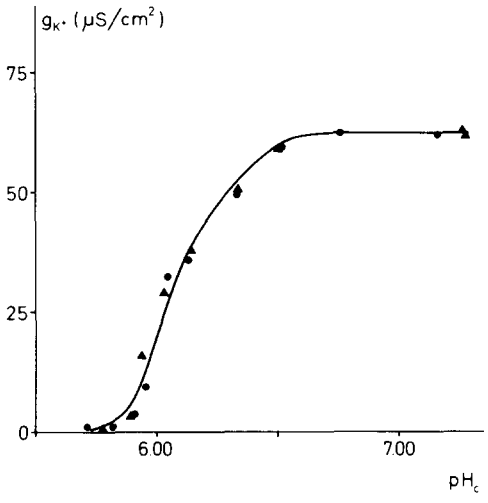

Fig. 6. The K^+-conductance (g_K) as a function of cellular pH (pH_c). The conductance was calculated from the K^+ net effluxes (J_K, Fig. 3) and the corresponding peak values of membrane potentials (V_m, Fig. 4) according to $I_K = J_K F = g_K(V_m - E_K)$; $F = 96500$ C/mol. (▲) represents the results from a parallel series of experiments. The abscissa is pH_c and the ordinate is g_K in $\mu S/cm^2$. The assumed area of 1 liter packed cells was $1.75 \cdot 10^7$ cm^2. The K^+-Nernst equilibrium potential (E_K) was calculated as explained in the text and legend to Fig. 2.

human red cell membrane they find that the dependence of channel activation on concentration of ionized calcium is compatible with the concept

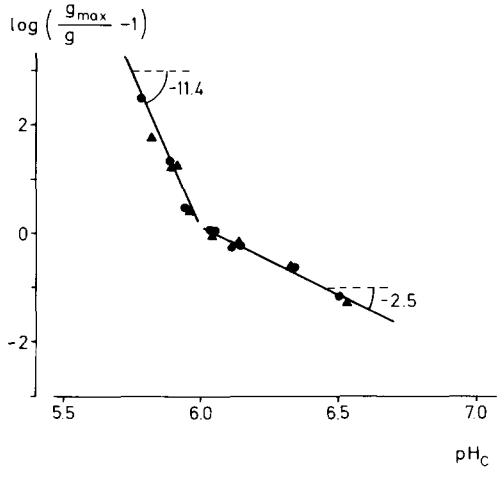

Fig. 7. A Hill-plot of the data presented in Fig. 6. The curve was drawn according to the best linear regressions to the values above and below $pH_c = 6.0$.

that the binding of two Ca-ions is necesary to open a single channel. In experiments in which a single Ca^{2+}-activated K^+-channel from rat muscle membranes was incorporated into a planar lipid bilayer, Moczydlowski and Latorre [23] find that activation of the conductance pathway most probably takes place by interaction of the channel with two Ca-ions. It is remarkable, that the conductance of the K^+-channel (delayed rectifier) of squid axon membrane [24] and the inward rectifier of starfish oocytes [25] have been shown likewise to be steep functions of the intracellular pH. Both of these K^+-channels are voltage (but not Ca^{2+}) activated. It is concluded that most probably two [24], respectively, three groups [25] are titrated resulting in a decrease in conductance without significantly affecting the kinetic parameters.

From $pH_c = 6.0$ and downwards the extremely high Hill-coefficient of about 11 most probably reflects an additional effect of increasing the proton concentration. One possibility would be that below $pH_c = 6.0$ we begin to titrate carboxylic acid groups of importance for the quaternary structure of the channel protein, for example resulting in an increasing cooperativity in a possible polymeric configuration of the channel.

If as mentioned above, half maximal Ca^{2+}-sensitive K^+-conductance at physiological concentrations of intracellular ionized Ca is found at a higher pH_c-value, then the pH_c-dependence of K^+-conductance might be of physiological importance. Recently, Pace [26] and Cook et al. [27] have shown that in pancreatic β-cells the Ca^{2+}-sensitive K^+-conductance decreases with a lowering of the intracellular pH, and they suggest that in this way metabollically induced decreases in intracellular pH may be important in modulating the dynamic pacemaker current assumed to regulate the production of insulin.

Another cell-type where the pH_c-dependence of the Ca^{2+}-sensitive K^+-conductance might be of importance is the skeletal muscle cell, where during anaerobic, glycolytic phases substantial acidification of the intracellular milieu takes place [28,29].

Acknowledgements

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