Glutamic acid 327 in the sheep $\alpha 1$ isoform of Na^+, K^+ -ATPase is a pivotal residue for cation-induced conformational changes

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The cation binding characteristics of the mutant E327A formed in the sheep $\alpha 1$ isoform of the Na^+, K^+ -ATPase were examined using [^3H]ouabain binding as a function of monovalent cation concentrations. Equilibrium competition binding assays in the presence of Mg^{2+} , inorganic phosphate and various amounts of unlabelled ouabain indicated that both wild-type sheep $\alpha 1$ protein and the E327A mutant expressed in 3T3 cells had similar affinities for ouabain ($K_D = 1.53$ and 1.31 nM respectively). Sodium inhibition of ouabain binding appeared competitive in both enzymes. However, binding of three Na^+ ions was required to explain the steep character of the Na^+ inhibition curve for the wild-type Na^+, K^+ -ATPase ($K_i = 12.8 \pm 1.6$ mM), whereas the binding of two Na^+ ions was detected for the mutant E327A ($K_i = 19.2 \pm 2.5$ mM). Potassium inhibition of [^3H]ouabain binding displayed a partially competitive nature with Hill co-

efficients of 2 for both wild-type sheep $\alpha 1$ ($K_i = 0.743 \pm 0.044$ mM) and E327A ($K_i = 0.875 \pm 0.067$ mM). At concentrations of K^+ above 10 mM, the sheep $\alpha 1$ competition curve levelled off whereas the inhibition curve for E327A displayed a stimulation in ouabain binding. This stimulation in [^3H]ouabain binding also occurred with Rb^+ , Cs^+ and Li^+ , but was never observed with choline or Na^+ , suggesting that this effect was not due to ionic strength. From these [^3H]ouabain-binding studies, it is obvious that the mutant enzyme E327A in the presence of Mg^{2+} , P_i and ouabain, interacts with monovalent cations in a unique fashion. One interpretation of these data is that the glutamic acid residue at position 327 is involved in a conformational transition induced by the binding of monovalent cations to the Na^+, K^+ -ATPase and that this transition is inhibited by the mutation of E327A.

INTRODUCTION

Na^+, K^+ -ATPase couples the transport of monovalent cations across the plasma membrane with the hydrolysis of ATP. During the catalytic cycle, three sodium ions are pumped out of the cell for every two potassium ions transported into the cell (reviewed in [1,2]). The enzyme mechanism of Na^+, K^+ -ATPase is comprised of numerous conformational changes in the protein induced by the binding of ligands (i.e. Na^+ , K^+ and ATP). One intermediate in the cycle involves a phosphorylated enzyme complex, a characteristic shared by all P-type ATPases [i.e. sarcoplasmic reticulum (SR) Ca^{2+} -ATPase, H^+, K^+ -ATPase and Mg^{2+} -ATPase]. Na^+, K^+ -ATPase is unique among the ATPases in that it is inhibited by cardiac glycosides (such as ouabain and digoxin) making the understanding of its mechanism both clinically and physiologically relevant.

Na^+, K^+ -ATPase binds cardiac glycosides with highest affinity under two different incubation conditions: media containing relatively high concentrations of Mg^{2+} and inorganic phosphate with no added monovalent cations, and media containing ATP, Mg^{2+} and high concentrations of Na^+ . In the present work, we report studies of the effects of monovalent cations on [^3H]ouabain binding under Mg^{2+} - P_i conditions. These monovalent cation effects have previously been described in reference to purified Na^+, K^+ -ATPase and are hypothesized to report functionally important conformational alterations of the enzyme (reviewed in [3]).

The amino acids of the Na^+, K^+ -ATPase involved in binding

and translocating cations have not yet been identified. One theory suggests that negatively charged residues within the membrane are important as neutralizing groups to carry the cations across the lipid bilayer. Thus, these transmembrane, anionic residues have been targets in chemical modification and site-directed mutagenesis studies.

Chemical labelling experiments performed with the hydrophobic, carboxyl-specific reagent, dicyclohexylcarbodiimide, have implicated two glutamic acid residues within the transmembrane domains which may be involved in neutralizing and transporting monovalent cations [4]. These residues are positioned at 327 and 955 in the amino acid sequence of the sheep $\alpha 1$ isoform. Site-directed mutagenesis, expression and kinetic characterization of modifications made at positions E955 and E956 showed that these mutant enzymes have no marked reduction in their cation dependence of ATPase activity [5]. In contrast, when E327 was altered either to a leucine or a glutamine residue, a decrease in both the apparent sodium-dependence and potassium-dependence of ATPase activity was observed [6]. These mutagenesis studies involved the expression of a ouabain-resistant form of Na^+, K^+ -ATPase (rat $\alpha 2^*$ isoforms) in cells which possess an ouabain-sensitive endogenous protein (e.g. HeLa cells) and growing these transfected cells in the presence of the drug. This selection scheme is limited in that if the cells do not possess an active ouabain-resistant enzyme, the cells do not survive the ouabain selection. Hence, any mutation rendering the expressed enzyme severely impaired will result in no stable cell lines. This was the case when E327 was altered to alanine [7].

Abbreviations used: E327A, a mutant sheep $\alpha 1$ isoform of Na^+, K^+ -ATPase in which the glutamic acid at position 327 is altered to an alanine residue; rat $\alpha 2^*$, a mutant rat isoform of Na^+, K^+ -ATPase in which the following changes have been introduced to make the protein ouabain-resistant: L111R and N122D; G418, geneticin (a neomycin analogue); SR, sarcoplasmic reticulum; AC_{50} , concentration producing half-maximal activation.

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Although these original site-directed mutagenesis studies revealed that E327 was extremely important in the catalytic turnover of Na^+, K^+ -ATPase, only functional, ouabain-resistant mutant proteins can be analysed by ATPase activity measurements.

Our objective in this study was to establish an expression and assay system by which functionally impaired enzymes could be probed to identify which step in the catalytic cycle was affected by the mutated site. The expression system we utilized had previously been developed to study mutations which might alter the affinity of Na^+, K^+ -ATPase for ouabain [8]. This system involved construction of the functionally impaired mutation in the ouabain-sensitive sheep $\alpha 1$ cDNA and co-transfecting this mutant cDNA along with a neomycin-resistance gene into a ouabain-resistant cell line (NIH 3T3 mouse cell line). Thus, successfully transfected cells were detected by their ability to survive in the presence of 400 $\mu\text{g}/\text{ml}$ of G418. This expression system does not require that the transfected Na^+, K^+ -ATPase be capable of transporting cations. With this expression system, [^3H]ouabain binding could be employed as a convenient, highly sensitive and highly specific probe for the exogenously expressed sheep $\alpha 1$ Na^+, K^+ -ATPase without interference from the endogenous mouse enzyme. Moreover, monovalent cation effects on [^3H]ouabain binding would demonstrate conformational changes induced by cation interactions with the exogenously expressed Na^+, K^+ -ATPase enzyme.

In this study, clonal NIH 3T3 cell lines were established that expressed either a wild-type or the E327A mutation of the sheep $\alpha 1$ isoform of Na^+, K^+ -ATPase. [^3H]Ouabain binding was studied in the presence of Mg^{2+} and inorganic phosphate (P_i). Characterization of [^3H]ouabain binding as a function of monovalent cation concentrations revealed several unique differences between the wild-type and the E327A mutant enzymes in their response to Na^+ and K^+ .

EXPERIMENTAL

Materials

[^3H]Ouabain was purchased from DuPont–New England Nuclear and ouabain was from Calbiochem or Sigma. The specific radioactivity of [^3H]ouabain was determined as previously described [9] and was calculated to be 1.69×10^{-5} pmol/d.p.m. Cell culture supplies were purchased from Gibco, Life Technologies, Inc. and Fisher. All other reagents (NaCl , KCl , Tris base, HCl , phosphoric acid, MgCl_2) were from Fisher.

NIH 3T3 cell clones and tissue culture

G418-resistant cell lines expressing the wild-type sheep $\alpha 1$ cDNA and cDNA encoding the sheep $\alpha 1$ mutant E327A were established using the procedure previously described [8]. These cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum and 400 $\mu\text{g}/\text{ml}$ G418 [10].

Isolation of crude plasma membranes from NIH 3T3 cells

Crude membranes were isolated from transfected NIH 3T3 cells as previously described [8]. The crude membranes were treated with NaI for further purification [11].

Western-blot analysis

Proteins contained in the NaI -treated plasma membrane fraction

were separated on a 7.5% SDS/polyacrylamide gel and electroblotted on to pure nitrocellulose at 350 mA for 90 min in 25 mM Tris/ HCl (pH 8.0). The filter was probed with a 1:1000 dilution of M7-PB-E9, a sheep $\alpha 1$ -specific antibody which does not cross-react with mouse $\alpha 1$ Na^+, K^+ -ATPase [12,13]. An anti-(mouse IgG) horseradish peroxidase-conjugated secondary antibody was purchased from Calbiochem. The blots were developed using the Enhanced Chemiluminescence system of Amersham.

[^3H]Ouabain binding to crude membrane fragments isolated from NIH 3T3 cells

All ouabain-binding studies were conducted under the following conditions, unless otherwise indicated in the Figure legends: 5 mM MgCl_2 , 5 mM Tris-phosphate, 50 mM Tris/ HCl (pH 7.4) in a final volume of 0.5 ml, 37 °C, incubation time 6 h. The amount of protein used was varied depending on the specific activity of the membrane preparation but typically was in the range of 20–100 $\mu\text{g}/\text{assay tube}$. Experiments were carried out in plastic tubes. For the competition curves with unlabelled ouabain, eight concentrations of unlabelled ouabain (including zero) were used in triplicate. The concentrations of [^3H]ouabain and unlabelled ouabain are indicated in the Figures and the Results section. Aliquots of the reaction mixture were taken in every experiment to estimate the concentration of [^3H]ouabain. Ligand and enzyme concentrations were adjusted such that no more than about 10% of the added ouabain was bound. Thus the free ligand concentration was essentially equal to the added ouabain concentration in all of the experiments shown here and the total concentration was used in place of the free concentration in the equations described in the Results section. Following incubation, the samples were aspirated on to glass-fibre filters using the Brandel M24R Cell Harvester and the filters were washed with 4 $\times$ 5 ml of cold water. Filters were counted in RPI Budget-Solve in a Packard 2000CA scintillation counter with an efficiency of approximately 42%. Monovalent cation inhibition experiments were conducted under the same incubation conditions as described above. Eleven to fifteen concentrations of each cation (including zero) were used in duplicate to define the inhibition curve. The [^3H]ouabain concentrations are given in the Figure legends. Data were plotted and curve fits obtained using KaleidaGraph by Abelbeck Software.

RESULTS

Western-blot analysis of NIH 3T3 cell lines

A Western blot was performed to confirm that membrane fractions isolated from clonal NIH 3T3 cell lines co-transfected with sheep $\alpha 1$ cDNAs and a neomycin-resistance gene, expressed the sheep $\alpha 1$ proteins of interest. The blot was stained with the $\alpha 1$ subunit-specific monoclonal antibody M7-PB-E9 which is able to distinguish the transfected sheep $\alpha 1$ isoform from the endogenous mouse $\alpha 1$ isoform. A single band of protein at the molecular mass of approx. 110 kDa was probed by the monoclonal antibody M7-PB-E7. Figure 1 shows the band of protein representing the expression of the wild-type and E327A mutant proteins and the absence of this band in untransfected NIH 3T3 cells. The sheep $\alpha 1$ protein detected by Western-blot analysis was confirmed to be the E327A mutant by PCR amplification of genomic DNA and sequencing of the sheep $\alpha 1$ coding region. Genomic DNA from all mutant cell lines contained the E327A alteration (data not shown).

Figure 1 Western-blot analysis of NIH 3T3 membrane fractions

Immunoblot staining of membrane fractions isolated from NIH 3T3 clonal lines co-transfected with a neomycin-resistance gene and either a wild-type sheep $\alpha 1$ cDNA or a mutant sheep $\alpha 1$ E327A cDNA. Each lane contains 20 μ g of protein. The blot was stained with a sheep $\alpha 1$ subunit-specific monoclonal antibody M7-PB-E9 [12,13] and anti-(mouse IgG) horseradish peroxidase-conjugated secondary antibody. The arrows represent the positions of the prestained molecular-mass markers, phosphorylase b and BSA (Bio-Rad) which were used as references to identify the protein band which contained the Na⁺,K⁺-ATPase.

Ouabain competition curves in wild-type and E327A membranes

The binding constant for ouabain in the absence of cations had to be determined prior to studying the effects of monovalent cations on ouabain binding. Competition curves were carried out using [³H]ouabain concentrations (1.69–2.10 nM) approximately equal to the K_D for ouabain. The data were fitted to the simple competition model (Scheme 1 and eqn. 1):

Figure 2 Ouabain competition curves

[³H]Ouabain binding was measured in wild-type membranes (■) or E327A membranes (●) in the absence and presence of various concentrations of unlabelled ouabain as shown on the x-axis. The symbols represent the mean of triplicate determinations. S.E.M. values were calculated for each point but are smaller than the symbol size and are not visible in the Figure. Assay conditions were as described in the Experimental section. Sample sizes of 72 μ g of wild-type protein or 138 μ g of E327A protein were used per assay tube. The concentration of [³H]ouabain was 1.69 nM based on standards taken from the incubation mixture. The solid lines represent curve fits to eqn (1) as described in the text. The fitted parameters were: wild type ($K_D = 1.60 \pm 0.14$ nM; $E_T = 0.102 \pm 0.002$ nM; NS = 0.000585 ± 0.000017) and E327A ($K_D = 1.67 \pm 0.13$ nM; $E_T = 0.110 \pm 0.015$ nM; NS = 0.000365 ± 0.000012).

Scheme 1

$$B = \frac{E_T \left(\frac{[O]}{K_D} \right)}{1 + \frac{[O]}{K_D} + \frac{[I]}{K_D}} + NS[O] \quad (1)$$

where [I] is the concentration of unlabelled ouabain, E_T is the amount of enzyme, [O] is the concentration of [³H]ouabain, and NS is the proportionality constant for non-specific binding. Figure 2 shows a comparison of the competition curves for one membrane preparation from wild-type and E327A cells. Eqn (1) was fitted to the data with E_T , K_D and NS as adjustable parameters. The mean K_D values obtained for three different wild-type and three different E327A membrane preparations were 1.53 ± 0.08 nM and 1.31 ± 0.21 nM respectively. These average K_D values were used in the fits of the potassium and sodium inhibition curves described below. It is clear that under

Figure 3 Na⁺ inhibition curves

[³H]Ouabain binding was measured in wild-type membranes (●) or E327A membranes (■) in the presence of various concentrations of NaCl as shown on the x-axis. The symbols represent the mean of duplicate determinations. The error bars represent the range of the duplicate determinations and are not shown if smaller than the symbol size. Assay conditions were as described in the Experimental section. Sample sizes of 43 μ g of wild-type protein or 49 μ g of E327A protein were used per assay tube. The concentration of [³H]ouabain was 10.3 nM based on standards taken from the incubation mixture. The solid lines in the main Figure represent curve fits to eqn (2) as described in the text. The fitted parameters were: wild type ($K_i = 15.7 \pm 0.1$ mM; $E_T = 0.241 \pm 0.003$ nM) and E327A ($K_i = 24.2 \pm 0.4$ mM; $E_T = 0.289 \pm 0.003$ nM). The inset shows the same data fitted to a four-parameter logistic function: $B = (B_{max} - B_{min}) / \{1 + ([Na^+]^{n_H} / IC_{50}^{n_H})\}$ where B_{max} is the amount bound at zero sodium, B_{min} is the amount bound at saturating sodium, IC_{50} is the concentration of sodium giving half-maximal binding, and n_H is the pseudo-Hill coefficient. The fitted parameter values were: wild type ($IC_{50} = 26.0 \pm 0.5$ mM; $n_H = 2.83 \pm 0.06$) and E327A ($IC_{50} = 71.3 \pm 1.6$ mM; $n_H = 1.80 \pm 0.06$).

these binding conditions the E327A mutation does not significantly affect the affinity for ouabain binding. For these three experiments, non-specific binding, expressed as a percentage of total binding, was $1.21 \pm 0.26\%$ for the wild type and $0.95 \pm 0.11\%$ for E327A. This non-specific binding was almost entirely due to adsorption to the filters during the filtration step and, at least for the range of enzyme concentrations used in this study, was essentially independent of the amount of protein filtered. The average value for NS calculated from combined wild-type and E327A data was 0.000366 ± 0.000073 ($n = 6$).

To ensure that all Mg^{2+} and P_i sites on the enzymes were occupied under experimental conditions, activation curves for these ions were carried out in the presence of 50 mM Tris/HCl (pH 7.4) and 10 nM [3H]ouabain (data not shown). The apparent AC_{50} values for P_i in the presence of 5 mM Mg^{2+} were 0.18 ± 0.01 mM and 0.079 ± 0.002 mM for wild-type sheep $\alpha 1$ and E327A, respectively. The apparent AC_{50} values for Mg^{2+} at 5 mM P_i were 0.20 ± 0.05 mM and 0.26 ± 0.03 mM for wild-type sheep $\alpha 1$ and E327A, respectively. Thus, all subsequent experiments were performed with 5 mM P_i and 5 mM Mg^{2+} to ensure that the enzymes were saturated with respect to these ions.

Sodium inhibition curves for wild-type and E327A enzymes

We have consistently found, both in partially purified sheep kidney Na^+, K^+ -ATPase as well as in 3T3 membranes, that saturating concentrations of Na^+ decrease binding to the same extent as saturating concentrations of ouabain. Typical inhibition curves are shown in Figure 3. Experiments were conducted with three different wild-type and three different E327A membrane preparations. The inhibition curves were first fitted to a Hill equation which yielded average values for the Hill coefficient of 2.84 ± 0.09 for the wild type and 1.96 ± 0.12 for E327A. These fits suggested that three Na^+ ions were required to explain Na^+ inhibition in the wild-type membranes but only two ions appeared to be necessary for E327A. On the basis of this evidence we chose to fit the wild-type data to a simple competition model involving three Na^+ ions (Scheme 2).

Scheme 2

The amount of [3H]ouabain bound as a function of Na^+ concentration is given by eqn (2):

$$B = \frac{E_T \left(\frac{[O]}{K_D} \right)}{1 + \frac{[Na^+]}{K_1} + \frac{[Na^+]^2}{K_1^2} + \frac{[Na^+]^3}{K_1^3} + \frac{[O]}{K_D}} + NS[O] \quad (2)$$

Figure 4 K^+ inhibition curves in wild-type membranes

[3H]Ouabain binding was measured in wild-type membranes in the presence of various concentrations of KCl as shown on the x-axis. The symbols represent the mean of duplicate determinations. The error bars represent the range of the duplicate determinations and are not shown if smaller than the symbol size. Assay conditions were as described in the Experimental section. A sample size of 29 μ g of wild-type protein was used per assay tube. The concentration of [3H]ouabain was 2.14 nM (■) or 30.3 nM (●) based on standards taken from the incubation mixture. The solid lines represent curve fits to eqn (3) as described in the text. The fitted parameters were: for [3H]ouabain = 2.14 nM ($K_i = 0.916 \pm 0.024$ mM; $\alpha = 10.1 \pm 0.2$; $E_1 = 0.120 \pm 0.002$ nM) and for [3H]ouabain = 30.3 nM ($K_i = 0.817 \pm 0.039$ mM; $\alpha = 12.1 \pm 0.2$; $E_1 = 0.123 \pm 0.002$ nM).

The E327A data were fitted to the same model but modified to include only two Na^+ ions. The K_D values for ouabain were fixed at the values determined from the competition experiments described in the previous section (1.53 and 1.31 nM for wild type and E327A). NS was fixed at 0.000366. For the three membrane preparations, the average values of the binding constants for Na^+ were: 12.8 ± 1.6 mM for wild type and 19.2 ± 2.5 mM for E327A.

It is important to note that a partially competitive model, such as we have used to describe K^+ inhibition of ouabain binding (see below), would also fit the data if Na^+ were to decrease the affinity for ouabain to such an extent that no significant binding could occur at the labelled ligand concentrations that can be used in these assays. In this case a partially competitive model with a very large interaction factor would give the same results as a pure competition model. Although we have chosen to fit our Na^+ inhibition data to the competition model shown in Scheme 2, we do not mean to imply that Na^+ and ouabain necessarily bind to the exact same site.

Potassium inhibition curves for wild-type and E327A enzymes

In contrast to the situation with Na^+ , we have consistently found, both in partially purified sheep kidney Na^+, K^+ -ATPase and in sheep $\alpha 1$ expressed in 3T3 cells, that saturating concentrations of K^+ do not inhibit binding to the same extent as saturating concentrations of unlabelled ouabain, suggesting that a partially competitive model is more appropriate than a pure competitive model for fitting the K^+ inhibition curves. Typical curves are shown in Figure 4 (wild-type membranes) and Figure 5 (E327A membranes) using two different [3H]ouabain concentrations.

Figure 5 K⁺ inhibition curves in E327A membranes

[³H]Ouabain binding was measured in E327A membranes in the presence of various concentrations of KCl as shown on the x-axis. The symbols represent the mean of duplicate determinations. The error bars represent the range of the duplicate determinations and are not shown if smaller than the symbol size. Assay conditions were as described in the Experimental section. A sample size of 26 μ g of E327A protein was used per assay tube. The concentration of [³H]ouabain was 2.14 nM (■) or 30.3 nM (●) based on standards taken from the incubation mixture. The solid lines represent curve fits to eqn (4) as described in the text. The fitted parameters were: for [³H]ouabain = 2.14 nM ($K_i = 0.879 \pm 0.092$ mM; $\alpha = 2.76 \pm 0.11$; $K_D = 152 \pm 71$ mM; $\beta = 1.40 \pm 0.31$; $E_T = 0.117 \pm 0.003$ nM). The curve with 30.3 nM [³H]ouabain could not be fitted due to the small magnitude of the signal (the solid line is a smoothed curve, not a fit to the model).

Figure 6 Choline inhibition curves in wild-type and E327A membranes

[³H]Ouabain binding was measured in wild-type membranes (■) and E327A membranes (●) in the presence of various concentrations of choline chloride as shown on the x-axis. The symbols represent the mean of duplicate determinations. The error bars represent the range of the duplicate determinations and are not shown if smaller than the symbol size. Assay conditions were as described in the Experimental section. Sample sizes of 35 μ g of wild-type protein or 22 μ g of E327A protein were used per assay tube. The concentration of [³H]ouabain was 11.1 nM based on standards taken from the incubation mixture. The solid lines represent curve fits to a Hill equation.

These experiments were repeated with three different wild-type and three different E327A membrane preparations, including two different E327A clones. The patterns shown in Figures 4 and

5 were highly reproducible. The magnitude of the 'dip' in the E327A curve depended on the concentration of [³H]ouabain and was maximized by low-ligand concentrations (e.g. the maximum percentage inhibition was 68.4 %, 45.0 %, and 16.5 % at 0.76, 10.0, and 30.3 nM [³H]ouabain respectively).

For the wild-type enzyme, the inhibition curves were first fitted to a Hill equation which yielded an average Hill coefficient of 1.65 ± 0.05 . These fits suggested that two K⁺ ions were required to explain K⁺ inhibition in the wild-type membranes. On the basis of this evidence we chose to fit the wild-type data to a partially competitive (heterotropic negative cooperativity) model involving two K⁺ ions (Scheme 3).

Scheme 3

In this model, E is the enzyme, O is ouabain, K_D is the binding constant for ouabain, K_i is the binding constant for each K⁺ ion, and α is the interaction factor per K⁺ ion (that is, one K⁺ ion increases the binding constant for ouabain from K_D to αK_D and the second K⁺ ion increases ouabain's binding constant to $\alpha^2 K_D$). Thus, for an α value of 10, the K_D for ouabain will be increased by a factor of 100 at saturating K⁺. The amount of bound [³H]ouabain (B) as a function of K⁺ concentration is given by eqn (3):

$$B = \frac{E_T \left(\frac{[O]}{K_D} + \frac{[O][K^+]}{\alpha K_D K_i} + \frac{[O][K^+]^2}{\alpha^2 K_D K_i^2} \right)}{1 + \frac{[K^+]}{K_i} + \frac{[K^+]^2}{K_i^2} + \frac{[O]}{K_D} + \frac{[O][K^+]}{\alpha K_D K_i} + \frac{[O][K^+]^2}{\alpha^2 K_D K_i^2}} + NS[O] \quad (3)$$

E_T is the total enzyme concentration, [O] is the concentration of [³H]ouabain, and NS is the proportionality constant for non-specific binding. The K_D value for ouabain was fixed at the value determined from the competition experiments described in a previous section (1.53 nM). NS was fixed at 0.000366. The average parameter values for the wild-type enzyme determined from five experiments (with three different membrane preparations and three ranges of [³H]ouabain concentration, 2, 10 and 30 nM) were: $K_i = 0.743 \pm 0.044$ mM and $\alpha = 16.9 \pm 3.6$ ($n = 5$).

It is obvious from the data shown in Figure 5 that a different model for K⁺ inhibition must be operative in the E327A mutant compared with the wild type. The inhibitory phase of the curve occurred in exactly the same K⁺ concentration range as for the wild type. In addition, from an examination of the general shape of the curves, we concluded that the inhibition phase was clearly more steep than the activation phase. We therefore considered various models involving inhibition by two K⁺ ions and activation by a third K⁺ ion. The inhibition could be caused by a decreased affinity for ouabain (heterotropic negative cooperativity) as in the wild-type model, and the activation could be due to an

increase in ouabain affinity (heterotropic positive cooperativity). A model that fits our data quite well is shown in Scheme 4:

Scheme 4

The amount of bound [³H]ouabain as a function of K⁺ concentration is given by eqn (4):

$$B = \frac{E_T \left(\frac{[O]}{K_D} + \frac{[O][K^+]}{\alpha K_D K_i} + \frac{[O][K^+]^2}{\alpha^2 K_D K_i^2} + \frac{[O][K^+]^3}{\beta K_D K_a K_i^2} \right)}{1 + \frac{[K^+]}{K_i} + \frac{[K^+]^2}{K_i^2} + \frac{[K^+]^3}{K_a K_i^2} + \frac{[O]}{K_D} + \frac{[O][K^+]}{\alpha K_D K_i} + \frac{[O][K^+]^2}{\alpha^2 K_D K_i^2} + \frac{[O][K^+]^3}{\beta K_D K_a K_i^2}} + NS[O] \quad (4)$$

The K_D for ouabain was fixed at 1.31 nM and NS was fixed at 0.000366. Fitting the data from four experiments with three different E327A membrane preparations and concentrations of [³H]ouabain between 2 and 10 nM gave the following average values for the parameters: $K_i = 0.875 \pm 0.067$ mM; $\alpha = 3.37 \pm 0.33$; $K_a = 84 \pm 28$ mM; $\beta = 1.98 \pm 0.37$. All of the parameters are reasonably well described, but K_a and β have very large errors. This is because this portion of the curve is incomplete. We have found (data not shown) that concentrations of K⁺ above about 150 mM cause a progressive decrease in binding, indicating the operation of an additional inhibition mechanism at high concentrations (see also Figure 7). This could represent a non-specific effect due to high ionic strength (see next paragraph). Whatever the cause, this decrease in binding severely limits the extent to which the activation phase can be reliably modelled. Increasing the number of data points would in theory increase the precision of the model in this region, but this will be limited by the magnitude of the activation portion of the curve. As previously noted, the magnitude of the 'dip' in the curve is determined by the [³H]ouabain concentration. Simulation studies of Scheme 4 using the parameter values given above showed that the maximum inhibition approached a limiting value of about 83 % as the [³H]ouabain is decreased. Lowering the [³H]ouabain concentration, of course, decreases the amount bound so that the amount of enzyme used would have to be markedly increased to maintain a reasonable signal. This creates potential problems with ligand depletion as well as practical problems with supply of mutant enzyme. Since the major purpose of the present study was not to provide precise values for the binding parameters that define the activation phase, we have not pursued this issue further.

As noted above there is a potential problem with ionic strength effects at high concentrations of cations. This is clearly not the explanation for the 'dip' in the mutant curve since this occurs at

about 10 mM K⁺ whereas this concentration of Na⁺ has almost no effect. However, the inhibition with K⁺ that occurs above 150 mM could be due to non-specific effects since choline chloride also inhibits ouabain binding at high concentrations (Figure 6). The IC₅₀ values from fits to a Hill equation were 196 and 198 mM for wild type and mutant respectively. It should be emphasized, however, that this effect of choline is not necessarily non-specific. It is not impossible that choline may interact with Na⁺,K⁺-ATPase in the same way as monovalent cations. Indeed, it has been suggested that choline exerts a Na⁺-like effect on the enzyme [14]. If this were the case with choline, then it is also possible that the inhibition seen with high concentrations of K⁺ (> 150 mM) might also be due to a Na⁺-like effect.

Effects of other monovalent cations

In view of the clear distinction between the inhibition curves for K⁺ and Na⁺ on the E327A membranes, it was of interest to examine other cations. Figure 7 shows the inhibition curve for Rb⁺, a congener of K⁺. The patterns of Rb⁺ inhibition are virtually identical to that observed with K⁺ for both membrane preparations. The Rb⁺ inhibition parameters for the wild type were $K_i = 0.841 \pm 0.056$ mM, $\alpha = 7.03 \pm 1.96$ ($n = 3$). The initial inhibition phase for E327A had $K_i = 1.26 \pm 0.06$ mM, $\alpha =$

2.10 ± 0.12 ($n = 3$). The activation phase had $K_a = 21.0 \pm 3.5$ mM, $\beta = 1.52 \pm 0.09$. As with K⁺ and choline, concentrations of Rb⁺ > 150 mM exert an additional inhibition of binding in the E327A preparation. Thus, the initial inhibition phase for both K⁺ and Rb⁺ on wild type and E327A had a K_i of about 1 mM. The interaction factor α was lower for Rb⁺ than for K⁺, suggesting that each Rb⁺ bound reduced the affinity of the enzyme for ouabain to a lesser extent than each K⁺ bound.

Cs⁺ and Li⁺ were also examined as inhibitors of ouabain binding (data not shown). The mutant E327A displayed a triphasic Cs⁺ or Li⁺-induced inhibition-activation-inhibition pattern qualitatively similar to that observed for K⁺ and Rb⁺. The magnitude of the initial inhibition phase induced by Cs⁺ and Li⁺ was less pronounced compared with K⁺ and Rb⁺. Inhibition constants could not be determined due to an inability to adequately resolve the individual phases.

DISCUSSION

The mutation E327A is one of several mutations introduced into the Na⁺,K⁺-ATPase which renders the pump functionally impaired when tested as previously described [7]. In the present study we showed that when this mutation was incorporated into a sheep $\alpha 1$ cDNA and expressed in 3T3 cells, [³H]ouabain binding was easily detected. It is clear from the results shown in Figure 2 that under Mg-P_i conditions in the absence of monovalent cations, the wild-type and mutant enzymes bind [³H]ouabain with virtually identical affinities. The K_D of ouabain for the wild-type protein (1.53 nM) determined here by equilibrium competition binding assays agrees well with the value previously determined by on and off rate measurements (1.55 nM; [8]). The E327A mutant has not been previously examined by [³H]ouabain binding but showed a similar K_D for

Figure 7 Rb⁺ inhibition curves in wild-type and E327A membranes

[³H]Ouabain binding was measured in wild-type membranes (■) or E327A membranes (●) in the presence of various concentrations of RbCl as shown on the x-axis. The symbols represent the mean of duplicate determinations. The error bars represent the range of the duplicate determinations and are not shown if smaller than the symbol size. Assay conditions were as described in the Experimental section. Sample sizes of 57 μg of wild-type protein or 66 μg of E327A protein were used per assay tube. The concentration of [³H]ouabain was 1.58 nM based on standards taken from the incubation mixture. The solid lines represent curve fits to eqn (3) for wild type and eqn (4) for E327A. The points enclosed by the rectangle were not included in the fit. The fitted parameters were: for wild type ($K_i = 0.932 \pm 0.023$ mM; $\alpha = 9.82 \pm 0.13$; $E_i = 0.298 \pm 0.006$ nM) and for E327A ($K_i = 1.26 \pm 0.26$ mM; $\alpha = 2.11 \pm 0.19$; $K_a = 22.5 \pm 10.6$ mM; $\beta = 1.56 \pm 0.12$; $E_i = 0.311 \pm 0.007$ nM).

ouabain (1.31 nM) as the wild type. A conversion of the measured E_T values reported in the results section to density of ouabain-binding sites indicated that the wild-type and mutant enzymes were expressed at approximately the same levels. The fact that these two enzymes are virtually identical in terms of ouabain binding suggests to us that there are no major structural perturbations in the mutant that could explain low or absent ATPase activity. Thus, to reconcile the inability of the E327A mutant to support cell viability and the ability of the same mutant to bind ouabain with an unchanged affinity constant, we undertook a detailed characterization of [³H]ouabain binding as a function of monovalent cation concentrations. Our goal was to determine whether the cation binding characteristics of E327A could explain the inability of the mutant to support cell growth.

The conformation of wild-type Na⁺,K⁺-ATPase present in medium containing Mg²⁺ and P_i binds ouabain with high affinity. Addition of monovalent cations to this Mg²⁺-P_i medium, shifts the enzyme conformation such that a fraction of the total enzyme has a lower affinity for ouabain. As the concentration of monovalent cation is increased, the population of Na⁺,K⁺-ATPase in the conformation with a high affinity for ouabain approaches zero (for competitive inhibitors such as Na⁺) or a minimum value (for partially competitive inhibitors such as K⁺ or Rb⁺). Hence, we have examined this shift in enzyme conformation as a function of monovalent cations using [³H]ouabain binding as our probe. The amount of [³H]ouabain complexed to Na⁺,K⁺-ATPase decreases as the concentration of enzyme in the high-affinity conformation decreases or as the amount of monovalent cation increases. Both the number of cation sites and the ability of the cation to induce a conformational change in the enzyme determine the effects of the cation on [³H]ouabain binding. Hence, any mutation in Na⁺,K⁺-ATPase which alters

either the number of cation sites on the enzyme or any mutation which interferes with the cation-induced conformational changes of Na⁺,K⁺-ATPase can be detected by this assay system.

Under Mg-P_i conditions, Na⁺ inhibits ouabain binding to both the wild-type sheep α1 and the E327A protein (Figure 3). As far as could be determined, this inhibition was complete for both enzymes (i.e. high concentrations of Na⁺ inhibited to the same extent as the saturating concentrations of unlabelled ouabain used to define non-specific binding). Inhibition curves for both enzymes could be analysed by a pure competitive inhibition mechanism. Three Na⁺ ions were required to explain the steepness of the inhibition curve associated with wild-type sheep α1 protein, whereas only two Na⁺ ions were required for the mutant enzyme. Two possible explanations exist to explain this reduction in the number of Na⁺ ions which inhibit ouabain binding to the E327A mutant. First, if the glutamic acid which is mutated is located in the Na⁺-binding pocket, replacement of this amino acid might disrupt the binding of one Na⁺ ion. This implies that the complex E327A-Na₂⁺ has a greatly reduced affinity for ouabain. A second indirect explanation for the reduced number of Na⁺ ions which inhibit ouabain binding is that the E327A mutation stabilizes an enzyme conformation which does not distinguish between the uncomplexed form of the protein (E) and a Na⁺-complexed form of the protein (E-Na⁺) making the binding of one Na⁺ ion unobservable using ouabain binding as a probe. This explanation suggests that the complex E327A-Na₃⁺ is the form of the enzyme at saturating concentrations of Na⁺ which displays a major reduction in its affinity for ouabain. At this point, we cannot conclude whether the alteration in the Na⁺ inhibition characteristics of E327A is due to a direct effect on the Na⁺-binding pocket or due to an indirect conformational effect of the amino acid replacement.

The pattern of K⁺ inhibition of ouabain binding was clearly different in wild-type and E327A proteins (Figures 4 and 5). In the wild-type preparation, as in the purified sheep kidney enzyme, K⁺ inhibition is monophasic but incomplete (i.e. saturating concentrations of K⁺ do not inhibit to the same extent as saturating concentrations of unlabelled ouabain). The inhibition curves could be fitted to a partially competitive (heterotropic negative cooperative) model [15]. Two K⁺ ions were required to explain the steepness of the curves. According to the model, each K⁺ decreases the ouabain affinity by a factor of about 17, so at saturating K⁺ the affinity would be decreased 300-fold. The E327A mutant enzyme exhibits a triphasic K⁺ inhibition curve. The initial inhibition is incomplete; it occurs in the same K⁺ concentration range as in the wild type (1–10 mM), and it is followed by an activation phase (10–100 mM). These two portions of the curve can be reconciled by assuming the existence of the same two K⁺ inhibition sites as in wild type, but with an additional K⁺ activation site. Activation of binding occurs because the interaction factor for this site (β) is less than the interaction factor (α) for the inhibition site(s). Based on the models described, we calculated an apparent affinity for K⁺ for the inhibition response of about 0.8 mM for both enzymes. This value for the K_i of K⁺ is similar to the dissociation constant calculated for K⁺ interaction with membrane fragments from ox brain (which contains a mixture of isoforms) measured under similar conditions (0.94 mM; [16]). The activation phase of the E327A mutant at K⁺ concentrations above 10 mM is incomplete; however, this activation in our hands was very reproducible in many plasma membrane preparations of at least two clonal cell lines and is also observed in the presence of Rb⁺. This reproducibility suggests that this activation is a specific effect associated with the mutation E327A. Analysis of this activation phase of the K⁺ competition curves is somewhat complicated by

the appearance of the third phase at concentrations of K^+ greater than 150 mM. The inhibition that occurs at K^+ concentrations above 150 mM might be a non-specific ionic strength effect since choline chloride also inhibits both enzyme preparations in this concentration range (IC_{50} approximately 200 mM). Alternatively, K^+ and choline might interact with a specific binding site to produce this inhibition. In any event, it is obvious from the lack of effect of choline chloride in the low concentration range (1–100 mM) that the initial inhibition and activation in the K^+ curve is not due to ionic strength effects.

The initial inhibition phase of the K^+ competition curves for both the wild-type sheep $\alpha 1$ and the mutant E327A suggests that two K^+ ions bind to the enzyme with affinities similar to those calculated to stimulate ATPase activity (1.03 ± 0.22 mM; [6]). Thus, one could conclude that the two K^+ -binding sites associated with transport by the Na^+, K^+ -ATPase are still intact and functional when the glutamic acid at position 327 is altered to an alanine. However, what about the third K^+ site detected in the equilibrium curve for the E327A mutant which promotes ouabain binding? It seems highly unlikely that the side chain of alanine would create an additional K^+ -binding site. It is more likely that by altering this glutamic acid, a normally hidden monovalent cation site is exposed, to which binding of K^+ causes a shift in conformation to a high-affinity ouabain-binding form of Na^+, K^+ -ATPase. The inability of the E327A mutant to maintain the ionic gradient necessary to support cell viability may be due to this altered conformational transition observed with the appearance of the third K^+ site. However, due to the concentrations of K^+ in the cell culture conditions (extracellular $[K^+] \approx 5$ mM/intracellular $[K^+] \approx 140$ mM), if K^+ binding to this third site were responsible for the inability of the mutant to support cell viability, this site must be located on the cytoplasmic side of the protein due to the high K_a value ($\approx 82 \pm 28$ mM). In the [3H]ouabain-binding experiments, both sides of the membrane are exposed to the added K^+ and therefore it is possible that the two high-affinity K^+ sites are located on the extracellular surface of the E327A protein while the low-affinity site is present on the cytoplasmic surface of the mutant Na^+, K^+ -ATPase. Since the high-affinity external K^+ sites were not altered by the mutation, we speculate that the glutamic acid at position 327 of the sheep $\alpha 1$ isoform is probably not involved in K^+ binding or translocation but that this residue may be important in transitional steps between intermediate conformations of the Na^+, K^+ -ATPase which transiently expose cation-binding sites on either side of the plasma membrane.

The detailed mechanism of Na^+/K^+ transport by the Na^+, K^+ -ATPase is not known but is thought to involve the binding of the cations to their site(s) followed by one or more conformational changes in the protein which carry the cations to the opposite side of the cell membrane. The E327A mutant form of the enzyme cannot support cell viability [7], indicating a major defect in its ability to transport Na^+ and K^+ . The K^+ inhibition curves reported here suggest the exposure of a third K^+ site in the E327A mutant which can only be explained through altered conformational characteristics of the ATPase. The Na^+ inhibition curves indicate that two Na^+ ions interact with the mutant promoting a conformational change which reduces the affinity of the enzyme for ouabain. These Na^+ competition data suggest two possible functions for the altered glutamic acid; either the residue lies within the Na^+ -binding pocket or the residue is important conformationally in differentiating between a Na^+ -complexed form of the enzyme and an uncomplexed form. The common role

for the glutamic acid at position 327 in the sheep $\alpha 1$ isoform, suggested by both the K^+ and the Na^+ inhibition data, is that this residue plays a pivotal function in the cascade of conformational changes normally induced by the binding of monovalent cations. Altering the glutamic acid hinders conformational changes from occurring in the protein such that ATPase activity is not sufficient to produce the ionic gradient essential for cell viability.

This glutamic acid residue is conserved between the amino acid sequences of the SR Ca^{2+} -ATPase and the Na^+, K^+ -ATPase. Extensive site-directed mutagenesis studies have been done on this site (E309) in the Ca^{2+} -ATPase [17–19]. When the mutant E309A of Ca^{2+} -ATPase was expressed transiently in COS cells, this mutant was deficient in both its ability to transport Ca^{2+} and in ATPase activity. These findings are in agreement with the inability of this mutant in Na^+, K^+ -ATPase to support cell viability in the presence of ouabain [7]. Vilsen and Andersen went on to study the Ca^{2+} dependence of phosphorylation by both ATP and by P_i . In summary, they observed that the mutant E309A formed at least two intermediate conformations (E_1P and E_2P) but that neither conformation interacted with Ca^{2+} in the same fashion as the expressed wild-type Ca^{2+} -ATPase. In analogy, we suggest that Na^+ and K^+ cannot facilitate conformational changes in the mutant E327A that are essential for the normal function of the Na^+, K^+ -ATPase. Although the effects of cations on conformational changes in these two related enzymes have been evaluated using different methods, studies of both systems suggest that this conserved glutamic acid in the fourth transmembrane domain of the ATPases is pivotal for transitions between intermediate conformations of the catalytic cycle of active cation transport by P-type ATPases.

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REFERENCES

- Glynn, I. M. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., ed.), pp. 35–114. Plenum Press, New York
- Karlish, S. J. D., Goldshleger, R., Tal, D. M. and Stein, W. D. (1991) *Soc. Gen. Physiol. Ser.* **46**, 129–141
- Hansen, O. (1984) *Pharmacol. Rev.* **36**, 143–163
- Goldshleger, R., Tal, D. M., Moorman, J., Stein, W. D. and Karlish, S. J. D. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6911–6915
- Van Huysse, J. W., Jewell, E. A. and Lingrel, J. B. (1993) *Biochemistry* **25**, 8125–8132
- Jewell-Motz, E. A. and Lingrel, J. B. (1993) *Biochemistry* **32**, 13523–13530
- Feng, J. and Lingrel, J. B. (1994) *Biochemistry* **33**, 4218–4224
- Schultheis, P. J., Wallick, E. T. and Lingrel, J. B. (1993) *J. Biol. Chem.* **268**, 22686–22694
- Wallick, E. T. and Schwartz, A. (1988) *Methods Enzymol.* **156**, 201–213
- Price, E. M. and Lingrel, J. B. (1988) *Biochemistry* **27**, 8400–8408
- Lane, L. K., Copenhaver, G. H., Lindenmayer, G. E. and Schwartz, A. (1973) *J. Biol. Chem.* **248**, 7197–7200
- Ball, W. J., Schwartz, A. and Lessard, J. L. (1982) *Biochim. Biophys. Acta* **719**, 413–423
- Ball, W. J., Jr., Kirley, T. L. and Lane, L. K. (1988) *Methods Enzymol.* **156**, 87–101
- Skou, J. C. and Esmann, M. (1980) *Biochim. Biophys. Acta* **601**, 386–402
- Johnson, C. L., Schultheis, P. J., Lingrel, J. B. and Wallick, E. T. (1995) *Arch. Biochem. Biophys.* **317**, 133–141
- Hansen, O. and Skou, J. C. (1973) *Biochim. Biophys. Acta* **311**, 51–66
- Clarke, D. M., Loo, T. W. and MacLennan, D. H. (1990) *J. Biol. Chem.* **265**, 17405–17408
- Andersen, J. P. and Vilsen, B. (1992) *J. Biol. Chem.* **267**, 19383–19387
- Vilsen, B. and Andersen, J. P. (1992) *FEBS Lett.* **306**, 247–250