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Rapid Report

A voltage-activated cation transport pathway associated with the sodium pump

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In proteoliposomes containing reconstituted shark Na,K-ATPase, inside positive potentials open a cation conductance characterized by a voltage-dependence very similar to that found in mammalian erythrocytes. In both proteoliposomes and erythrocytes, the voltage-activated pathway is inhibited by external oligomycin, which traps the Na,K-ATPase in a Na-occluded E_1 form. These results indicate that a cation permeable pathway, activated by inside positive potentials, can be ascribed to the Na-K pump – possibly through interaction with its gating mechanism.

The cation permeability of the membrane of high-potassium (HK) mammalian red blood cells (RBC) increases progressively as the membrane potential is made > 20 mV positive inside the cells [1,2]. This phenomenon does not seem to be restricted to RBC because it has been observed in other non-excitable cells such as the *Xenopus* oocyte [3,4]. Recently, voltage-gated channel activity was found in patch clamped human RBC membranes [5]. The voltage-activated cation fluxes were absent in low-potassium (LK) RBC. Because the HK and LK RBC are characterized by a high and low number of Na-K pumps, respectively, Halperin et al. proposed that a voltage-activated transport pathway (VAP) may be associated with the pump [2]. This possibility is tested in the present investigation using liposomes with and without incorporated purified shark Na,K-ATPase [6,7].

The liposomes containing 130 mM NaCl were suspended in 130 mM NaCl and a diffusion potential positive inside was generated by adding KCl (10 mM) or LiCl (10 mM) in the presence of the K ionophore valinomycin or the Li ionophore AS701 [8], respectively. Alternatively, the weak acid FCCP was added in the presence of a proton gradient ($pH_i = 7.0$; $pH_o = 6.0$). The transmembrane potential was assessed with the fluorochrome oxonol VI [9].

In the presence of the either ionophore (valinomycin or AS701) the conductance of the respective cation (g_K or g_{Li}) is so overwhelmingly greater than g_{Na} and g_{Cl} that V_m should be effectively clamped at the K or the Li equilibrium potential (E_K or E_{Li}). This occurred in liposomes that did not contain incorporated Na-K pumps (Figs. 1A, 1B). In the presence of FCCP and a proton gradient V_m is expected to shift towards E_H because the membrane is so highly permeable to both the undissociated (nonionic) and the charged ($FCCP^-$) forms of the compound that both come rapidly to equilibrium so that $V_m = E_{FCCP^-} = E_H = (RT/F) \cdot (pH_i - pH_o)$. This was also observed in the liposomes without incorporated pumps (Fig. 1C). In contrast, when either the ionophores or FCCP were added to liposomes with incorporated pumps in the presence of an inwardly directed K, Li or pH gradient, the peak V_m was much less positive inside and rapidly dissipated with a time constant of ≈ 40 s (Figs. 2A, 2B and 2C). When experiments similar to those depicted in Fig. 2A were performed with liposomes containing Na-K pumps in which internal NaCl was replaced by sucrose (260 mM) or Tris-Cl (130 mM, Fig. 2C), rapid dissipation of V_m was not observed; rather a steady-state V_m developed. The value of V_m was smaller in vesicles with pumps than in vesicles without pumps because incorporation of the protein increases the ground permeability of the liposomes. Thus, the conductive pathway that dissipates V_m seems to be permeable to alkali cations but not, or much less so, to Tris or Cl. Using FCCP we found that the cation selectivity of the pathway is $K > Na \geq Li \gg Tris$ (manuscript in preparation).

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When V_m in reconstituted liposomes was estimated at increasing values of E_K (varied by adjusting the external K concentration outside liposomes containing 120 mM NaCl and 10 mM KCl), V_m followed E_K up to +20 mV. At E_K values above +20 mV, the peak V_m was progressively smaller and V_m dissipated progressively faster (inset to Fig. 2B). This shows that the cation conductance that dissipates V_m in liposomes with incorporated pumps opens at the same V_m value

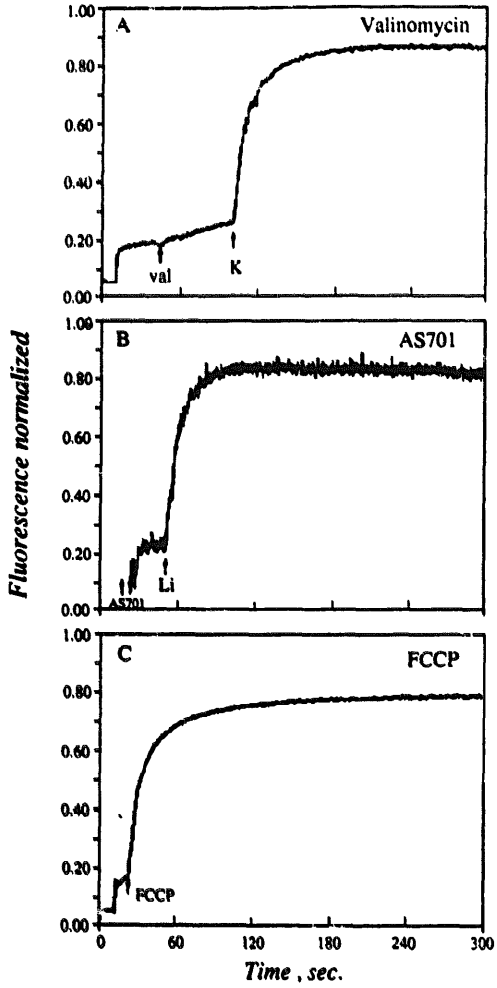

Fig. 1. Comparison of valinomycin-induced K diffusion potentials (A), AS701-induced Li diffusion potentials (B) and FCCP-induced H diffusion potentials (C) in liposomes without reconstituted Na,K-ATPase. All liposomes contained 130 mM NaCl, 2 mM $MgCl_2$ and 30 mM histidine, pH 7.0 at 23°C. 75 μl liposomes were added to 2 ml of 130 mM NaCl, 2 mM $MgCl_2$, 30 mM histidine pH 7.0 (A and B) or pH 6.0 (C) in a cuvette containing 530 nM of the potential sensitive dye oxonol VI. At the times indicated by the respective arrows, valinomycin (18 nM final, A), or AS701 (5 μM final, B) were added. Inside positive potentials were then induced by addition of 10 mM K to the vesicles with valinomycin (A), 10 mM Li to the vesicles with AS701 (B) or 400 nM FCCP for the vesicles at $pH_o = 6.0$ (C). The magnitude of the inside positive potentials was estimated by the fluorescence emitted at 660 nm at an excitation wavelength of 580 nm, using SPEX CM dual-wavelength spectrofluorometer. The specific conductance of the liposomes is very low: 4 nS/cm^2. Calibration with valinomycin indicates that the steady-state potential obtained with an infinite K gradient is $\approx$ 200 mV.

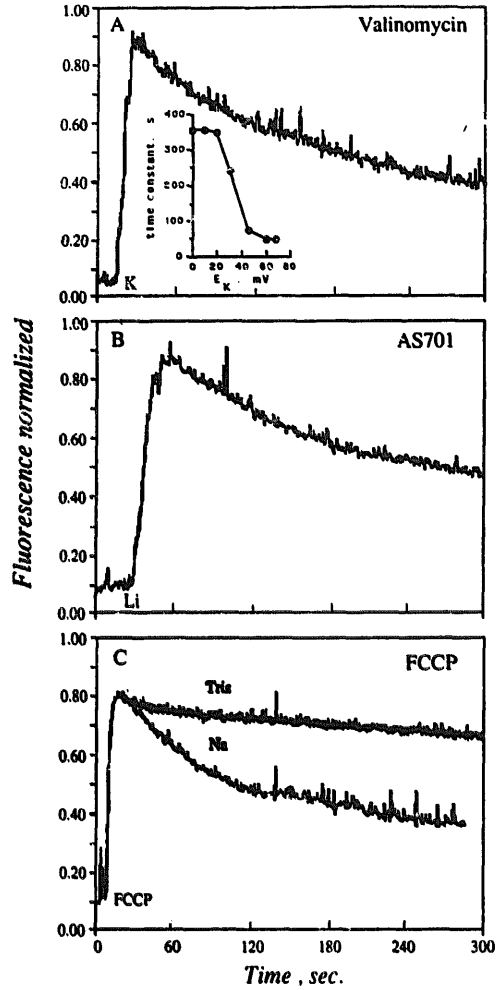

Fig. 2. Comparison of voltage decay after generation of valinomycin-induced K diffusion potentials (A), AS701 induced Li diffusion potentials (B) and FCCP-induced H diffusion potentials (C), in liposomes reconstituted with shark Na,K-ATPase. Experimental conditions were similar to those described in Fig. 1. Panel C (upper curve) also shows the effect of inside positive potentials when both internal and external NaCl was replaced by Tris-Cl. (Inset to Panel A) Time constant for the decay of valinomycin-induced potential in reconstituted liposomes at different values of E_K. The liposomes contained 120 mM NaCl, 2 mM $MgCl_2$, 30 mM histidine (pH 7.0) and 10 mM KCl. Different values of E_K($E_K = (RT/F) \cdot \ln([K]_o/[K]_i)$) were set by adjusting the external K concentration ($[K]_o$). The figure shows the time necessary for the potential to dissipate down to 1/e of the initial peak value. Peak V_m values were also progressively less positive inside as E_K increased.

as (+20 mV), and exhibits a voltage dependence similar to, (Fig. 4B, below) that found in RBC [1].

The most likely explanation for the dissipation of V_m in the vesicles containing Na-K pumps is the opening of a cation conductance through the pumps. An electrical breakdown of the vesicles causing collapse of the ion gradients and/or release of the oxonol dye can be excluded, because V_m dissipated only when the liposomes contained pumps and internal alkali cations. Dissipation through a complex of the internal cation with either the ionophores or FCCP can also be ex-

cluded because valinomycin did not affect the Li conductance nor did FCCP influence the conductance of the alkali cations contained inside the vesicles. Dissipation of V_m was not due to activation of any classical mode of the pump because the phenomenon was not dependent on the presence internal and/or external ATP and was insensitive to digitoxigenin (100 μM) which is lipid soluble and therefore inhibits the enzymatic and ion exchange activities of both inside-out and right-side out reconstituted pumps [6]. In contrast, oligomycin, an inhibitor of the pump that binds to its extracellular side [10] significantly decreased, and in some instances completely prevented, the dissipation of the valinomycin-induced potential (Fig. 3). As shown in Fig. 3, the inhibitory effect of oligomycin was more pronounced when it was present in the external medium, i.e., the extracellular side of the right side out oriented pumps. Oligomycin was also found to inhibit the VAP in human RBC: this compound, which inhibited 95% of the normal (ouabain-sensitive) operation of the pump (see legend Fig. 4), completely inhibited the voltage-activated Na fluxes in human RBC (Fig. 4A). Fig. 4B shows the effect of oligomycin on the membrane conductance of the human RBC membrane. It has been previously shown that when external NaCl is reciprocally substituted by sucrose in DIDS-treated RBC, V_m follows E_{Cl} up to $\approx +20$ mV; then V_m progressively deviates towards the equilibrium poten-

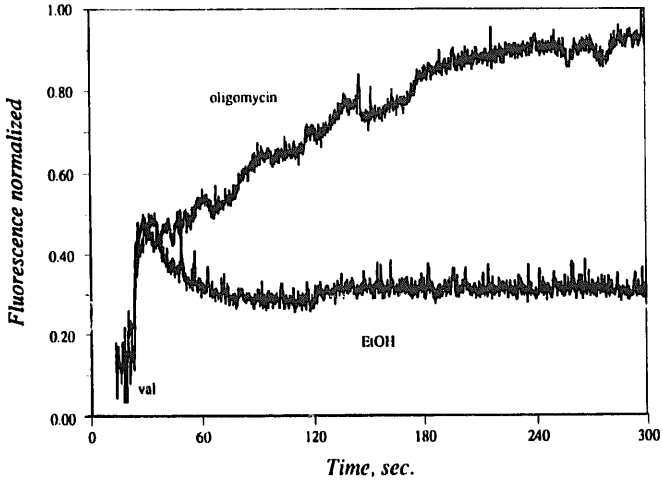

Fig. 3. The sided effect of oligomycin on the rate of dissipation of inside positive transmembrane potentials in liposomes with reconstituted Na,K-ATPase. The symmetry of orientation in these proteoliposomes, determined by the 'ouabain/ionophore' method described in Ref. 6, was as follows: 65% of the pumps were oriented as in the cells (right-side-out), 15% had the opposite orientation with the original cytoplasmic side exposed to the external medium (inside-out), and the remaining 20% was not oriented. The liposomes were prepared to contain 130 mM NaCl, 2 $MgCl_2$, 30 mM histidine (pH 7.0) and oligomycin 20 μg/ml. The figure shows the change in fluorescence that occurs in these liposomes upon addition of valinomycin (18 nM) and K (15 mM) to the external medium in the presence of either oligomycin (20 μg/ml final) dissolved in 5 μl ethanol (upper curve) or only 5 μl ethanol (bottom curve).

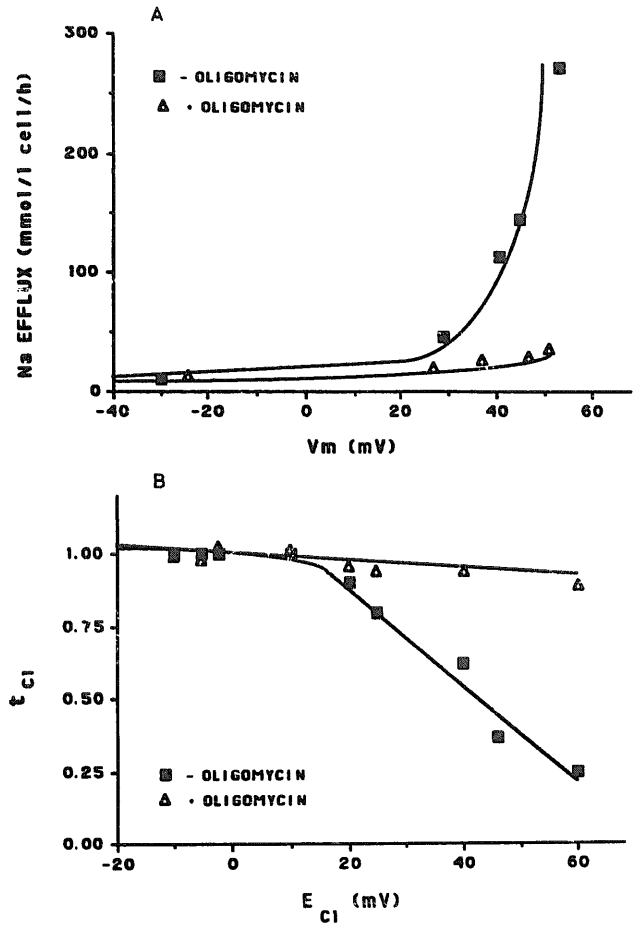

Fig. 4. (Panel A) The effect of oligomycin on voltage-activated Na efflux from valinomycin-treated human red cells. The red cells were prepared with nystatin to contain 100 mmol/l cell Na [17]. When Na loading was completed, the cells were preincubated for 1 h (4°C) with (Δ) and without (■) oligomycin (20 μg/ml) in a solution containing 140 mM NaCl, 1 mM $MgCl_2$, 10 mM Mops-Tris (pH 7.4), 10 mM glucose. Na efflux into Na-free media was measured by atomic absorption spectrometry, as described in Ref. 1. The ouabain-sensitive Na efflux at 10 mM K_o (representing Na_i-K_o exchange, the normal mode of the pump) was 8.9 and 0.3 mmol/l cell per h in the absence and presence of oligomycin, respectively (> 95% inhibition by oligomycin). To assay the voltage-activated fluxes, the cells were suspended in media of different KCl concentrations (reciprocal choline substitution) containing: 130–0 mM KCl, 0–130 choline chloride (KCl/choline chloride in mM: 130/0; 100/30; 80/50; 40/100; 10/120; 0/130), 1 mM $MgCl_2$, 10 μM DIDS and 10 μM methazolamide. The fluxes were measured by centrifugation through phthalate, as described in Refs. 1 and 17. V_m was calculated from E_H in unbuffered medium containing 50 μM CCCP [$V_m = E_H = 60\ (pH_i - pH_o)$] [1]. (Panel B) Effect of oligomycin on the membrane conductance of the human RBC membrane. t_{Cl}, the relative chloride conductance, was calculated at different values of E_{Cl} in cells preincubated with and without oligomycin. The cells were incubated at 37 °C in media of different NaCl concentrations (sucrose substitution) exactly as described in Fig. 7 of Ref. 1 and V_m was measured as described above. At each value of E_{Cl} ($E_{Cl} = (RT/F) \cdot \ln([Cl]_i/[Cl]_o)$), t_{Cl} was calculated as described in Ref. 1.

tial for cations (E_{Cat}) [1,2]. This is very similar to the dissipation of V_m observed in reconstituted liposomes; the observation is consistent with the opening of the

VAP above +20 mV, with the consequent increase of the relative cation conductance (t_{Cat}) and decrease of the relative chloride conductance (t_{Cl}) reponsible for the shift of V_m($V_m = t_{Cl} \cdot E_{Cl} + t_{Cat} \cdot E_{Cat}$). In contrast, when oligomycin was added to the media, t_{Cl} above +20 mV did not deviate significantly from its initial value of 1, confirming that oligomycin inhibits the VAP in human RBC (Fig. 4B).

The results of the experiments reported here indicate that the cation permeable pathway opened by inside positive potentials, the VAP, is most likely associated with the Na-K pump. The insensitivity of the pathway to the digitalis compounds and ATP suggests that its opening can occur in the non-phosphorylated pumps, as opposed to the leak-channel conductance found in Na,K-ATPase incorporated into planar lipid bilayers [11]. The inhibitory effect of oligomycin indicates that inside positive potentials may open the VAP by interaction with the gating mechanism of the pump, the occlusion/deocclusion step. This is consistent with the evidence that oligomycin traps the pump in the E_1-Na occluded state and prevents the transition towards E_2 [12,13], considered the voltage-sensitive step of the pump cycle [14]. In addition, the predominant effect of external oligomycin indicates that the VAP is associated with the pumps oriented right-side out: similarly to the original observation in RBC, the VAP in reconstituted pumps seems to be activated only when V_m is positive inside.

In conclusion, it is very likely that the VAP may represent a channel formed in the Na-pump when inside positive potentials induce a conformational change that either increases the main time of opening or freezes the pump in a state with both ion-gates open. The less likely possibility that a voltage-sensitive, oligomycin-inhibitable cation permeable channel different from the pump could be co-reconstituted in the liposomes cannot be completely excluded at present, even though the ATPase preparation employed is very highly purified. The findings presented here raise the intriguing possibility that the VAP may represent the same, or part of the same channel through which cations pass during pumping [15,16]. If so, this would also raise the question of how this voltage-sensitive pump channel would be controlled in excitable cells which routinely develop transient depolarizations during impulse propagation, although these occur at much higher frequency, and are of a smaller magnitude, than the potentials imposed experimentally by the addition of ionophores.

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