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A CALMODULIN ACTIVATED Ca^{2+}-DEPENDENT K^+ CHANNEL IN HUMAN ERYTHROCYTE MEMBRANE INSIDE-OUT VESICLES

LEON PAPE † and BERIT I. KRISTENSEN

Zoophysiological Laboratory B, August Krogh Institute, 13, Universitetsparken, DK-2100 Copenhagen Ø (Denmark)

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The role of calmodulin in stimulating active calcium transport in the human red cell membrane is well documented. In contrast, efforts to characterize the effect of calmodulin on the Ca^{2+}-dependent K^+ channel in erythrocyte membranes have given rise to conflicting reports. These studies have indicated that experimental conditions may play a critical role in preserving the Ca^{2+}-dependent K^+ channels in erythrocyte inside-out vesicles. With these observations in mind, a double-labelling study of simultaneous active Ca^{2+} and passive Rb^+ uptake in red-cell inside-out vesicles was undertaken. Addition of calmodulin and ATP to a suspension of inside-out vesicles containing 1 mM K^+ caused a Ca^{2+}-dependent increase in both the rate of active calcium transport and Rb^+ uptake. The initial Rb^+ isotope flux was increased 3-fold over the rate observed in the absence of calmodulin. The $k_{1/2}$ for activation of K^+ permeability was approx. $5 \cdot 10^{-7}$ M Ca^{2+} as compared to 10^{-6} M Ca^{2+} for active Ca^{2+} transport. Addition of the calmodulin antagonists pimozide and chlorpromazine blocked calmodulin activation of the Ca^{2+}-dependent K^+ channel. The observation that activation of the K^+ channel occurs at Ca^{2+} concentrations which are lower than those required for maximum stimulation of the calcium pump suggests that these processes are dependent on two states of the calmodulin molecule, characterized by a lower or higher amount of Ca^{2+} bound to calmodulin.

Introduction

For some time, our interest has been directed towards understanding the process whereby an increase in intracellular calcium causes the red-cell membrane to become highly permeable to potassium. The phenomenon, first described by Gárdos in 1958 in experiments with metabolically depleted red cells [1], has been extensively investigated since then. Red cells are normally very impermeable to calcium and contain a highly effective active calcium transport system. Therefore, calcium-dependent potassium permeability changes have primarily been studied using ATP-depleted cells or red-cell ghosts. An alternative method has been the use of the divalent cation ionophore A23187.

Using microelectrodes to measure single-cell membrane potentials in the giant red cells of *Amphiuma means* [2] and potential-sensitive fluorescent probes in human red cells [3], it was possible to demonstrate a calcium-dependent increase in potassium permeability and hyperpolarization of the cell membrane when these otherwise unperturbed red cells were exposed to increased extracellular calcium concentrations. Based on these observations together with an analysis of the experimental results accumulated in recent years, particularly with regard to calmodulin's calcium-dependent role in activating active calcium transport, Lassen et al. [4] proposed a model for a calmodulin-mediated calcium-dependent change in red-cell K^+ permeability. According to the model,

† Deceased January 2nd, 1984.

the red-cell membrane can exist in three calmodulin-related states depending on the level of intracellular free calcium concentrations. At normal low levels, calmodulin is inactive. An increase in intracellular calcium and consequent binding of Ca^{2+} to calmodulin results in an activated calmodulin which then binds to the membrane, shifting it to a state characterized by an increase in potassium permeability (opening of the Ca^{2+}-dependent K^+ channels) and hyperpolarization of the membrane. The hyperpolarization causes a further increase in intracellular calcium and increased Ca^{2+}-calmodulin binding which causes K^+ channels to close and stimulates maximum Ca^{2+}-pumping. This sequence, implying that the Ca^{2+}-dependent K^+ channels undergo spontaneous closing before the activated Ca^{2+} pump reduces the intracellular Ca^{2+} concentration to its initial low level, was suggested in earlier studies [5] and confirmed in a recent study by Vestergaard-Bogind [6].

While much experimental evidence has accumulated verifying the role of Ca^{2+}-activated calmodulin in stimulating Ca^{2+}-ATPase and active translocation of calcium, there is as yet no direct evidence that this system is also involved in calcium-dependent changes in erythrocyte membrane potassium permeability as proposed in the model described above. Sanchez et al. [7] and Szebeni [8] observed that chlorpromazine, a calmodulin-blocking phenothiazine, inhibited calcium-activated K^+ conductance. Recently Lackington and Orrego [9] reported that a number of drugs known to inhibit Ca^{2+}-calmodulin activation of human erythrocyte Ca^{2+}-ATPase also inhibit Ca^{2+} activated K^+ conductance.

The present studies were undertaken in an effort to test more directly the possibility that calcium-activated calmodulin is involved in opening of calcium-dependent K^+ channels in the human erythrocyte membrane.

Materials and Methods

Preparation of inside-out vesicles

Inside-out vesicles were prepared from freshly-drawn human blood according to the method described by Steck and Kant [11], with the exception that final separation was carried out without the use of a dextran gradient. Final vesiculation was performed in a 1 mM K_2SO_4 buffer in order to load the vesicles with 1 mM K^+. The sidedness and ratio of inside-out vesicles to right-side-out vesicles were determined by measuring the acetylcholinesterase activity in the presence and absence of the detergent Triton X-100. The percentage of inside-out vesicles varied from 55–80% based on the accessibility of cholinesterase. Membrane protein was determined by the method of Lowry et al. [12].

Calmodulin

Calmodulin was purified from outdated human erythrocytes as described by Jarrett and Penniston [13] and stored as lyophilized powder at −24°C.

Ca^{2+} and Rb^+ flux determination

The experiments were carried out at 37°C. The sample of inside-out vesicles was preincubated at 37°C for 5 min in washing buffer, comprising 0.5 mM EGTA/5 mM KCl/2 mM NaCl/2 mM $MgCl_2$/2 mM Tris-HCl (pH 7.4). After centrifugation, the supernatant was discarded and 500 μl of prewarmed flux medium containing ^{45}Ca and ^{86}Rb was immediately added to the pellet of inside-out vesicles (0.24 mg inside-out vesicle protein/ml suspension). 75 μl samples of the mixed suspension were withdrawn and filtered after 15 s, 30 s, 90 s, 3 min and 5 min of incubation. The samples were filtered under vacuum using Millipore filters of 1 cm diameter (HA, pore size 0.45 μm; Millipore Corp., Bedford, MA), which had been prewashed with washing buffer. The inside-out vesicles-containing filters were immediately washed with 3 × 500 μl ice-cold washing buffer. Besides the isotopes, the basic flux medium contained 0.5 mM EGTA, 5 mM KCl, 2 mM NaCl, 2 mM $MgCl_2$, 2.25 mM ATP, 0.1 mM ouabain, 5 μg/ml calmodulin (or no calmodulin), 2 mM Tris-HCl (pH 7.4) and different levels of $CaCl_2$ [14]. The calcium activity of each solution was checked using a calcium-sensitive electrode.

Radioactivity

The washed filters were transferred to minivials containing 2.5 ml of Filter-count™ Packard scintillation fluid. After the filters were dissolved the samples were counted in a Liquid Scintillation Spectrometer (Packard model 3330). A sample of

the remaining vesicle suspension was assayed for specific activity of the isotopes. ^{45}Ca and ^{86}Rb were purchased from RISØ, Research facility, Roskilde, Denmark. The specific activities were 10 – 14 mCi/mg Ca and 1.5 – 4 mCi/mg Rb.

Results

As an initial phase in the investigation of the calcium-dependent K^+ channel in erythrocyte membrane inside-out vesicles, the vesicles were examined for their capacity to translocate calcium under activation of the membrane Ca^{2+}-pump by calmodulin. The results of this study are shown in Fig. 1, and demonstrate that there is a time-dependent accumulation of ^{45}Ca which is a function of the free calcium ion concentration in the suspension medium and requires the presence of calmodulin. Calculated initial flux rates for active calcium transport as a function of pCa are shown in Fig. 2 and indicate a maximum rate at about 10^{-4} M Ca^{2+}. At higher free calcium concentrations the rate decreases sharply. $k_{1/2}$ for the calculated Ca^{2+}-flux rates is close to 1 μM Ca^{2+}. This is in agreement with the value for activation of red-cell membrane Ca^{2+}-ATPase reported by Scharff and Foder [15].

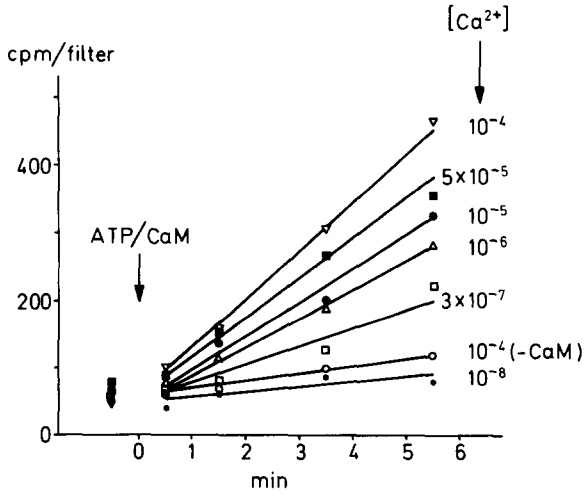

Fig. 1. Active ^{45}Ca uptake by erythrocyte inside-out vesicles as a function of time. At $t = 0$, ATP and calmodulin (CaM) were added to the inside-out vesicles flux suspension. See text for experimental details. Ca^{2+} concentrations are indicated at the end of each uptake curve. Abscissa, time in min; ordinate, ^{45}Ca activity in cpm/10 μg inside-out vesicle.

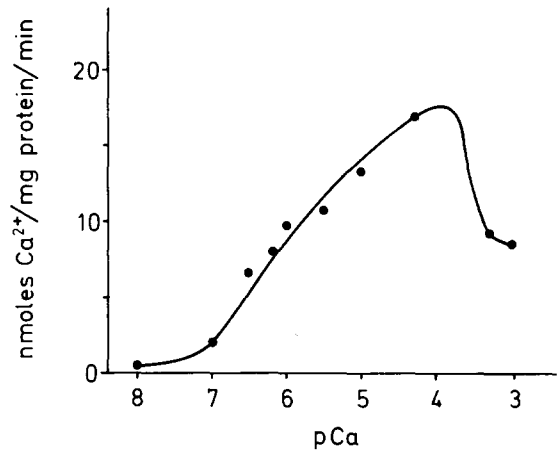

Fig. 2. Calculated initial ^{45}Ca uptake rate in inside-out vesicles as a function of Ca^{2+} concentration in the suspension buffer. Abscissa, p*Ca*; ordinate, flux rate in nmol Ca^{2+}/mg inside-out vesicle per min.

Having verified the presence of a calmodulin-stimulated active calcium transport in the vesicle preparation, the effect of calcium-calmodulin on K^+ accumulation was examined using the potassium analogue rubidium. Experiments were carried out using both ^{86}Rb and ^{45}Ca as a double label in order to correlate potential changes in K^+ permeability with simultaneous activation of the calcium pump. In the double-labelling studies, calmodulin-stimulated active transport was identical to that observed using ^{45}Ca alone. Inside-out vesicle accumulation of ^{86}Rb as a function of time and calcium

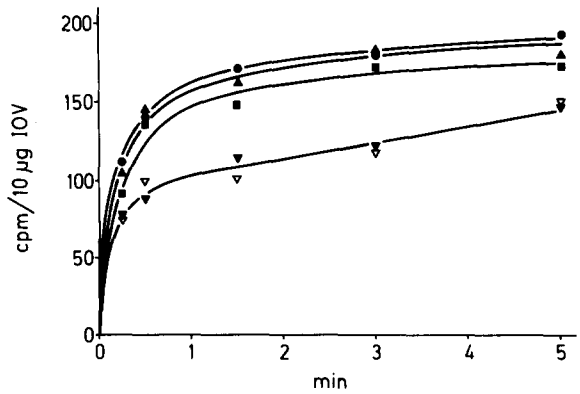

Fig. 3. ^{86}Rb uptake in inside-out vesicles as a function of time and Ca^{2+} concentration. At $t = 0$, ATP and calmodulin (CaM) were added. See text for experimental details. Ca^{2+} concentration: ●, 10^{-6} M; ▲, $6 \cdot 10^{-7}$ M; ■, 10^{-4} M; ▼, 10^{-8} M; ▽, 10^{-8} M ÷ CaM. Abscissa, time in min; ordinate, ^{86}Rb activity in cpm/10 μg inside-out vesicles (IOV).

concentration in the presence of calmodulin is shown in Fig. 3. ^{86}Rb uptake at a free Ca^{2+} concentration of 10^{-8} M was the same with and without calmodulin present and possibly represents K^+ diffusion which is non-calcium dependent.

In order to maintain active calcium transport during ^{86}Rb uptake studies, it was necessary to have ATP and Mg^{2+} present in the suspension medium. Szebeni [8] suggested that magnesium inhibits calcium-sensitive K^+ transport in erythrocyte inside-out vesicles, but lack of details makes it difficult to evaluate the circumstances. Therefore, ^{86}Rb uptake was also examined in the absence of Mg^{2+}. No difference in either initial rates or amount accumulated with or without Mg^{2+} was observed at Ca^{2+} concentrations less than 10^{-4}.

As a further test of the role of calmodulin in activation of the Ca^{2+}-dependent K^+ channel, ^{86}Rb accumulation was measured with and without calmodulin as well as with calmodulin and the calmodulin antagonists pimozide or chlorpromazine present. The effect of these conditions with 10^{-6} M Ca^{2+} in the suspension medium is shown in Fig. 4. From these curves, it is clear that the calmodulin stimulated Ca^{2+}-dependent uptake of ^{86}Rb is almost completely inhibited by either pimozide or chlorpromazine. The uptake curve in the absence of calmodulin shows an interesting

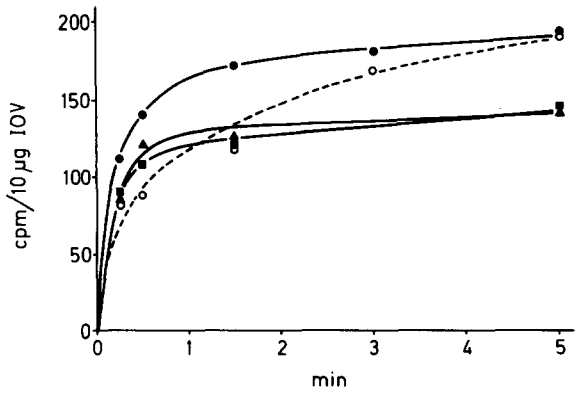

Fig. 4. The effect of calmodulin (CaM) deletion and chlorpromazine or pimozide on ^{86}Rb uptake in inside-out vesicles at 10^{-6} M Ca^{2+}. +CaM, ●———●; −CaM, ○———○; +CaM + 50 µM pimozide, ▲———▲; +CaM + 50 µM chlorpromazine, ■———■. Coordinate axes are the same as in Fig. 3.

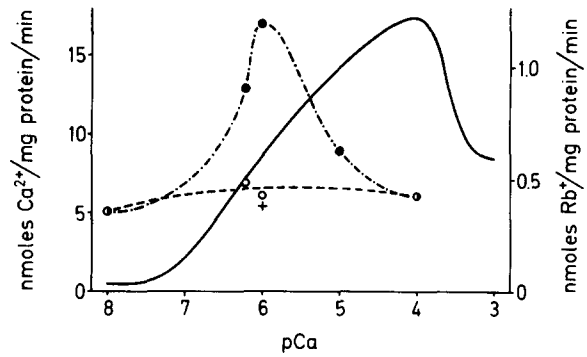

Fig. 5. Calculated initial uptake rates for ^{86}Rb in erythrocyte inside-out vesicles as a function of Ca^{2+} concentration. +CaM, ●–·–·–●; −CaM, ○- - - -○. For the purpose of comparison, Fig. 2 is superimposed (———). The initial ^{86}Rb uptake rate in the presence of pimozide or chlorpromazine is indicated by the symbol +. Abscissa, p*Ca*; ordinate, nmol Rb^+/mg inside-out vesicle per min (right) and nmol Ca^{2+}/mg inside-out vesicle per min (left).

variation in comparison with the uptake observed in the presence of pimozide or chlorpromazine. Although the initial flux rate is reduced and comparable to that seen in the presence of the calmodulin antagonists, the amount of Rb^+ accumulated over the 5 min sampling period, instead of reaching a plateau after 2 min, increased continuously and approached the amount accumulated in the presence of calmodulin. This type of uptake in the absence of calmodulin was also obtained with $6 \cdot 10^{-7}$ and $1 \cdot 10^{-4}$ M Ca^{2+}. Calculated initial isotope flux rates for active calcium transport and calmodulin-activated ^{86}Rb uptake are assembled in Fig. 5. According to these data ^{86}Rb fluxes have a $k_{1/2}$ of approx. $5 \cdot 10^{-7}$ M and reach a maximum at calcium concentrations which are lower than those required to maximize active calcium transport.

Discussion

A great deal of interest has been directed towards examining the role of calcium-activated calmodulin in stimulating active Ca^{2+}-transport in the human red cell. These studies have given relatively consistent results which have served to clarify many of the parameters associated with this system. In this regard, the use of inside-out vesicles to investigate phenomena which are associated with the nominally unavailable cytoplasmic surface of

the plasma membrane has provided much valuable information. Studies of the Ca^{2+}-dependent K^+ channel using inside-out vesicles, have, however, given rise to conflicting results. Sze and Solomon [16] reported a calcium-induced potassium pathway in erythrocyte inside-out vesicles with a $k_{1/2}$ for activation of 0.2 mM Ca^{2+}. They expressed disappointment that the vesicles did not exhibit the same specific Ca^{2+}-induced K^+ leak that characterizes adenosine-triphosphate-depleted intact red cells, and concluded that the conformation of one class of Ca^{2+} binding sites depends on specific environmental conditions. Szebeni, in a comparable study [8], observed Ca^{2+}-sensitive K^+ transport in red cell inside-out vesicles at a Ca^{2+} concentration of 50 μM. In commenting on the difference, Szebeni postulated that dextran gradient centrifugation, a common procedure in the preparation of inside-out vesicles, caused loss of the K^+ permeability response to low Ca^{2+} concentrations. In addition, he found that rate constants for rubidium fluxes were dependent on K^+ concentrations in the suspension medium, and were strongly reduced for KCl concentrations above 5–10 mM.

The first experimental indication that calmodulin might be involved in the activation of the Ca^{2+}-dependent K^+ channel came from the work of Sarkadi et al. [17]. They reported that addition of a cytoplasmic protein extract stimulated rubidium uptake by erythrocyte inside-out vesicles. Lew et al. [18] were unable to reproduce these results and found that normally prepared inside-out vesicles (method of Steck and Kant [11]) exhibited little or no Ca^{2+}-activated K^+ flux. They then described conditions for one-step preparation of inside-out vesicles which favoured preservation of the Ca^{2+}-activated Rb^+ flux and reported that with these vesicles, activation occurred in the range of 2–10 μM Ca^{2+} and exhibited 'all-or-none' characteristics. The possibility that calmodulin is involved in the Ca^{2+}-dependent K^+ permeability changes in red cell membranes has been further supported by reports that calmodulin antagonists block the calcium-activated increase in K^+ permeability in erythrocytes [9] and inside-out vesicles [7].

It is clear from the above discussion that, while there is presumptive evidence that calmodulin plays a role in activation of the Ca^{2+}-dependent K^+ channel in the erythrocyte membrane, direct experimental verification is highly dependent on experimental conditions. The present study was carried out so as to anticipate those factors which have been shown to be critical. For example, studies have demonstrated that activation of the Ca^{2+}-dependent K^+ channel requires the presence of extracellular K^+ [19,20]. Therefore, vesicles were prepared in the presence of potassium sulphate buffers so that the vesicles contained K^+ (K^+-inside-out vesicles). A preliminary study indicated that activation of the Ca^{2+}-dependent K^+ channel does not occur in vesicles containing Na^+. In addition, final separation of inside-out vesicles in the preparative phase was carried out without the use of a dextran gradient.

Rubidium uptake in K^+-inside-out vesicles under the conditions described in the present work, revealed two important aspects.

(1) Initial flux rate. In the absence of calmodulin, rubidium was taken up with an initial flux rate of 0.36 nmol Rb^+/mg inside-out vesicle per min. The uptake rate was independent of calcium concentration in the range 10^{-8}–10^{-4} M Ca^{2+}. Addition of calmodulin (5 μg/ml) to the suspension medium caused a Ca^{2+}-dependent increase in the initial rate of Rb^+ uptake which reached a maximum of 1.2 nmol Rb^+/mg inside-out vesicle/min at 10^{-6} M Ca^{2+}. This rate was more than 3-times the rate observed in the absence of calmodulin. At calcium concentrations greater than 10^{-6} M Ca^{2+} the uptake rate was diminished. Addition of the calmodulin antagonists pimozide or chlorpromazine (50 μM) to the calmodulin containing suspension medium caused the initial rate to fall to that observed in the absence of calmodulin. The $k_{1/2}$ for activation of Rb^+ initial uptake rates in the presence of calmodulin was approx. 0.5 μM Ca^{2+}. Simultaneous calmodulin stimulation of active calcium transport reached a maximum at 10^{-4} M Ca^{2+}, with a corresponding $k_{1/2}$ of approx. 1 μM Ca^{2+}. These $k_{1/2}$ values for calmodulin-stimulated Ca^{2+}-dependent K^+ permeability increase and activated Ca^{2+}-pump are consistent with the $k_{1/2}$ values for the calcium-dependent binding of calmodulin to red-cell inside-out vesicles reported previously by the present authors [10].

(2) Total accumulation. Although the initial flux rates in the absence of calmodulin were not dependent on the Ca^{2+} concentration, the amount of rubidium accumulated in the course of the 5 min

sampling period did show a Ca^{2+}-dependent variation. At 10^{-8} M Ca^{2+}, the amount taken up reached a relatively constant level after approximately 2 min of about 95 pmol Rb^+/mg inside-out vesicle. When the Ca^{2+} concentration of the suspension was increased, instead of leveling off, the amount of rubidium taken up continued to increase and approached the amount accumulated in the presence of calmodulin at comparable calcium concentrations. For example, the amount taken up after 5 min at 10^{-6} M Ca^{2+}, with and without calmodulin, was approx. 132 pmol Rb^+/mg inside-out vesicle. The increase in total Rb^+ uptake without calmodulin at Ca^{2+} concentrations greater than 10^{-8} M may be due to a Ca^{2+}-dependent K^+ leak which does not require calmodulin. However, not only the initial flux rate but the amount accumulated in the presence of pimozide or chlorpromazine were comparable to those obtained with 10^{-8} M Ca^{2+}. This finding suggests that the increase in the amount of rubidium accumulated in the absence of calmodulin at Ca^{2+} concentrations greater than 10^{-8} M may be due to endogenous membrane-bound calmodulin which is not removed during vesicle preparation.

If it is true that Rb^+ transport is indistinguishable from K^+ transport, and that membrane protein in the form of inside-out vesicles is unaltered in its transport properties, it is possible to extrapolate from Rb^+ flux rates observed in inside-out vesicles to K^+-flux rates which would be expected in intact red cells. Assuming that the flux rate at 10^{-6} M Ca^{2+} in the presence of calmodulin antagonists represents Rb^+ transport which is not dependent on calcium, the maximum net calmodulin/Ca^{2+}-activated Rb^+ initial flux rate is 49.2 nmol Rb^+/mg inside-out vesicle per h. Based on standard values for membrane protein per red cell, 1 mg inside-out vesicle corresponds to $1.1 \cdot 10^{-4}$ liter red cells, so that the equivalent Rb^+ flux is 0.45 mmol Rb^+/liter red cells per h. 0.5 μCi/ml ^{86}Rb, with a specific activity of 3 mCi/mg Rb^+, was used per experiment, with a resultant K^+ to Rb^+ concentration ratio of $2.5 \cdot 10^3$. Therefore, the expected maximum K^+ flux rate is $1.12 \cdot 10^3$ mmol K^+/liter red cells per h.

In conclusion, the present study supports the contention that erythrocyte inside-out vesicles contain a calcium-dependent K^+ channel which requires calmodulin for activation, and resembles the Gárdos channel in intact human red cells. The observation that activation of the channel occurs at Ca^{2+} concentrations which are lower than those required for maximum stimulation of the calcium pump suggests that these processes are dependent on two states of the calmodulin molecule, characterized by a lower or higher amount of Ca^{2+} bound to calmodulin.

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