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Mutational analysis of Glu^{771} of the Ca^{2+}-ATPase of sarcoplasmic reticulum
Effect of positive charge on dephosphorylation

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Abstract The glutamic acid residue Glu^{771} in the fifth transmembrane segment M5 of the Ca^{2+}-ATPase of rabbit fast twitch muscle sarcoplasmic reticulum was substituted with lysine, alanine, and glycine by site-directed mutagenesis. Mutant Glu^{771}→Lys was unable to occlude Ca^{2+}, and Ca^{2+} did not inhibit phosphorylation from P_i or activate phosphorylation from ATP of this mutant. Mutants Glu^{771}→Ala and Glu^{771}→Gly were likewise unable to occlude Ca^{2+}, but Ca^{2+} in the millimolar concentration range activated phosphorylation from ATP and inhibited phosphorylation from P_i of these mutants. The dephosphorylation of the ADP-insensitive E2P phosphoenzyme intermediate of mutants Glu^{771}→Ala and Glu^{771}→Gly was found to be blocked, whereas the dephosphorylation proceeded rapidly for mutant Glu^{771}→Lys. This finding suggests a role of the positive charge of the lysine in induction of dephosphorylation, supporting the hypothesis that the side chain of Glu^{771} participates in the countertransport of two protons per Ca^{2+}-ATPase cycle.

Key words: Calcium; Proton countertransport; Glutamate; Lysine; Mutant

1. Introduction

The Ca^{2+}-ATPase of sarcoplasmic reticulum utilizes the free energy derived from hydrolysis of ATP to drive active uptake of two Ca^{2+} per enzyme cycle. The Ca^{2+} transport reaction has long been known to be inhibited at alkaline pH, and recent evidence obtained with Ca^{2+}-ATPase solubilized and reconstituted into tight proteoliposomes [1–3] has demonstrated that protons are ejected from the liposomes during Ca^{2+} transport, suggesting that the Ca^{2+}-ATPase may mediate proton countertransport in exchange with Ca^{2+}, possibly in a way similar to the K^+/Na^+ exchange mediated by the Na^+,K^+-ATPase. A stoichiometry of two H^+ exchanged for two Ca^{2+} in each ATPase cycle seems to be favored at neutral pH and is in accordance with the electrogenicity of the pump [2,3]. It is, however, not yet clear whether this stoichiometry is obligatory and whether the countertransported protons are bound by groups on the Ca^{2+}-ATPase protein during their translocation. If so, these protein groups might be the same as those participating in Ca^{2+} translocation. Studies of the phosphoenzyme intermediates and CrATP-induced Ca^{2+} occlusion in mutants of the Ca^{2+}-ATPase have pointed to five membrane buried residues with oxygen containing side chains, Glu^{309}, Glu^{771}, Asn^{796}, Thr^{799}, and Asp^{800}, as likely Ca^{2+} ligands in the occluded transport intermediate [4–6]. Because the dephosphorylation of the E2P phosphoenzyme intermediate was partially or completely blocked in mutants Glu^{309}→Gln, Glu^{771}→Gln, and Asn^{796}→Ala, we furthermore hypothesized that the residues Glu^{309}, Glu^{771}, and Asn^{796} might participate in the countertransport of H^+ (or H_3O^+) [4,5,7]. This would be analogous to the Na^+,K^+-ATPase, in which the dephosphorylation of E2P is believed to be activated by binding of extracellular K^+. Our previous study indicated that the block of dephosphorylation was most prominent in the Ca^{2+}-ATPase mutant Glu^{771}→Gln [4]. If indeed this residue is involved in the binding of protons or hydroxonium ions to be countertransported, it might be expected that the signal transmission to the phosphorylation site triggering dephosphorylation would depend on the presence of positive charge near the Glu^{771} side chain. Assuming that an exchange stoichiometry of 2 H^+ per 2 Ca^{2+} is obligatory, dephosphorylation should not occur unless both of the protons to be countertransported are bound.

In the present work, the role of Glu^{771} in Ca^{2+} binding has been further analysed, and the hypothesis that this residue participates also in proton countertransport has been examined by studying the functional consequences of substituting lysine, alanine, and glycine for Glu^{771}. The lysine substitution adding two positive charges relative to the charge of the glutamate side chain was chosen to mimick the binding of two protons (or H_3O^+) to be countertransported, or the binding of one calcium ion. The alanine substitution serves as a control and in addition mimicks the situation existing if only one proton were bound. Moreover, by comparison with the previously characterized mutant Glu^{771}→Gln [4], the alanine mutation tests whether both oxygens of the Glu^{771} side chain are equally important. The glycine substitution was carried out to study the role of steric factors associated with the presence of a side chain.

2. Materials and methods

The methods employed in this study have been described in detail elsewhere [4,6,8,9]. In brief, mutations were introduced into the rabbit fast twitch muscle Ca^{2+}-ATPase cDNA using the site-specific mutagenesis method of Kunkel [10]. The presence of the correct mutation was confirmed by nucleotide sequencing according to Sanger et al. [11].

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Abbreviations: CrATP, β,γ-bidentate chromium(III) complex of ATP; E2P, phosphoenzyme intermediate with luminally facing transport sites and ADP-insensitivity of the phosphoryl aspartyl bond; EGTA, [ethylenebis(oxyethylene-nitrilo)] tetraacetic acid; HPLC, high-performance liquid chromatography; M1-M10, putative transmembrane segments numbered from the NH_2-terminal end of the peptide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate.

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The entire Ca^{2+}-ATPase cDNA containing the desired mutation was cloned into vector pMT2 [12] for expression in COS-1 cells [13]. Microsomes were prepared from transfected cells and assayed for expression of the exogenous Ca^{2+}-ATPase by immunoblotting [9]. Ca^{2+} transport was measured by Millipore filtration [9], at 37°C to avoid precipitation of calcium oxalate outside the vesicles at the highest Ca^{2+} concentrations. The phosphoenzyme intermediates formed from [γ-^{32}P]ATP or $^{32}P_i$ were analysed as a function of Ca^{2+} concentration and by kinetic experiments in which the phosphorylated enzyme was diluted into a medium containing non-radioactive P_i to study the rate of dephosphorylation [4]. The phosphorylated acid-precipitated microsomal protein was washed and subjected to SDS polyacrylamide gel electrophoresis at pH 6.0 followed by autoradiography of the dried gels and quantitation by liquid scintillation counting of gel slices.

Measurement of Ca^{2+} occlusion was carried out essentially as described [6] except for the use in the present study of an HPLC column with larger pore size (TSK G 4000 SW, 7.5 mm × 300 mm, equipped with a guard column). The isolated microsomal membranes were incubated for 1 h at 37°C in 300 μl of a reaction mixture containing $^{45}Ca^{2+}$ concentrations varying between 10 μM and 10 mM, 1 mM CrATP, 5 mM Mg^{2+}, 0.1 M NaCl, and 50 mM TES/Tris buffer (pH 7.0). Following this incubation, the membranes were solubilized by addition of the non-ionic detergent $C_{12}E_8$ at a concentration of 5 mg/ml. Insoluble material was removed by centrifugation for 30 minutes in a Beckman Airfuge at 130,000 × g, and 250 μl of the supernatant was subjected to molecular sieve HPLC. The eluant contained 5 mg $C_{12}E_8$/ml, 0.1 M NaCl, 50 mM TES (pH 7.0), 1.5 mM non-radioactive $^{40}CaCl_2$ (to replace exchangeable Ca^{2+}), 1.0 mM EGTA, and 10 mM $MgCl_2$. Fractions of 0.5 ml were collected for analysis of radioactivity by liquid scintillation counting.

3. Results

3.1. Ca^{2+} transport and Ca^{2+} occlusion

Neither of the mutants Glu^{771}→Lys, Glu^{771}→Ala, or Glu^{771}→Gly was able to transport Ca^{2+} at Ca^{2+} concentrations up to 200 μM, which is the experimental upper limit of the Ca^{2+} transport assay. Lack of Ca^{2+} transport has previously been demonstrated for mutant Glu^{771}→Gln [4,14]. The formation of a Ca^{2+} occluded transport intermediate was studied using the previously developed technique [6] involving incubation with CrATP in the presence of $^{45}Ca^{2+}$ followed by HPLC separation of the expressed Ca^{2+}-ATPase from other Ca^{2+} binding proteins. Occlusion experiments were carried out at Ca^{2+} concentrations ranging from 10 μM to 10 mM. The data obtained with the mutants Glu^{771}→Lys, Glu^{771}→Ala, and Glu^{771}→Gly at 10 mM Ca^{2+} are shown in Fig. 1. Neither of the mutants was able to occlude Ca^{2+} at any of the Ca^{2+} concentrations tested. Lack of Ca^{2+} occlusion has previously been demonstrated for mutant Glu^{771}→Gln at Ca^{2+} concentrations up to 1 mM [6]. In the present study, additional measurements were carried out with the latter mutant at 10 mM Ca^{2+}, but even in this condition no Ca^{2+} occlusion was detected (not shown).

3.2. Ca^{2+} dependency of phosphorylation

Fig. 2 shows that the mutants Glu^{771}→Ala, Glu^{771}→Gly, and Glu^{771}→Gln were able to become phosphorylated from ATP, albeit with a reduced apparent affinity for activating Ca^{2+}. The $K_{0.5}$ value for Ca^{2+} activation of mutant Glu^{771}→Ala was similar to the $K_{0.5}$ value of approximately 0.5 mM previously determined for mutant Glu^{771}→Gln [4,5], i.e. the apparent Ca^{2+} affinity was about 500-fold lower than that displayed by the wild-type enzyme. It is noteworthy that maximum phosphorylation was attained at a Ca^{2+} concentration at which there was no measurable Ca^{2+} occlusion (Fig. 1). Mutant Glu^{771}→Gly displayed a slightly higher $K_{0.5}$ value for Ca^{2+} activation than

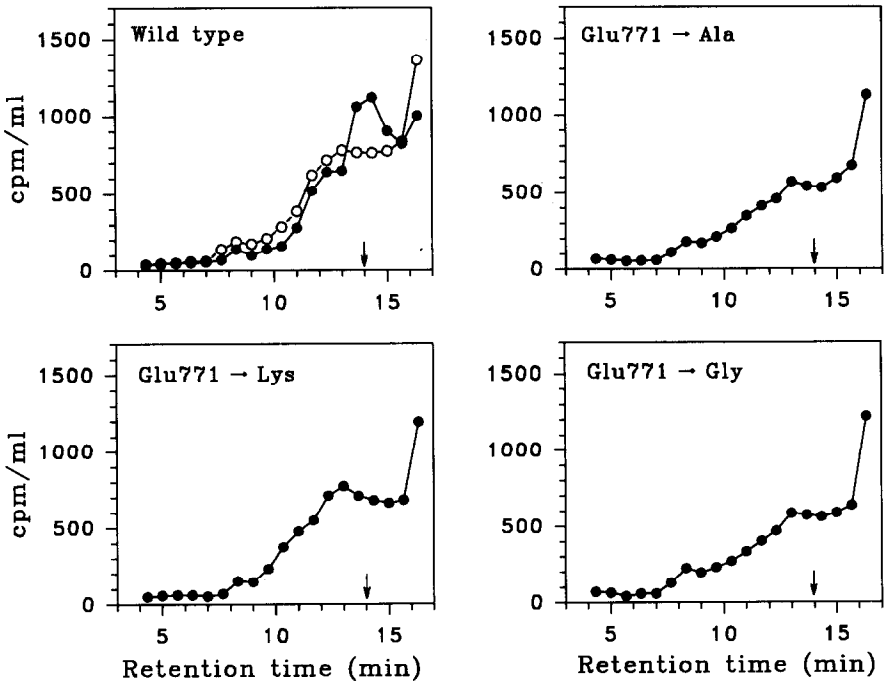

Fig. 1. HPLC-measurement of CrATP-induced Ca^{2+}-occlusion in the presence of 10 mM Ca^{2+}. Microsomal membranes from COS-1 cells transfected with cDNA encoding the wild-type Ca^{2+}-ATPase or mutant Glu^{771}→Ala, Glu^{771}→Gly, or Glu^{771}→Lys (●), or from cells transfected with expression vector without insert (○) were incubated in the presence of 10 mM $^{45}Ca^{2+}$ and CrATP, followed by solubilization and size-exclusion HPLC as described in section 2. Equivalent amounts of expressed wild-type or mutant Ca^{2+}-ATPase, as determined by phosphorylation capacity from $^{32}P_i$, were applied to the column in each experiment. The arrow indicates the elution position corresponding to monomeric Ca^{2+}-ATPase purified from fast skeletal muscle.

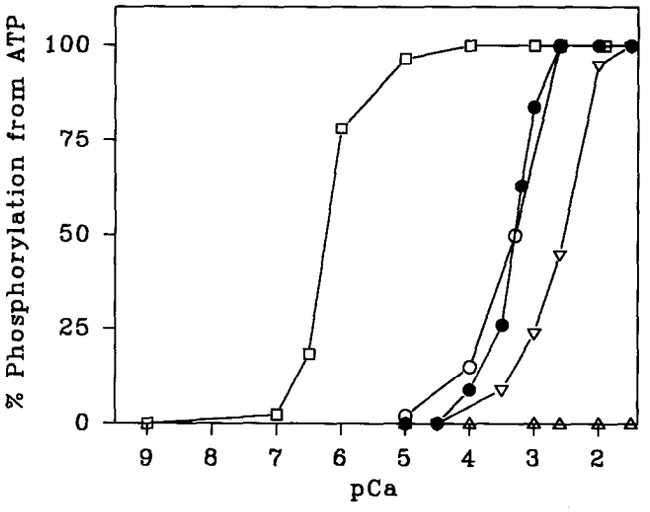

Fig. 2. Calcium dependency of phosphorylation from ATP. Phosphorylation of the Ca^{2+}-ATPase in microsomal fractions isolated from COS-1 cells transfected with mutant or wild-type Ca^{2+}-ATPase cDNA was carried out at 0°C for 15 s in the presence of 50 mM MOPS buffer (pH 7.0), 80 mM K^+, 5 mM Mg^{2+}, 2 μM [γ-^{32}P]ATP, and the indicated Ca^{2+} concentrations. (□) Wild-type Ca^{2+}-ATPase; (○) mutant $Glu^{771}\rightarrow Gln$ (data reproduced from [5] for comparative use); (●) mutant $Glu^{771}\rightarrow Ala$; (▽) mutant $Glu^{771}\rightarrow Gly$; (△) mutant $Glu^{771}\rightarrow Lys$.

mutant $Glu^{771}\rightarrow Ala$. The mutant with the positively charged substituent $Glu^{771}\rightarrow Lys$ was, on the other hand, completely unable to become phosphorylated from ATP up to the highest Ca^{2+} concentration tested (25 mM).

Like the wild-type Ca^{2+}-ATPase, the mutants $Glu^{771}\rightarrow Ala$, $Glu^{771}\rightarrow Gly$, and $Glu^{771}\rightarrow Lys$ could be phosphorylated 'backdoor' by inorganic phosphate (P_i) in the absence of Ca^{2+} (Fig. 3). In the wild-type enzyme, Ca^{2+} binding with high-affinity prevents this phosphorylation. The inhibition by Ca^{2+} of the phosphorylation from P_i may, therefore, provide additional information on the function of the Ca^{2+} sites. As seen in Fig. 3, Ca^{2+} was able to inhibit phosphorylation from P_i of mutants $Glu^{771}\rightarrow Ala$ and $Glu^{771}\rightarrow Gly$ with apparent affinities very similar to those observed for Ca^{2+} activation of phosphorylation from ATP (Fig. 2). In addition, there was no inhibitory effect of Ca^{2+} on phosphorylation from P_i of mutant $Glu^{771}\rightarrow Lys$ at any Ca^{2+} concentration tested.

3.3. *Dephosphorylation of the E2P phosphoenzyme intermediate*

In the Ca^{2+}-ATPase reaction cycle, the release of Ca^{2+} at the luminal side of the membrane is associated with formation of an ADP-insensitive phosphoenzyme intermediate, E2P, that appears to be identical to the phosphoenzyme intermediate formed in the backdoor reaction with P_i [15]. This is the phosphoenzyme intermediate which would be supposed to bind protons (or H_3O^+) at the luminal surface in a proton countertransport mechanism of the Ca^{2+}-ATPase. If proton countertransport were an obligatory part of the Ca^{2+}-ATPase cycle, the dephosphorylation should be regulated by the binding of the protons to be countertransported. In the present study, the dephosphorylation of the E2P intermediate was examined for mutants $Glu^{771}\rightarrow Ala$, $Glu^{771}\rightarrow Gly$, and $Glu^{771}\rightarrow Lys$ in kinetic experiments in which the phosphorylation with $^{32}P_i$ was terminated by dilution of the phosphorylated sample in excess nonradioactive P_i. As can be seen in Fig. 4, the dephosphorylation rates of mutants $Glu^{771}\rightarrow Ala$ and $Glu^{771}\rightarrow Gly$ were strongly reduced relative to the dephosphorylation rate of the wild type. By contrast, dephosphorylation of mutant $Glu^{771}\rightarrow Lys$ proceeded at a high rate.

4. Discussion

In the normal transport cycle of the wild-type Ca^{2+}-ATPase, the two calcium ions bind at cytoplasmically facing high-affinity transport sites through a stepwise mechanism in which the binding of the first calcium ion is sufficient to prevent phosphorylation from P_i whereas the simultaneous binding of both calcium ions is needed for Ca^{2+} occlusion and for activation of phosphorylation from ATP [16–18]. The present data suggest that mutant $Glu^{771}\rightarrow Lys$ is completely unable to bind at least the first calcium ion in the sequence. Thus, Ca^{2+} did not inhibit phosphorylation from P_i of this mutant, and there was no activation by Ca^{2+} of phosphorylation from ATP or occlusion of Ca^{2+}, at Ca^{2+} concentrations corresponding to more than 10,000-fold the Ca^{2+} concentration giving half-maximum effect in the wild type. On the other hand, Ca^{2+} appeared to bind at both sites in mutants $Glu^{771}\rightarrow Ala$ and $Glu^{771}\rightarrow Gly$, albeit with a 500–2,000-fold reduction in affinity, as demonstrated by the effect of high concentrations of Ca^{2+} on phosphorylation from ATP and P_i. There was no significant difference between the apparent Ca^{2+} affinity of mutant $Glu^{771}\rightarrow Ala$ and that determined previously for mutant $Glu^{771}\rightarrow Gln$ [4,5], suggesting that the carboxamide oxygen of the glutamine substituent is of little importance for Ca^{2+} binding in the $Glu^{771}\rightarrow Gln$ mutant. It is possible that in the wild-type enzyme the bound Ca^{2+} interacts only with the oxygen atom which was removed upon replacement of glutamate with glutamine. There seems to be an unspecific requirement for a side chain since the Ca^{2+} affinity of mutant $Glu^{771}\rightarrow Gly$ was somewhat lower than that of mutants $Glu^{771}\rightarrow Ala$ and $Glu^{771}\rightarrow Gln$.

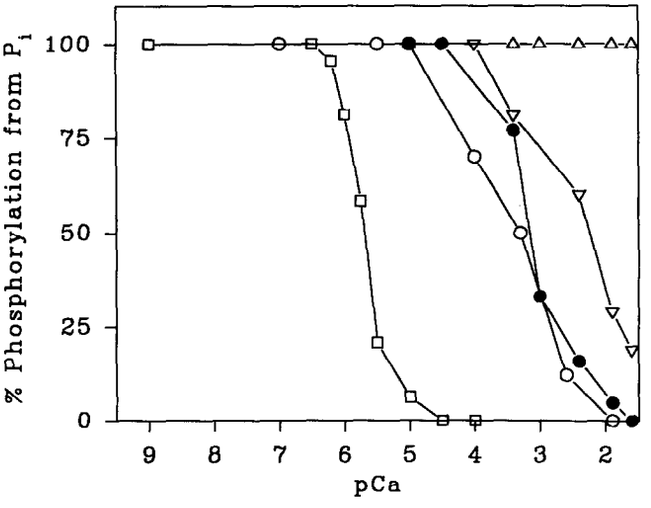

Fig. 3. Calcium dependency of phosphorylation from P_i. Phosphorylation was carried out for 10 min at 25°C in the presence of 100 μM $^{32}P_i$, 100 mM TES/Tris (pH 7.0), 5 mM Mg^{2+}, 20% (v/v) dimethylsulfoxide and the indicated Ca^{2+} concentrations set with EGTA. (□) Wild-type Ca^{2+}-ATPase; (○) mutant $Glu^{771}\rightarrow Gln$ (data reproduced from [5] for comparative use); (●) mutant $Glu^{771}\rightarrow Ala$; (▽) mutant $Glu^{771}\rightarrow Gly$; (△) mutant $Glu^{771}\rightarrow Lys$.

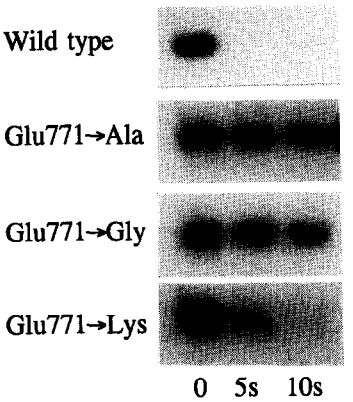

Fig. 4. Dephosphorylation of E2P formed by phosphorylation with P_i. Wild-type and mutant Ca^{2+}-ATPases were phosphorylated at 25°C for 10 min in the presence of 100 μM $^{32}P_i$, 100 mM TES/Tris (pH 7.0), 5 mM Mg^{2+}, 2 mM EGTA, and 20% (v/v) dimethylsulfoxide. Following cooling of the sample to 0°C, dephosphorylation was initiated by 20-fold dilution of an aliquot into ice-cold medium containing 50 mM MOPS (pH 7.0), 80 mM K^+, 5 mM Mg^{2+}, 1 mM non-radioactive P_i, and 2 mM EGTA, and acid quenching was performed 5s or 10s after the dilution. The acid-quenched samples containing equivalent amounts of expressed Ca^{2+}-ATPase were subjected to SDS-polyacrylamide gel electrophoresis at, pH 6.0, and the autoradiograms of the dried gels are shown.

The prevention of Ca^{2+} binding in mutant Glu^{771}→Lys may result from the bulkiness of the lysine side chain and/or electrostatic repulsion of the calcium ion by the positive charge of the amino group, in line with the hypothesis that Glu^{771} is an integral part of the Ca^{2+} binding structure. The finding that Ca^{2+} was not occluded in mutants Glu^{771}→Ala and Glu^{771}→Gly, even at Ca^{2+} concentrations at which these mutants were clearly able to bind Ca^{2+}, suggests that occlusion demands the very stable interaction of the calcium ions with the site reflected in the normal high Ca^{2+} affinity of the wild type.

One of the aims of the present study was to test the hypothesis that the side chain of Glu^{771} participates in the binding of H^+ (possibly H_3O^+) to be countertransported. The removal of the negative charge by substitution of the glutamate with either glutamine, alanine, or glycine led to enzymes for which the dephosphorylation of the E2P phosphoenzyme intermediate was blocked, while the mutant in which lysine was substituted for Glu^{771} was able to dephosphorylate rapidly. Thus, the presence of the positive charge of the lysine side chain seems to induce dephosphorylation. For the wild-type enzyme, the processing of the phosphoenzyme as well as the overall turnover of the pump can be blocked by increasing the pH [19]. The mutagenesis data support the hypothesis that normally the binding of positively charged H^+ or H_3O^+ in the vicinity of the Glu^{771} side chain is required for dephosphorylation of E2P and thus for turnover of the pump. A stoichiometry of at least two positive charges bound near Glu^{771} appears to be obligatory for dephosphorylation, because a single positive charge (corresponding to the neutral substituents Gln, Ala, and Gly) was insufficient to induce dephosphorylation. On the basis of the present data it may thus be inferred that countertransport of at least two H^+ (or H_3O^+) in exchange for the two Ca^{2+} taken up per enzyme cycle is obligatory at neutral pH, and that Glu^{771} plays a key role in this countertransport, as well as in the binding and occlusion of Ca^{2+}.

It is interesting that recently chemical modification of the homologous counterpart in Na^+,K^+-ATPase, Glu^{779}, was shown to result in loss of the ability to bind and occlude Na^+ as well as K^+ [20,21]. The Ca^{2+}-ATPase and the Na^+,K^+-ATPase may work by similar mechanisms, possibly involving consecutive exposure of the glutamic acid residue in transmembrane segment M5 on either side of the membrane to mediate the transport of cations in both directions.

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