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Biochimica et Biophysica Acta 1189 (1994) 181–188

BBA Biochimica et Biophysica Acta

Proton ejection as a major feature of the Ca^{2+}-pump

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(Received 22 June 1993)

Abstract

H^+ ejection and Ca^{2+} uptake promoted by the sarcoplasmic reticulum (SR) Ca^{2+}-pump are similarly stimulated by millimolar Mg^{2+}. This cannot be assigned to enhanced Ca^{2+} uptake and H^+ displacement from internal metal binding sites since: (1) loading SR vesicles with high Mg^{2+} concentrations does not impair H^+ ejection; (2) loading SR vesicles with Mn^{2+} does not depress H^+ ejection occurring during Mn^{2+} uptake; (3) H^+ ejection occurs even when Ca^{2+} accumulation inside the vesicles is prevented with Ca^{2+} ionophores. It is concluded that the Ca^{2+}-pump promotes an active Ca^{2+}/H^+ countertransport stimulated by Mg^{2+}. Finally, a mechanism for Ca^{2+} translocation is proposed in basic physico-chemical terms.

Key words: Calcium pump; Proton ejection; Sarcoplasmic reticulum

1. Introduction

The sarcoplasmic reticulum (SR) Ca^{2+}-pump promotes active uptake of Ca^{2+} at expense of energy driven from ATP splitting. This system has been extensively studied and several structural aspects of the ATPase and kinetic features of the catalytic cycle have been established (for reviews, see Refs. 1 and 2), but the molecular pumping mechanism remains to be elucidated in clear physico-chemical terms.

Proton ejection from SR vesicles during the uptake of Ca^{2+} has been clearly established [3–10]. However, clear-cut evidence that H^+ is actively transported by the pump is difficult to verify, owing the passive permeabilities of SR for several ions, namely H^+. Proton movements have been supposed to compensate for Ca^{2+} uptake, rendering the transport mechanism electrically silent [6,11], or as resulting from H^+ release from internal binding sites in exchange with Ca^{2+} [5,12].

Hitherto, studies using impermeant reconstituted proteoliposomes [10] have provided the best evidence that H^+/Ca^{2+} countertransport is promoted by the pump. In the present work this possibility is confirmed using native SR vesicles and it is shown that the active movements of Ca^{2+} and H^+ are similarly dependent on Mg^{2+} from the external medium. Additionally, a model for Ca^{2+} translocation is proposed.

2. Materials and methods

Sarcoplasmic reticulum (SR) vesicles were prepared as described elsewhere [13], except that isolation and resuspension media always contained 4 μM PMSF and 2.5 mM DTT. Purity of SR preparations was checked by SDS-polyacrylamide gel electrophoresis. Protein concentration was determined by the biuret method [14] calibrated with serum albumin. Aliquots (0.5 ml) suspended in media containing 0.1 M KCl, 1 M sucrose and 20 mM Tris-HCl (pH 7.0) were rapidly frozen in liquid nitrogen and stored at −80°C. These preparations retain full activities of Ca^{2+} uptake and ATP hydrolysis for several months.

Loading with Mg^{2+} was done by overnight incubation at 0–4°C of freshly isolated SR vesicles in 0.1 M KCl, 5 mM Hepes (pH 7.0) and $MgCl_2$ at the desired final concentration inside the vesicles. A similar procedure was used to load SR vesicles with maleate buffer by overnight incubation with 0.1 M KCl and maleate (pH 6.0) at the desired concentration.

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Abbreviations: ATP, adenosine triphosphate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SR, sarcoplasmic reticulum.

SSDI 0005-2736(93)E0304-S

H^+ ejection was monitored at 25°C with a pH electrode in 2 ml of assay medium containing 0.1 M KCl, 1.0 mg protein, Mg^{2+} as desired and 0.5 mM Hepes (pH 6.0). This low buffer capacity medium has been used for technical reasons to provide high sensitivity of measurements. At this pH, ATP hydrolysis does not yield scalar protons [3]. Reactions were started by adding 0.1 mM MgATP and stimulated with 100 μM Ca^{2+}. Other particular additions are as stated in the legends to figures. At the end of each experiment the electrode was calibrated with repeated additions of 30 nmol standard HCl. Controls without SR were always performed to account for proton release by ATP due to cation complexation; this is particularly important when using low (< 5 mM) Mg^{2+} concentrations.

Ca^{2+} uptake was monitored with a Ca^{2+} electrode of the neutral carrier type generously supplied by Dr. W. Simon (Laboratorium für Organische Chemie, Zurich). The reactions were carried out at 25°C in 2 ml of assay medium containing 0.1 M KCl, 100 μM Ca^{2+}, 0.3 mg protein, Mg^{2+} as desired and 5 mM Hepes (pH 6.0). Reactions were started with 200 nmol MgATP and, at the end of each experiment, the electrode was calibrated with repeated additions of 30 nmol Ca^{2+}. These experimental conditions (added Ca^{2+} and protein) were such that at the steady state of Ca^{2+} uptake the concentration of Ca^{2+} remaining in solution was well above (at least 40 times) the detection limit of the electrode. Under these conditions, the response of the electrode remains virtually linear along the course of Ca^{2+} uptake. Controls without SR were always performed to account for any Ca^{2+} complexation by ATP.

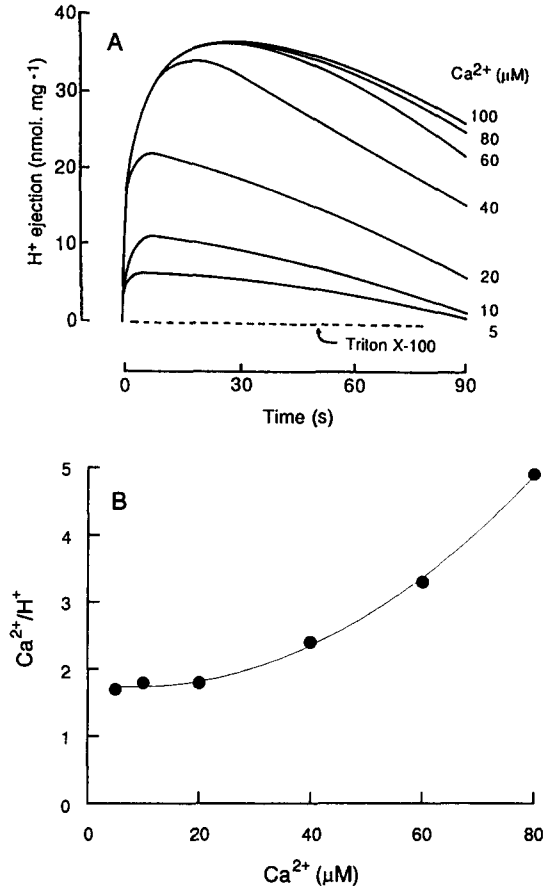

Fig. 1. H^+ ejection promoted by Ca^{2+}. (A) Reactions were carried out at 25°C in 2 ml of assay medium containing 0.1 M KCl, 5.0 mM $MgCl_2$, 1.0 mg protein, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). Ca^{2+}-activated reactions were started by addition of $CaCl_2$ as indicated on the traces. The dashed line shows the effect of SR preincubation for 5 min with 0.1% Triton X-100 (100 μM added Ca^{2+}). (B) Ca^{2+}/H^+ ratios calculated from A.

3. Results

3.1. Stimulation of H^+ ejection by Ca^{2+} and Mg^{2+}

Stimulation of the sarcoplasmic reticulum Ca^{2+}-pump with Ca^{2+} results in a sudden acidification of the assay medium followed by a slow phase of alkalinization (Fig. 1). Disrupting the vesicles with Triton X-100 abolishes H^+ ejection; since ATP hydrolysis is not impaired by treatment with Triton X-100, the acidification observed in the absence of detergent cannot be assigned to acid-base equilibration of released phosphate and ADP. This acidification may be due either to ejection of H^+ from the vesicles or to removal of OH^- from the assay medium. For simplicity, the observed pH drop will be referred here as ejection of H^+ from the vesicles.

The amount of ejected H^+ depends on the amount of added Ca^{2+} (Fig. 1A). For added Ca^{2+} below 60 μM, H^+ ejection fast phase is immediately followed by a slow alkalinization phase. The amount of ejected protons is linear up to 40 μM of added Ca^{2+}. Considering the amount of Ca^{2+} taken up by the vesicles, a ratio Ca^{2+}/H^+ of about 1.8 can be estimated for added Ca^{2+} lower than 40 μM. For higher Ca^{2+} concentrations, Ca^{2+}/H^+ ratio increases; however, the value of 1.8 is probably closer to the real stoichiometry, since for high Ca^{2+} concentrations the onset of alkalinization phase occurs early with a consequent underestimation of H^+ ejection. This stoichiometry deviates from the value of 1 reported by Levy et al. in proteoliposomes [10] probably as a consequence of the higher permeability of native SR vesicles as compared with proteoliposomes.

Fig. 2 shows the effect of millimolar Mg^{2+} concentrations on Ca^{2+} uptake and H^+ ejection. Calibration traces show that the pH and Ca^{2+} electrodes have a very fast response and can be considered to report accurately reaction kinetics. Mg^{2+} stimulates the initial rates of H^+ ejection and Ca^{2+} uptake. This increased pump activity results in higher amounts of H^+ ejected and Ca^{2+} taken up in later stages of the reactions.

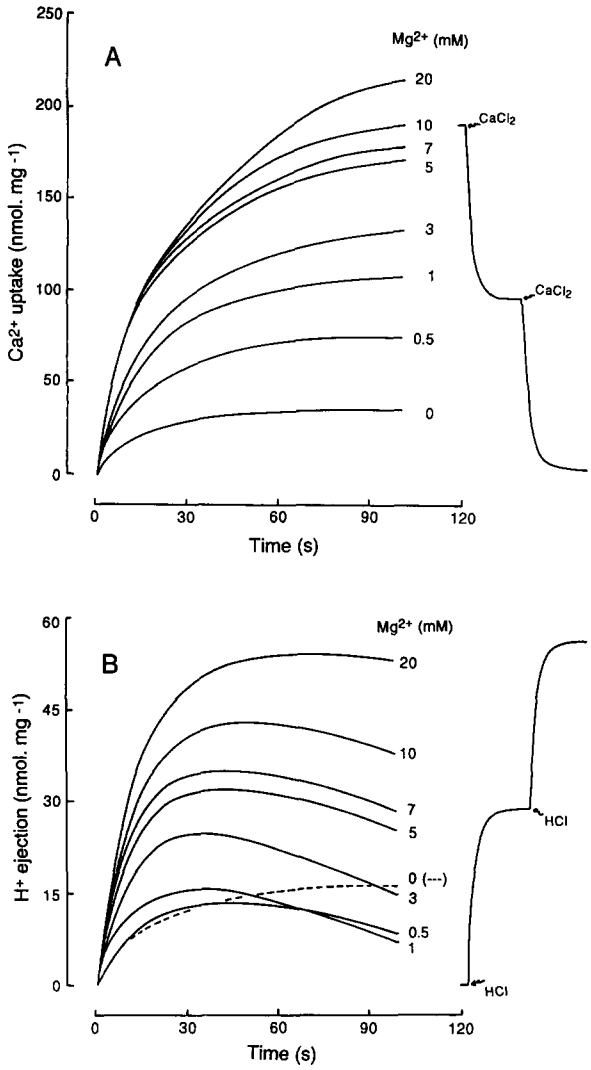

Fig. 2. Ca^{2+} uptake and H^+ ejection as a function of Mg^{2+} concentration. (A) Ca^{2+} uptake reactions were carried out at 25°C in 2 ml of assay medium containing 0.1 M KCl, 100 μM $CaCl_2$, 0.3 mg protein, $MgCl_2$ as indicated and 5.0 mM Hepes (pH 6.0). The reactions were started with 200 nmol MgATP. At the end of each experiment, the electrode was calibrated with repeated additions of 30 nmol Ca^{2+} (traces on the right). (B) H^+ ejection was followed at 25°C in 2 ml of assay medium containing 0.1 M KCl, 1.0 mg protein, $MgCl_2$ as indicated, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). The reactions were started by addition of 100 μM $CaCl_2$. At the end of each experiment the electrode was calibrated with repeated additions of 30 nmol standard HCl (traces on the right).

Since MgATP is used as substrate, stimulation of the pump is due to free Mg^{2+}. In the absence of added Mg^{2+} (background Mg^{2+} is about 5 μM, as measured by atomic absorption spectroscopy), only a small amount of Ca^{2+} is taken up and net uptake stops after 1 min. For these conditions, a small ejection of H^+ is observed without subsequent alkalinization. It should be stressed that the measurements were carried out at pH 6.0 to avoid scalar protons from ATP hydrolysis. However, the general features of Ca^{2+} transport remain similar to those observed at the more physiological pH of 7.0 [15]. The initial rates cannot be quantitatively measured in our methodology and, therefore, the affinity constants could not be calculated. However, the affinities for Ca^{2+} relating the asymptotic values of Ca^{2+} uptake or H^+ ejection are similar (about 20 μM Ca^{2+} for half maximal values).

3.2. H^+ ejection by Mg^{2+}-loaded vesicles

It is possible that some of the Ca^{2+} taken up by the vesicles may bind to internal non-specific binding sites that exchange protons for Ca^{2+}. Therefore, the increased H^+ ejection for high Ca^{2+} or Mg^{2+} concentrations could be in principle assigned to the higher Ca^{2+} uptake rates observed in these conditions. To ascertain this possibility, we carried out experiments in which SR vesicles were previously loaded with Mg^{2+} to saturate the putative metal binding sites.

When Mg^{2+} is raised outside and inside the vesicles (Figs. 3 A, B), H^+ ejection and Ca^{2+} uptake profiles are similar to those obtained with unloaded vesicles. It should be emphasized that Mg^{2+} inside the vesicles is unable to promote either H^+ ejection or Ca^{2+} uptake by SR.

3.3. H^+ ejection promoted by Mn^{2+}

Mn^{2+}, when present in millimolar concentrations, is actively accumulated by SR vesicles [15,16]. Uptake of Mn^{2+} is accompanied by H^+ ejection from the vesicles (Fig. 4), similarly as observed for Ca^{2+} uptake, except that no alkalinization phase is observed. The amount of H^+ ejected is about 4-times higher as compared with the amount observed during Ca^{2+} uptake. Loading the vesicles with high Mn^{2+} concentrations slightly depresses the total amount of H^+ ejected but has no effect on the initial rates of H^+ ejection. As in the case of Mg^{2+}, Mn^{2+} inside the vesicles does not promote H^+ ejection.

3.4. H^+ ejection in the presence of Ca^{2+} ionophores

Lasalocid or A23187 (calcimycin) added after the initial fast phase of H^+ ejection promote a rapid alkalinization of the assay medium back to the pH measured before Ca^{2+} addition. However, equilibrium is not readily reached, since a second, slow phase of H^+ ejection is observed (Fig. 5A). In the absence of added Ca^{2+} and in the presence of ATP, Ca^{2+} ionophores induce H^+ ejection from the vesicles (Fig. 5B). This ejection of H^+ is slower than that promoted by Ca^{2+} but has a similar profile: an initial fast phase of acidification followed by alkalinization. Adding Ca^{2+} after treatment with Ca^{2+} ionophores induces a residual burst of H^+.

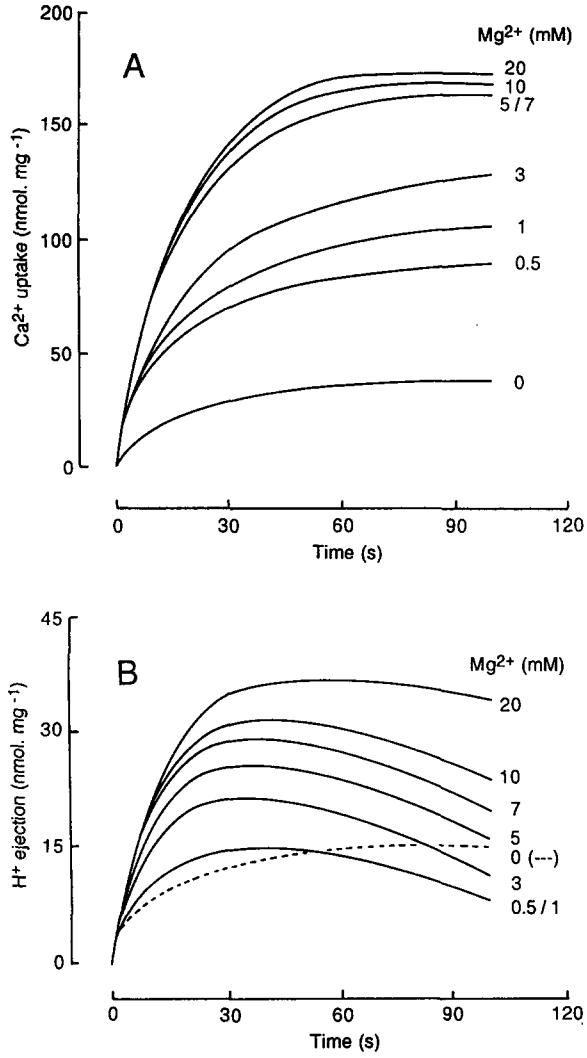

Fig. 3. Mg^{2+} dependence of Ca^{2+} uptake and H^+ ejection in Mg^{2+}-loaded vesicles. (A) Ca^{2+} uptake was followed at 25°C in 2 ml of assay medium containing 0.1 M KCl, 100 μM $CaCl_2$, 0.3 mg protein and 5 mM Hepes (pH 6.0). The reactions were started with 200 nmol MgATP. (B) H^+ ejection reactions were carried out at 25°C in 2 ml of assay medium containing 0.1 M KCl, 1.0 mg protein, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). The reactions were started by addition of 100 μM $CaCl_2$. Numeric labels on traces refer to $MgCl_2$ concentration inside and outside the vesicles.

An identical residual burst of H^+ is observed upon Ca^{2+} addition for concentrations of lasalocid above 20 μM (Fig. 6), meaning that maximal permeability to Ca^{2+} is attained at 20 μM lasalocid but, probably, recycling of Ca^{2+} through the ionophore proceeds at a slower rate than Ca^{2+} transport by the pump. However, under these conditions ATP splitting proceeds until exhaustion of ATP is attained (i.e., no steady state is observed) [15,16] and, therefore, no significant Ca^{2+} accumulation inside the vesicles is expected.

Ejection of H^+ promoted by lasalocid in the absence of Ca^{2+} depends also on Mg^{2+} concentration. Mg^{2+} outside markedly stimulates the rate and the

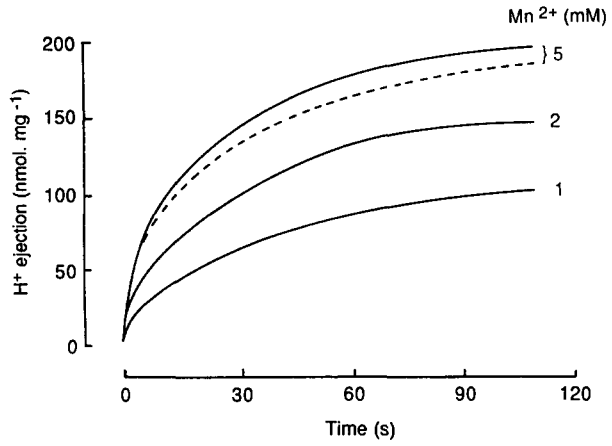

Fig. 4. H^+ ejection promoted by Mn^{2+}. H^+ ejection reactions were carried out at 25°C in 2 ml of assay medium containing 0.1 M KCl, 1.0 mg protein, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). The reactions were started by addition of $MnCl_2$. Dashed line represents H^+ ejection from vesicles loaded with 10 mM $MnCl_2$.

amount of H^+ ejection promoted by lasalocid (Fig. 7) and similar results are obtained with Mg^{2+}-loaded vesicles.

3.5. H^+ ejection as a function of buffering capacity inside the vesicles

In previous experiments (e.g., Fig. 2B), about 30 nmol of H^+ are ejected per mg of SR vesicles in the presence of 5 mM Mg^{2+}, and higher amounts are recorded with increasing Mg^{2+}. If the source of protons were the internal medium, a vast alkalinization inside the vesicles could be predicted with consequent

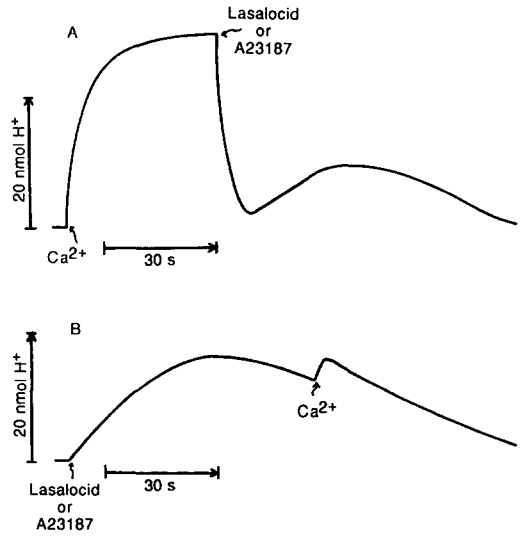

Fig. 5. Effect of Ca^{2+} ionophores on the time-course of H^+ ejection. The reactions were followed at 25°C in 2 ml of assay medium containing 0.1 M KCl, 5.0 mM Mg^{2+}, 1.0 mg protein, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). Lasalocid (40 μM), A23187 (15 μM) and $CaCl_2$ (100 μM) were added as indicated.

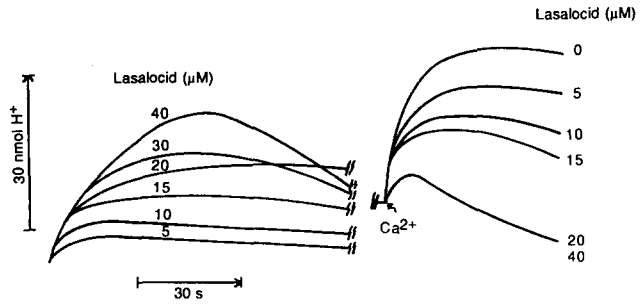

Fig. 6. Dependence of H^+ ejection on lasalocid concentration. The reactions were carried out at 25°C in 2 ml of assay medium containing 0.1 M KCl, 5.0 mM Mg^{2+}, 1.0 mg protein, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). The reactions were started by addition of lasalocid. Ca^{2+} (100 μM) was added as indicated. For the sake of clarity, time-courses of H^+ ejection after Ca^{2+} addition are depicted as starting from the same level.

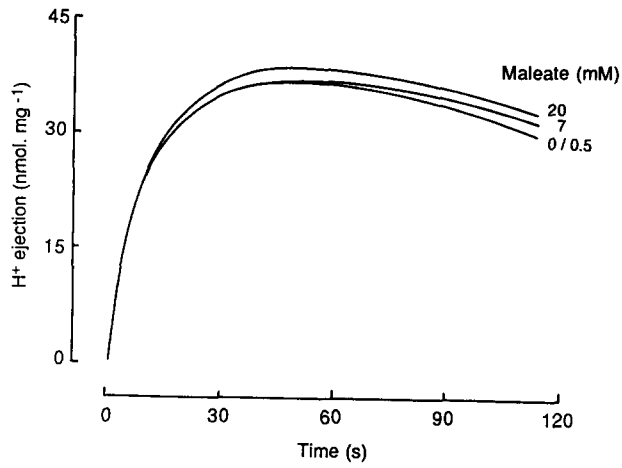

Fig. 8. Effect of the buffer capacity inside the vesicles on H^+ ejection. The reaction were followed at 25°C in 2 ml of assay medium containing 0.1 M KCl, 5.0 mM Mg^{2+}, 1.0 mg protein, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). Maleate concentrations inside the vesicles are as indicated. H^+ ejection was started by addition of 100 μM $CaCl_2$.

denaturation of the pumping system. Furthermore, endowing the vesicles with high buffer capacity, and thus providing an internal source of H^+, should stimulate H^+ ejection. However, as is shown in Fig. 8, increasing the concentration of maleate buffer inside the vesicles up to 20 mM does not have any significant effect on H^+ ejection. Although maleate may bind Ca^{2+} with low affinity, the uptake of Ca^{2+} by maleate-loaded vesicles is similar to that observed with unloaded vesicles (not shown). Any Ca^{2+} binding by maleate would provide the internal medium with additional released protons from the buffer. Therefore, alkalinization observed inside the vesicles during Ca^{2+} transport [4,10] is probably limited, not as extensive as predicted from the amount of ejected protons.

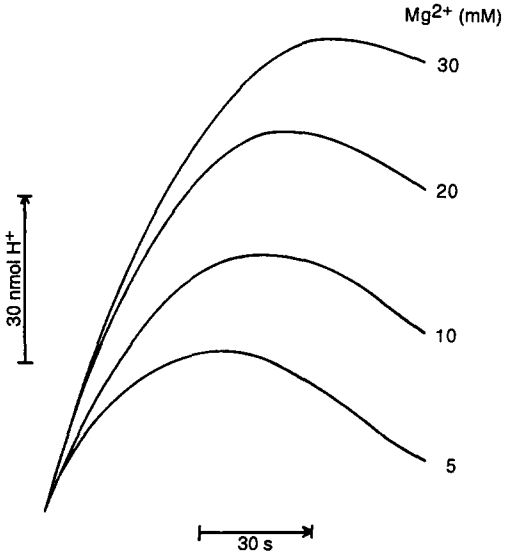

Fig. 7. Mg^{2+} dependence of H^+ ejection promoted by lasalocid. The reactions were carried out at 25°C in 2 ml of assay medium containing 0.1 M KCl, 1.0 mg protein, $MgCl_2$ as indicated, 0.1 mM MgATP and 0.5 mM Hepes (pH 6.0). Reactions were started by addition of lasalocid (40 μM). Identical results are obtained when Mg^{2+} concentration is equal inside and outside the vesicles.

4. Discussion

Several authors have described H^+ ejection from sarcoplasmic reticulum vesicles concurrent with Ca^{2+} uptake [3–10]. However, various interpretations and different explanations have been proposed. Generally, the explanations fall into two categories: (1) countertransport of H^+ is actively promoted by the pump as an intrinsic property of the molecular pumping mechanism; (2) conversely, H^+ ejection is passive, occurring indirectly because of Ca^{2+} accumulation inside the vesicles; accordingly, efflux of H^+ occurs to compensate charge displacement due to Ca^{2+} entry or as a passive diffusion resulting from H^+ released from unspecific internal binding sites in exchange with Ca^{2+}.

The stimulation of H^+ ejection observed when Ca^{2+} or Mg^{2+} concentration in the assay medium is raised (Figs. 1A and 2) could be interpreted in terms of an increased Ca^{2+} uptake and displacement of H^+ from putative internal binding sites. Fig. 3 clearly shows that H^+ ejection occurs when vesicles are loaded with high Mg^{2+} concentrations and significant H^+ ejection is observed even for 20 mM Mg^{2+} inside. Since these high concentrations of Mg^{2+} should saturate unspecific metal binding sites, the observed H^+ ejection cannot be assigned to displacement of H^+ from these sites. The question remains, however, if one considers that H^+ arises from specific Ca^{2+} internal binding sites. The ideal experiment would involve loading the vesicles with Ca^{2+}; however, this obviously should de-

press Ca^{2+} uptake and H^+ ejection due to reversal of the pump. In order to address this problem, Ca^{2+} was replaced with Mn^{2+} (Fig. 4). The pump also accumulates Mn^{2+} and the capacity of the vesicles for Mn^{2+} is about three times that observed for Ca^{2+}, with consequent increased H^+ ejection. Therefore, it is possible to load the vesicles with high concentrations of Mn^{2+} that should saturate putative internal binding sites; these concentrations remain, however, significantly lower than those reached inside the vesicles at transport steady state. The experiments with Mn^{2+} rule out the hypothesis that H^+ arises from internal metal binding sites since loading the vesicles with the transported cation does not depress initial rates of H^+ ejection.

Another line of evidence that H^+ does not arise from internal metal binding sites is given by the observation that H^+ ejection is promoted by Ca^{2+} ionophores in the absence of added Ca^{2+} (Figs. 5B and 6). Upon ionophore addition, maximal stimulation of the pump activity by residual Ca^{2+} is observed and this activity proceeds to ATP exhaustion without building of an inhibitory transmembrane Ca^{2+} gradient [16]. Therefore, H^+ ejection induced by ionophores must be consequence of a Ca^{2+}/H^+ countertransport induced by uptake of residual Ca^{2+} at a faster rate than the Ca^{2+}/H^+ exchange promoted by the ionophore. Since Ca^{2+} concentration inside the vesicles does not increase significantly and, therefore, displacement of H^+ from internal metal binding sites is unlikely to occur, H^+ ejection is assigned to Ca^{2+}/H^+ countertransport intrinsic of the molecular Ca^{2+} pump mechanism.

ATP hydrolysis in the presence of lasalocid is not affected by millimolar concentrations of Mg^{2+} [15]. Since no Ca^{2+} accumulation is measurable, no direct evidence can be provided about the effect of Mg^{2+} on the pump efficiency when Ca^{2+} ionophores are present. However, H^+ ejection induced by lasalocid is greatly stimulated by Mg^{2+} (Fig. 7). Since similar results are obtained with unloaded and Mg^{2+}-loaded vesicles, enhanced H^+ ejection evoked by Mg^{2+} outside is not consequence of a Mg^{2+}/H^+ exchange promoted by ionophores, but of increased pumping efficiency in the presence of Mg^{2+}. This is also reflected by the increased amount and rates of H^+ ejection and Ca^{2+} uptake observed in the absence of ionophores and in the presence of high Mg^{2+} concentrations (Figs. 2 and 3), suggesting that Mg^{2+} is essential to couple ATP splitting to ion transport.

The amount of ejected protons cannot be compensated by alkalinization inside the vesicles since predictable alkalinization would be dramatic with consequent denaturation of the pumping system. Several authors [4,7,8] reported that a transient alkalinization occurs inside the vesicles during Ca^{2+} uptake, but the extension of this alkalinization is limited and does not account for the extruded H^+. Data of Fig. 8 clearly indicate that the increased buffering capacity inside the vesicles does not stimulate H^+ ejection. Furthermore, low-affinity binding of Ca^{2+} by maleate might be expected with consequent release of H^+, but this is not reflected in an increased H^+ ejection, suggesting that the source of protons is not the internal aqueous medium. Therefore, protons are putatively originated from acid-base groups located internally not involved in Ca^{2+} binding. Alternatively, acidification of the external medium can occur at expense of OH^- removal into the pump system, an effect compensated by deprotonation of protein groups. Additionally, intrinsic buffering systems already present inside the native vesicles could explain the lack of effect of added buffers.

Ca^{2+}/H^+ ratios could not be accurately calculated since H^+ ejection is underestimated due to the rapid onset of an alkalinization phase. However, the value of 1.8 for Ca^{2+}/H^+ ratio found when low Ca^{2+} is present in the medium (Fig. 1B) is probably close to the true stoichiometry, meaning that H^+ ejection does not fully compensate for charge displacement due to Ca^{2+} accumulation. Other authors [5,10], using different approaches, namely reconstituted vesicles impermeant to H^+ where no alkalinization phase is observed, never found reliable values for Ca^{2+}/H^+ lower than 1.0. On the other hand, several lines of evidence indicate that Ca^{2+} transport is electrogenic, occurring without full charge compensation [17–20]. This could account for the observed alkalinization phase, which is apparently a puzzling phenomenon, since it starts before a steady state for Ca^{2+} uptake is reached. Assuming that Ca^{2+} uptake creates an electric transmembrane gradient, negative outside, this should attract H^+ to the surface of the vesicles and accelerate H^+ diffusion to the inside. Since this may occur before a steady state for Ca^{2+} uptake is reached, the time-course profiles for H^+ ejection should be similar to those of Fig. 2. Also, the inexistence of an alkalinization phase when Mg^{2+} is absent outside the vesicles (Fig. 2B) is putatively related with the low amount of Ca^{2+} taken up, not enough to promote the electrical gradient needed to concentrate ejected H^+ at the membrane surface. Efforts to elucidate the exact nature of the alkalinization phase will deserve our future attention.

4.1. Proposed mechanism of Ca^{2+} translocation

Several proposed mechanisms of Ca^{2+} translocation involve extensive movements of protein segments required for internalization of Ca^{2+}-binding sites never identified [21,22]. Actually, only very limited conformational alterations have been reliably established [23–25]. Other mechanisms postulate a coordinated movement of dimeric units for the alternate exposure of Ca^{2+}-

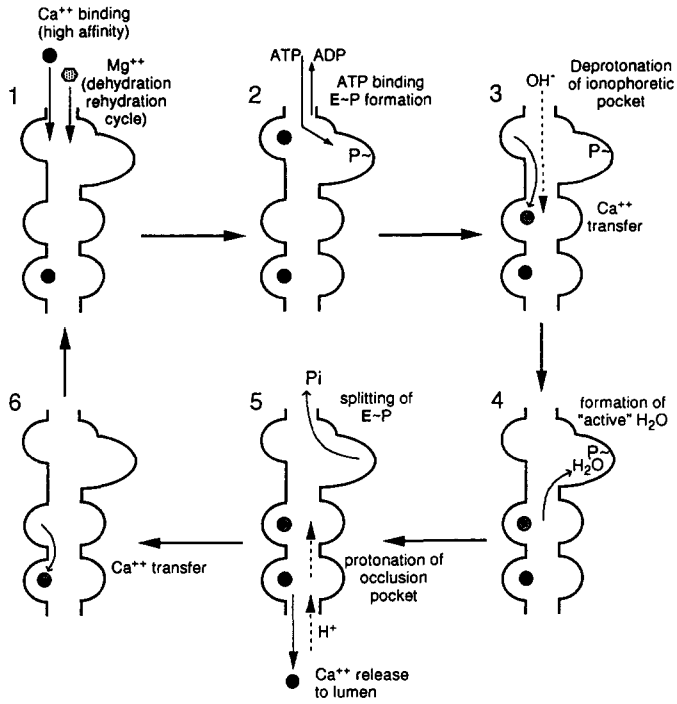

Fig. 9. Mechanism of Ca^{2+} translocation. Main steps and essential features of the proposed mechanism are depicted. For clarity, translocation of only one Ca^{2+} is illustrated in the scheme.

binding sites to the exterior and to the lumen of SR [26]. However, it has been shown that Ca^{2+} translocation occurs in the monomeric state of the pump [27–31].

Our proposal is based in a monomeric ATPase promoting Ca^{2+} translocation without extensive conformational changes. The mechanism, summarized in Fig. 9, is based on unidirectional and asymmetric movements of H^+ and OH^-, creating an electromotive force that pushes Ca^{2+} along an ionophoretic pathway. Enzyme phosphorylation assists sequential deprotonation of pockets in a translocation channel promoting the transfer of Ca^{2+} from the outer side of the pump to internally located occlusion sites (steps 1–3); protons thus originated and OH^- imported from the outside (the concept of 'active water') are responsible for splitting the aspartyl anhydride, allowing reprotonation of occluded pockets with subsequent release of Ca^{2+} to lumen (steps 4–6).

This model accounts for several features of the pump well documented in literature:

(1) Ca^{2+} bound to 'high-affinity' accessible binding sites is exchangeable, showing that these sites are superficial; binding of Ca^{2+} to these sites does not require ATP and, on the other hand, binding of ATP does not require bound Ca^{2+}; however, pump phosphorylation requires bound Ca^{2+} at these sites [32–37]

(2) With E ~ P formation, Ca^{2+} is internalized in putative Ca^{2+} occlusion sites, located in the membranous moiety of the polypeptide (non-exchangeable Ca^{2+}) [33–37].

(3) Deprotonation of occlusion pockets is favored by dehydration of the phosphorylation site promoted by E ~ P formation. Dehydration is indeed a must since the presence of water would promote immediate hydrolysis of E ~ P without deprotonation of occlusion sites and, therefore, wasting energy (ATP hydrolysis without Ca^{2+} translocation). Therefore, hydrofobicity of the active site is essential to couple ATP splitting to Ca^{2+} transport. Water activity changes during enzyme turnover and a critical role of these changes on pumping activity have been established [23,38–42].

Additionally the model accounts for other findings not accounted for or not satisfactorily explained by other models:

(1) It allows for Ca^{2+}/ATP stoichiometries lower than 2 if reaction conditions lead to a deficient dehydration of the active site (point 3, above). Although no conclusive evidence for variable stoichiometry has been provided, several reports imply that the pump mechanism does not require a fixed stoichiometry, namely when pseudosubstrates replace ATP [43], Ca^{2+} is replaced by Sr^{2+} [44], or when free Mg^{2+} concentration is low [15,45]. Since several factors may determine the dehydration/rehydration cycle of the pump, Mg^{2+} is a possible candidate.

(2) Extensive conformational changes need not to be invoked for Ca^{2+} translocation. Indeed, extensive net changes in the secondary structure do not occur during pump activity [23–25]. The absence of gross structural changes is hard to reconcile with transmembrane movement of Ca^{2+} binding sites. The model can operate with limited segmental conformational changes.

(3) The Ca^{2+}-pump is completely operational as a monomer [27–31].

(4) Proton 'ejection' is an essential feature of this model.

Acknowledgement

This work was supported by INIC.

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