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Effects of anions on the Ca^{2+}, H^+ and electrical gradients formed by the sarcoplasmic reticulum ATPase in reconstituted proteoliposomes

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The chaotropic character of several ions determines their partition on membrane interfaces with aqueous media as predicted by the Hofmeister series. However, specific characteristics of each individual ion determine its ability to cross the membrane and to influence Ca^{2+}, H^+ and electrical gradients produced by the sarcoplasmic reticulum (SR) Ca^{2+} pump in reconstituted proteoliposomal vesicles. Specific effects of this kind may be relevant to a variety of biological systems, including the excitation–contraction coupling of muscle fibers in which SR plays a prominent role.

Anion; Sarcoplasmic reticulum, Ca^{2+} pump

1. INTRODUCTION

The observation by Hofmeister [1] of a systematic difference in the effects of neutral salts on the solubility of proteins has been extended to many systems [2,3]. The common mechanistic feature appears to be related to a stronger or weaker interaction of the ions with water as compared with other available surfaces, with the following order: SO_4^{2-}, $HPO_4^{2-} < Cl^- < ClO_4^- < SCN^-$, where the chaotropic (water-structure breaking) character of the anions increases from left to right. The chaotropic character of these anions promotes their partition at the interface of membrane bilayers [4] with aqueous media.

We have studied the effects of several neutral salts on an ATP-dependent Ca^{2+} transport system obtained by reconstituting proteoliposomal vesicles [5] with sarcoplasmic reticulum (SR) ATPase and exogenous phospholipids. The advantage of the proteoliposomes over native SR vesicles is their ability to maintain H^+ and electrical gradients, in addition to Ca^{2+} gradients.

2. EXPERIMENTAL

Preparation of SR vesicles and reconstitution of proteoliposomes was carried out as described by Lévy et al. [5]. ATP dependent Ca^{2+} uptake by the proteoliposomes, lumenal alkalinization and formation of electrical gradient were monitored [6] by spectrophotometric methods using the indicators murexide, pyranine and oxonol VI.

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3. RESULTS AND DISCUSSION

Addition of ATP to a suspension of reconstituted proteoliposomes is followed by prolonged Ca^{2+} transport activity, resulting in high asymptotic levels of Ca^{2+} accumulation by the proteoliposomes. Optimal activation of transport activity requires 1–100 μM Ca^{2+}, 1–10 mM Mg^{2+}, and 100 mM neutral salt. We now find that if, in the presence of constant Ca^{2+} and Mg^{2+} concentrations, the neutral salt composition of the medium is varied, the asymptotic level of Ca^{2+} accumulation also varies even though the initial velocity of transport does not vary significantly (Fig. 1). The order of effectiveness of the corresponding ions is as follows: $NH_4^+ < K^+$, SO_4^{2-}, $NO_3^- < Cl^- < ClO_4^- < SCN^-$, CH_3COO^-. We then measured ATPase activity in the presence of ionophores which render the proteoliposomes leaky to Ca^{2+}, H^+ and K^+, thereby preventing ATPase inhibition by accumulated Ca^{2+} or other transmembrane gradients. We found that ATP hydrolysis is not affected by various salts with the same pattern as the Ca^{2+} accumulation. Therefore, direct effects of the related ions on the ATPase activity do not account for the different levels of Ca^{2+} accumulation by intact proteoliposomes.

We found previously that a transmembrane electrical potential develops upon addition of ATP to proteoliposomes in a reaction mixture sustaining Ca^{2+} transport. A steady-state potential of approximately 50 mV is reached in the presence of 100 mM K_2SO_4 as the principal neutral salt [6]. Paradoxically, a more stable potential is obtained in the presence of the Ca^{2+} ionophore A23187 which allows efflux of accumulated Ca^{2+} from the vesicles through an electroneutral $Ca^{2+}/2H^+$ exchange [6]. Thereby, ATPase inhibition by the rise of both lumenal Ca^{2+} and lumenal pH is prevented, per-

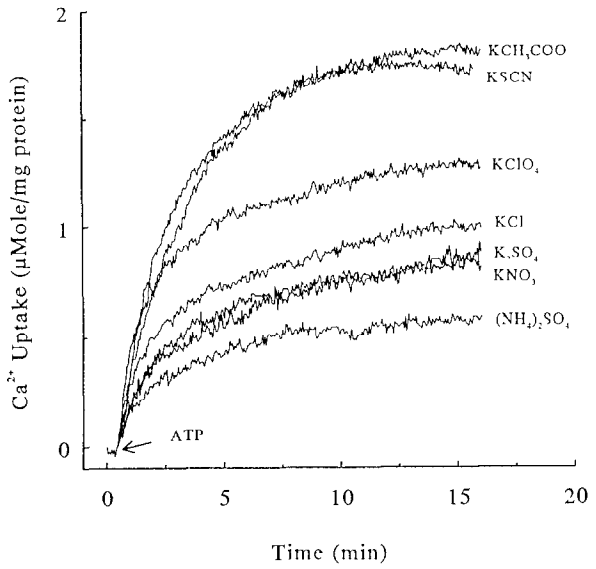


Fig. 1. ATP-dependent Ca^{2+} uptake by proteoliposomal vesicles. The vesicles were obtained by reconstituting SR Ca^{2+}-ATPase and exogenous phospholipids as described by [5]. Ca^{2+} uptake was obtained in a reaction mixture containing 10 mM PIPES, pH 7.0, 100 mM K_2SO_4 (or neutral salts as indicated in the figure), 5 mM $MgSO_4$, 60 μM $CaCl_2$, 2 mM phospho(enol)pyruvate, 25 U pyruvate kinase/ml, 25 U LDH/ml, 150 μM NADH, 100 μM murexide, and 20 μg SR protein/ml. The reaction was started with 0.1 mM ATP and followed by monitoring murexide differential absorption (λ550/487).

mitting continuous steady-state cycling of the pump and related net charge transfer. We now find that if $KClO_4$ is added to a final concentration of 10 mM, the electrical potential falls rapidly to a much lower level (Fig. 2). KSCN has a slightly lower effect, while KNO_3 and NH_4Cl have much lower effects. KH_2PO_4, KCl, and KCH_3COO do not produce any change. It is then apparent that the order of effectiveness of the ions subjected to experimentation is as follows: K^+, SO_4^{2-}, CH_3COO^-, HPO_4^{2-}, $Cl^- < NO_3^- < NH_4^+ 1 < < SCN^- < < < ClO_4^-$. This is in contradiction to the order of these ions in stimulating Ca^{2+} transport (see above). For instance, CH_3COO^- has the highest effect on the asymptotic levels of Ca^{2+} accumulation, and no effect on the electrical potential.

It is known that a H^+ gradient is formed as a consequence of Ca^{2+} transport and H^+ countertransport by the SR ATPase [5,7,8]. It is shown in Fig. 3A that activation of the Ca^{2+} pump by the addition of ATP in the presence of optimal Ca^{2+}, Mg^{2+}, K^+ and SO_4^{2-} concentrations produces alkalinization of the lumen of the proteoliposomes in parallel with Ca^{2+} uptake. Addition of valinomycin, which collapses the electrical potential [6] by rendering the membrane permeable to K^+, does not change the Ca^{2+} accumulation level significantly. On the other hand, addition of FCCP, which collapses the H^+ gradient by increasing the membrane permeability H^+, increases the Ca^{2+} accumulation level. We now find that collapse of the H^+ gradient can also be produced by addition of KCH_3COO and KSCN (Fig. 3b). In fact, in some cases, the lumenal pH is brought to a lower level than the initial pH, due to diffusion of weak acid without corresponding buffer. Addition of KH_2PO_4, KCl and KNO_3 is not followed by significant changes, and addition of NH_4Cl produces further alkalinization of the lumen of the proteoliposomes. Based on these observations, it is apparent that the order of effectiveness of the related ions is: $NH_4^+ < < K^+$, HPO_4^{2-}, Cl^-, SO_4^{2-}, $NO_3^- < < SCN^- < CH_3COO^-$, where NH_4^+ increases, and SCN^- and CH_3COO^-, reverse the lumenal alkalinization produced by Ca^{2+} and H^+ countertransport.

The basic mechanistic device of the Ca^{2+} transport ATPase is a transition of its specific cation binding characteristics from

$$2Ca^{2+}_{out} + E \cdot H_{2\,out} \rightleftharpoons E \cdot Ca_{2\,out} + 2H^+_{out} \quad (1)$$

to

$$2Ca^{2+}_{in} + E \cdot H_{2\,in} \rightleftharpoons E \cdot Ca_{2\,in} + 2H^+_{in} \quad (2)$$

where E is the enzyme, and the transition from (1) to (2) is produced by ATP through enzyme phosphorylation. Since K_1 is $\sim 10^{12}$ M^{-2}, and K_2 is $\sim 10^6$ M^{-2} with respect to Ca^{2+} at neutral pH, the transition occurs with expenditure of ~ 9 kcal per cycle ($RT \ln(K_2/K_1)$), which are provided by ATP [9]. Starting with μM Ca^{2+} concentrations outside and inside the vesicles, each cycle (dotted

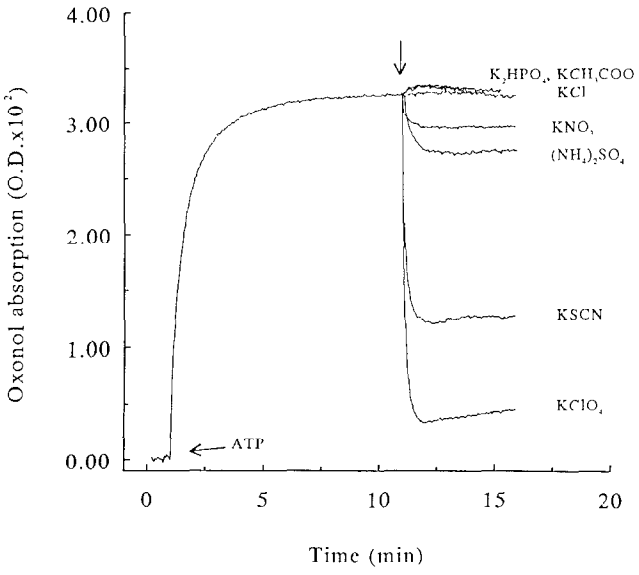
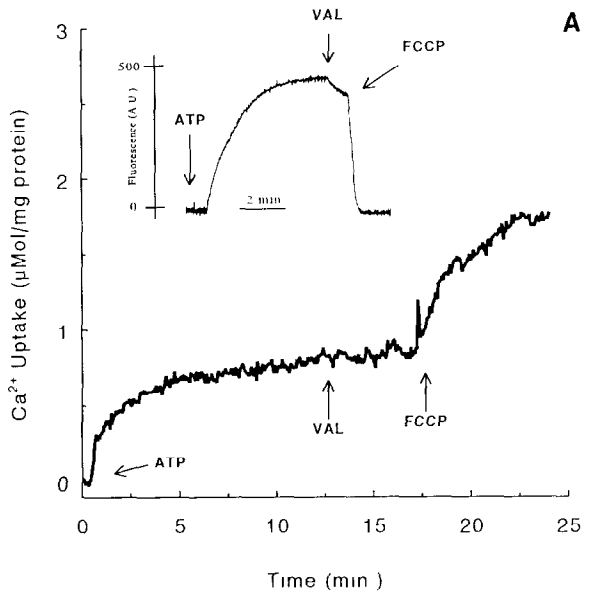


Fig. 2. Transmembrane electrical potential in proteoliposomal vesicles. A steady-state potential was developed in a reaction mixture containing 10 mM PIPES, pH 7.0, 100 mM K_2SO_4, 5 mM $MgSO_4$, 1 μM oxonol VI, 5 μg SR protein/ml, and 1 μM A23187. The reaction was started with 0.5 mM ATP, and the electrical potential followed by monitoring oxonol differential absorption (λ625/603). When indicated KSCN and $KClO_4$ were added to yield a 10 mM concentration, and the other neutral salts indicated in the figure to reach a 20 mM concentration.



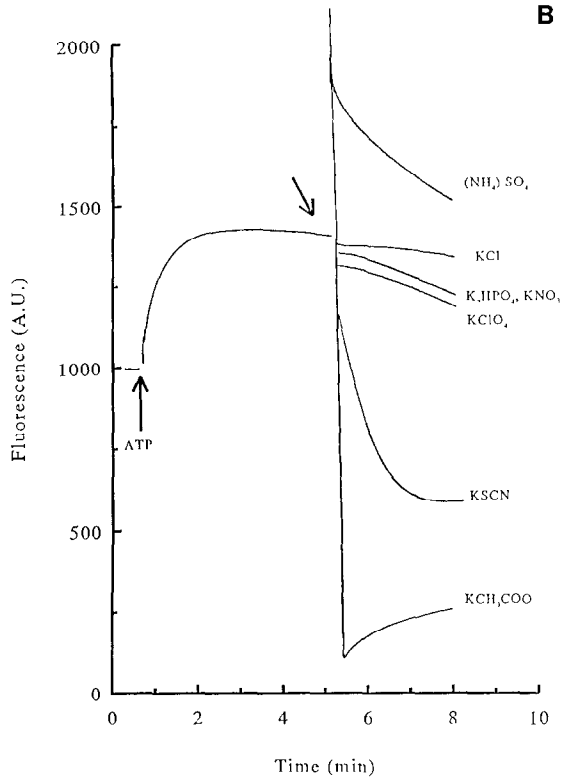


Fig. 3. Lumenal alkalinization in proteoliposomal vesicles. (A) Extrusion of lumenal H^+ was obtained under conditions permitting ATP-dependent Ca^{2+} accumulation as in Fig. 1. In this case, however, proteoliposomal vesicles filled with 200 μM pyronine were used in order to monitor lumenal pH changes by measuring the pyronine fluorescence intensity ($\lambda_{ex\ 460}$; $\lambda_{em\ 510}$). When indicated, valinomycin and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine (FCCP) were added to reach 0.5 μM concentrations. (B) Various neutral salts were added after reaching a steady-state H^+ gradient, as indicated in Fig. 2. The pH of the concentrated salt solution was adjusted to 7.0 before addition to the reaction mixture.

lines) results in transport of 2 Ca^{2+} into the vesicles, outward countertransport of $2H^+$, and inward transfer of net positive charge [6]. Repeated cycling is permitted by hydrolytic cleavage of P_i from EP and is inhibited by rise of Ca^{2+} or fall of H^+ concentrations in the lumen of the vesicles. Our present experiments with the proteoliposomal system lead to the following conclusions (Fig. 4).

(1) Collapse of the electrical potential by the addition of $KClO_4$ (Fig. 2) indicates that ClO_4^- anion crosses the proteoliposomal membrane and offsets the excess positive charge. The lack of a ClO_4^- effect on Ca^{2+} transport suggests that the transport's rate-limiting steps are not sensitive to electrical potential, and the additional thermodynamic burden created by the observed electrical potential (~ 50 mV) does not exceed the free energy output of the coupled chemical reaction.

(2) Rise of the lumenal H^+ concentration following the addition of KCH_3COO (Fig. 3) indicates that in this case the protonated form (CH_3COOH), rather than the anion, crosses the membrane and delivers H^+, but no net charge upon dissociation in the lumen of the vesicles. It also demonstrates that in fact Ca^{2+} transport is very sensitive to the lumenal concentration of H^+ as predicted by Eqn. 2.

(3) In the case of KSCN, both SCN^- and HSCN are able to cross the proteoliposomal membrane as shown by reversal of *both* electrical and H^+ gradients (Figs. 2 and 3), although an effect on Ca^{2+} transport (Fig. 1) is produced only by the protonated form.

(4) The species crossing the membrane following ad-

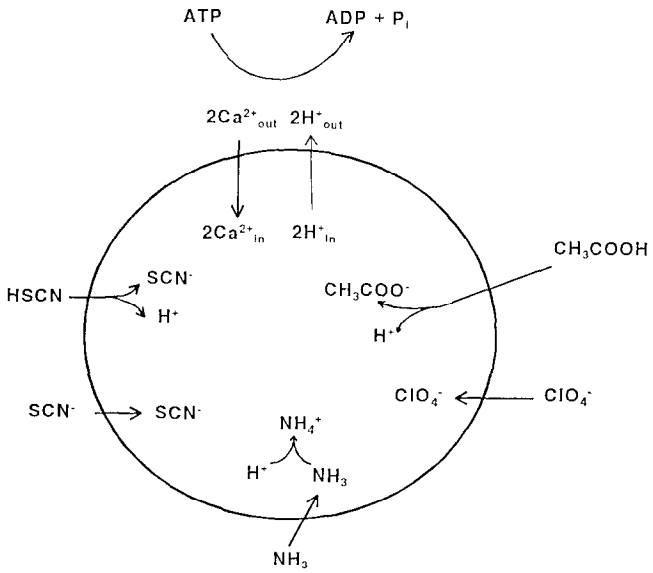


Fig. 4. Diagram of ionic fluxes in proteoliposomal vesicles. The Ca^{2+} pump translocates 2 Ca^{2+} inward and 2 H^+ outward with expenditure of 1 ATP, CH_3COOH and HSCN enter the vesicles and, upon dissociation, produce 1 lumenal H^+ and no net charge. SCN^- and ClO_4^- enter the vesicles contributing net negative charge. NH_3 enter the vesicles and binds 1 H^+.

dition of $(NH_4)_2SO_4$ is evidently NH_3, which reduces further the lumenal H^+ concentration (Fig. 3) upon H^+ acquisition to form the NH_4^+ cation. Thereby, Ca^{2+} transport is inhibited (Fig. 1) as a consequence of lumenal alkalinization.

(5) Although the chaotropic character of ions provides a common explanation for their partition at the interface of various systems with water, including membrane bilayers [4], other specific features of each ion (e.g. p*K*) produce functional effects which must be determined individually.

In addition to their general relevance, our observations on proteoliposomal vesicles reconstituted with SR ATPase may be of specific relevance to several studies [10–16] demonstrating a potentiating effect of ClO_4^- on the process that couples membrane excitation to contractile tension in muscle fibers.

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