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Evidence against involvement of the human erythrocyte plasma membrane Ca^{2+}-ATPase in Ca^{2+}-dependent K^+ transport

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Two tests were performed to assess the relationship between the Ca^{2+}-activated K^+ channel and the Ca^{2+}-pumping ATPase in human erythrocytes. Antibodies against the purified ATPase inhibited the ATPase in resealed erythrocytes, but had no effect on the K^+ channel (as assessed by Rb^+ efflux). Reconstituted liposomes containing the purified active Ca^{2+}-pumping ATPase showed no Ca^{2+}-activated Rb^+ influx. Both of these results suggest that some molecule other than the Ca^{2+}-ATPase is responsible for the K^+ channel.

The identity of the molecule(s) responsible for the Ca^{2+}-activated K^+ channels in erythrocytes and excitable cells has long been an object of speculation. Both the $(Na^+ + K^+)$-ATPase and the Ca^{2+}-pumping ATPase of plasma membranes have been suggested for this role [1]. In particular, a number of recent papers have made observations suggesting that the Ca^{2+}-pumping ATPase might be responsible for the K^+-channel activity: four different laboratories have reported that calmodulin activates the K^+ channel [2–6]. Since the Ca^{2+}-pumping ATPase is the only erythrocyte membrane protein to which calmodulin binds tightly and specifically [7,8], an activation by calmodulin of the channel in erythrocytes would suggest that the channel and the ATPase are due to the same molecule. A more recent study of the effect of phenothiazines on Ca^{2+}-dependent K^+ efflux in ghosts has indicated that calmodulin is probably not involved, but that phenothiazines inhibit the K^+ efflux by acting directly on the Ca^{2+} ATPase [9].

Because of these observations, we carried out two types of experiments designed to test whether the Ca^{2+}-pumping ATPase is responsible for the Ca^{2+}-activated K^+ channel: We reconstituted the purified Ca^{2+}-ATPase into liposomes and tested for increased permeability of the liposomes to the K^+ analog Rb^+, and we tested for possible inhibition of the Ca^{2+}-dependent K^+ channel by antibodies directed against the Ca^{2+}-ATPase. The results of both of these studies indicated that the Ca^{2+}-ATPase is probably not responsible for the Ca^{2+}-activated K^+ channel.

Resealed human erythrocyte ghosts were prepared according to the method of Yingst and Hoffman [10]. Ten ml of fresh human blood was drawn into heparin and the erythrocytes were washed three times at 4°C with 20 mM Hepes-Na (pH 7.4), 0.2 mM EGTA-Na (pH 7.4) and 150 mM NaCl. The cells were then suspended at 20% hematocrit in the washing buffer containing 1 mg/ml of chloramphenicol and incubated at 37°C with shaking (70 oscillations/min) for 24 h to

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tes, 2-([2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]-amino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IgG, immunoglobulin G.

deplete the cells of ATP. After incubation, cells were again washed three times with the washing buffer and finally centrifuged at 12000 × *g* for 10 min. One volume of packed cells was lysed in 20 volumes of lysis buffer (20 mM Hepes-Tris (pH 7.0), 50 μM EGTA-Na (pH 7.0) and 2 mM $MgCl_2$) at 0°C. Different dilutions of the anti-Ca^{2+}-ATPase-IgG or IgG from nonimmune rabbit serum were also included in the lysis buffer. The cells were mixed rapidly on ice with the lysis medium and at the end of 1 min, 100 μCi of ^{86}RbCl was added for each ml of packed cells. At the end of 5 min of total lysis time, 1/10 volume of a mixture containing 0.3 M NaCl and 1.27 M KCl was added to restore the tonicity of the medium for resealed vesicle formation. The resealed ghosts were washed two times with suspension medium (20 mM Hepes (pH 7.4), 5 mM EDTA-Na (pH 7.4), 6 mM KCl, 147 mM NaCl) by centrifugation at 48000 × *g* maximum for 10 min. The resealed ghosts were suspended in the above medium (4 ml for each ml of packed cells) and incubated at 37°C for 15 min to release ^{86}Rb from that portion of the vesicles which were leaky. At the end of incubation, the released ghosts were washed twice with the suspending medium and the pellet saved on ice before being used for the incubation to measure ^{86}Rb efflux.

The ^{86}Rb efflux measurements were carried out in a total volume of 10 ml. The medium inside the resealed ghosts contained the lysis buffer with or without anti-ATPase-IgG. The IgG used here was prepared as previously described [11]. The external medium was the same suspension buffer as mentioned above. In the control flasks, the external medium was 3.7 mM $MgCl_2$; in the experimental flasks, it was 2.38 mM $CaCl_2$ in addition to 3.7 mM $MgCl_2$. This resulted in a free Mg^{2+} concentration of 1 mM and a free Ca^{2+} contentration of 30 μM. The total assay volume was 10 ml; 0.1 ml of resealed vesicles were added with a 1 ml syringe, so that the final hematocrit was 1%. After 5 min preincubation at 37°C with gentle shaking (70 oscillations/min), efflux was started by the addition of 10 μl of 2 mM calcium ionophore (A23187) in ethanol (final concentration 2 μM). Two ml aliquots were withdrawn at zero time, chilled and immediately centrifuged at 48000 × *g* for 15 min. At the end of the 20 min incubation period, more such aliquots were treated in the same way. Half ml portions of the supernatants were mixed with 10 ml of scintillation fluid for the measurement of radioactivity. A portion of the total reaction mixture was also taken to determine the total amount of ^{86}Rb trapped in the resealed ghosts. The percent ^{86}Rb radioactivity released was determined; the time-dependent portion of the Rb^+ release was almost totally dependent on the presence of Ca^{2+}. In addition to the controls lacking Ca^{2+}, a complete set of controls was done with IgG not containing the antibody against the ATPase. The number reported here take as 100% the Ca^{2+}-dependent release in the presence of non-immune IgG. The Ca^{2+}-ATPase activity in the resealed ghosts was measured by monitoring the release of ^{32}P from [γ-^{32}P]ATP. The reaction mixture, in a final volume of 0.5 ml, contained 50 mM Tes-triethanolamine buffer (pH 7.4), 5 mM EGTA-triethanolamine (pH 7.4), 1 mM EDTA-triethanolamine (pH 7.4), 6 mM $MgCl_2$ (free Mg^{2+} concentration, 0.44 mM), 5.44 mM $CaCl_2$ (free Ca^{2+} concentration, 10 μM), 6 mM ATP, 0.05% Triton X-100, 10 μg calmodulin and about 40 μg of resealed ghost protein. the Triton X-100 was employed to lyse the resealed ghosts for complete expression of enzyme activity. Incubations were done at 37°C for 30-min periods and inorganic phosphate was determined by extraction of the phosphomolybdate complex into an organic phase. Calcium and calmodulin stimulated activity was determined by subtracting the value in the absence of calcium and calmodulin from that in their presence. The percent Ca^{2+}-ATPase activity left was determined by dividing the activity in the presence of anti-Ca^{2+}-ATPase-IgG by the activity in the presence of non-immune IgG and multiplying the result by 100.

For the study of Rb^{86} uptake, Ca^{2+} ATPase purified from human erythrocytes [12] was incorporated into liposomes by the cholate dialysis procedure [13]. The lipids used contained phosphatidylcholine and phosphatidylserine in the ratio of 4 : 1. Approximately 100 μg of the Ca^{2+}-ATPase was used for each 50 mg of lipid. During the solubilization of the lipids, 80 mM sodium cholate, 90 mM NaCl, 20 mM Hepes-Na (pH 7.6), 1 mM $MgCl_2$, 50 μM $CaCl_2$, and 5 mM KCl were present. The final dialysis buffer contained 25 mM

Hepes-Na (pH 7.4), 10 mM KCl, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol and 0.02 mM EDTA-Na. The lipid concentration in the final liposome suspension was about 16.3 mg/ml.

For the study of uptake of ^{86}Rb by the liposomes, the reaction mixture in a final volume of 4 ml contained 25 mM Hepes-Na (pH 7.5), 0.798 mM EDTA-Na (pH 7.5), 0.012 mM EGTA-Na (pH 7.5), 1.5 mM $MgCl_2$, 0.4 μCi $^{86}RbCl$, 1 mM sodium ascorbate (pH 7.5), 1 mM phenazine methosulfate and 20 μl hemolysate (1 : 2.2 dilution of the packed erythrocytes) per milliliter. When they were present, $CaCl_2$ was 0.672 mM and calmodulin 80 μg/ml. Hemolysate was included to provide any required factors which might have been purified away from the ATPase; the artificial electron donor system (ascorbate plus phenazine methosulphate) was included because it has been reported to increase the sensitivity of K^+ transport to Ca^{2+} by about 5-times [14]. The final concentration of free Ca^{2+} was 98 μM. After incubation of the mixture for different times at 37°C, a 250-μl aliquot was taken out and passed immediately through a 1 ml Dowex® AG 50W-X (20–50 mesh, Tris form) column coated with 1% bovine serum albumin in 0.25 M sucrose [15]. The free ^{86}Rb was retained by the resin, while that which had diffused into the liposomes came through with the liposomes themselves. The columns were washed with three chilled portions of 0.75 ml 0.25 M sucrose. The eluate was collected in a vial and mixed with scintillation fluid for the measurement of ^{86}Rb uptake by the liposomes.

One test of the possibility that the Ca^{2+}-pumping ATPase has K^+-channel activity was to incorporate specific antibodies against the pump into resealed red cells, under conditions which inhibited the Ca^{2+}-ATPase. We have already shown that our antibodies inhibit the Ca^{2+}-ATPase in erythrocyte inside out vesicles [11] and that affinity-purified antibodies inhibit (from the inside only) in resealed erythrocytes [16]. In the experiment shown in Fig. 1, we used IgG which had not been affinity purified; as the figure shows, when it was incorporated into resealed ghosts it inhibited the Ca^{2+}-pumping ATPase. Under similar conditions, this IgG had no effect on the Ca^{2+}-activated Rb^+ efflux from the ghosts. Under these conditions, Rb^+ uses the same channel as K^+, so that

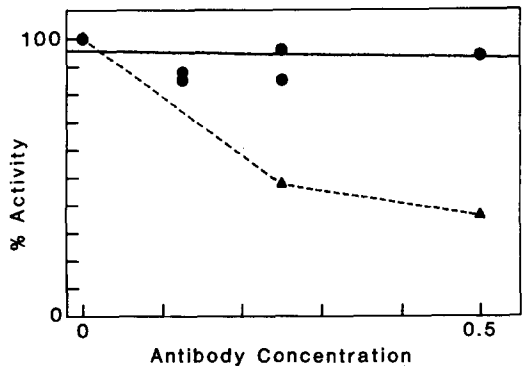

Fig. 1. Effect of antibodies against the Ca^{2+}-ATPase (triangles and dashed line) and Rb^+ efflux (circles and solid line) in resealed erythrocytes. The Ca^{2+}-ATPase and Rb^+ efflux were measured in separate samples. The antibody concentration is expressed relative to the concentration in the original serum. The solid line is a linear least-squares fit to the Rb^+ efflux data, while the dashed line simply connects the ATPase data points. Individual Rb^+ efflux points are shown, while the ATPase points are the average of duplicate determinations. These duplicates agreed closely; the standard deviation of the points was ±2.2% or less.

Rb^{2+} efflux is a good measure of K^+-channel activity [17]. This experiment alone cannot eliminate the possibility being tested, since the antibodies might conceivably bind to the Ca^{2+} pump and still allow passage of K^+ even though Ca^{2+} transport is abolished.

Another test for a relationship between the pump and the channel was performed by reconstitution of the Ca^{2+}-ATPase into liposomes. Such reconstituted proteoliposomes carry out Ca^{2+} transport and Ca^{2+}-ATPase nearly identical to that seen in the red cell [13]. Fig. 2 shows the passive Rb^+ influx into such liposomes as a function of time. From the experiment shown in Fig. 2 and others, it was evident that the Rb^+ which leaked into the liposomes showed no dependence on Ca^{2+} and calmodulin. The data shown in Fig. 2 was pooled with that from two other similar experiments and the percent change in Rb^+ influx due to addition of Ca^{2+} and calmodulin was calculated. The mean change in Rb^+ influx was −2.0% with a standard deviation of 8.2% ($n = 25$). Thus, it was evident that no Ca^{2+} activated influx of Rb^+ into liposomes was mediated by the Ca^{2+}-ATPase. Under the conditions used, the ATPase shows good Ca^{2+}-pumping capability.

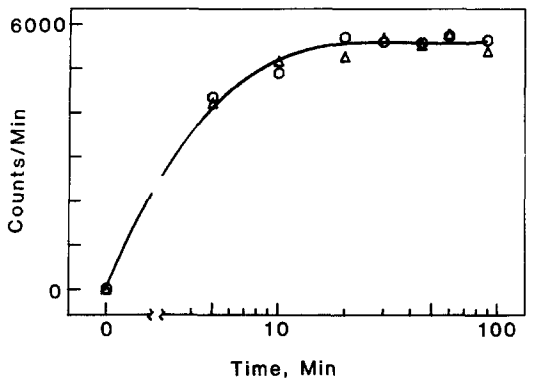

Fig. 2. Rb^+ influx into proteoliposomes containing the Ca^{2+} pump. The circles represent the data accumulated in the absence of Ca^{2+} and calmodulin, while the triangles represent the data in the presence of Ca^{2+} and calmodulin. No significant changes in influx due to Ca^{2+} and calmodulin were observed.

Negative results of the type reported here cannot totally rule out the possibility that the Ca^{2+}-ATPase is responsible in some way for the Ca^{2+}-activated K^+ channel. Only a conclusive demonstration of a separate molecule with K^+ channel activity can finally settle the issue. Our experiments, and the reconstitution experiments of Karlish et al. [18] with the $(Na^+ + K^+)$-pumping ATPase suggest that neither of the plasma membrane ion pumps cause the K^+-channel activity. The effects of phenothiazines on the K^+ channel observed by Plishker [9] were probably due to a direct action of such drugs on the channel itself, rather than on the ATPase.

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