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A Glu^{329}→Gln variant of the α-subunit of the rat kidney Na^+,K^+-ATPase can sustain active transport of Na^+ and K^+ and Na^+,K^+-activated ATP hydrolysis with normal turnover number

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An allelic variant of the ouabain-insensitive rat kidney Na^+,K^+-ATPase α_1-isoform was identified by chance in a cDNA library. The variant differed from the wild-type rat kidney Na^+,K^+-ATPase by a single G-to-C base substitution in the cDNA, which on amino acid level gave rise to a glutamine in place of the glutamate residue Glu^{329} previously suggested as a likely donator of oxygen ligands for Na^+ and K^+ binding. The variant cDNA was transfected into COS-1 cells and the transfectants expanded with success into stable cell lines that were able to grow in the presence of a concentration of ouabain highly cytotoxic to the parental cells containing only the endogenous COS-1 cell Na^+,K^+-ATPase. Under these conditions, the viability of the cells depended on the cation transport mediated by the ouabain-insensitive Glu^{329}→Gln variant, whose cDNA was shown by polymerase chain reaction amplification to be stably integrated into the COS-1 cell genome. The maximum specific ATP hydrolysis activity of isolated plasma membranes of the Glu^{329}→Gln variant did not differ significantly from that of plasma membranes containing the wild type. A method was established for measurement of the phosphorylation capacity of the expressed Glu^{329}→Gln variant and wild-type enzyme, and it was thereby demonstrated that the variant had a turnover number similar if not identical to that of the wild-type.

Glutamate; Allelic variant; Na^+,K^+-pump; Cation site; Polymerase chain reaction

1. INTRODUCTION

The active transport of Na^+ and K^+ by the Na^+,K^+-ATPase in the plasma membranes of animal cells provides the basis for a number of essential cell functions, some of which are associated with cell growth homeostasis [1]. Understanding how the free energy of hydrolysis of ATP is transduced into active exchange of 3 Na^+ for 2 K^+ across the plasma membrane requires knowledge of the structure of the ATP- and cation-binding sites as well as their interactions. The characterization of the structure of the cation sites would help to answer fundamental questions such as: (i) whether the same residues bind to Na^+ and K^+ as proposed in the ping-pong sequential transport model; (ii) whether the cation and ATP sites are adjacent or quite separate in the protein structure; (iii) whether the cation sites are charged or neutral; (iv) which factors confer cation selectivity on the protein.

This communication describes the cloning and functional expression of an allelic variant of the α_1-isoform of Na^+,K^+-ATPase. This variant, identified accidentally in a rat kidney cDNA library, differed from the wild-type enzyme by having a glutamine in place of the glutamic acid residue at position 329. Glu^{329} is highly conserved within the family of P-type cation-transporting ATPases, to which the Na^+,K^+-ATPase belong, and site-directed mutagenesis studies have shown that the homologous residue of another family member, the sarcoplasmic reticulum Ca^{2+}-ATPase, is essential to Ca^{2+} transport [2–4]. Moreover, based on chemical modification studies using DCCD it was recently suggested that Glu^{329} is essential to cation occlusion and transport in the Na^+,K^+-ATPase [5]. In the present study, a stable COS-1 cell line expressing the Glu^{329}→Gln variant was constructed, and surprisingly the functional analysis revealed that the negative charge of Glu^{329} is non-essential for overall Na^+- and K^+-transport and ATPase activity.

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; kb, kilobase pair; Na^+,K^+-ATPase, sodium plus potassium-activated adenosine triphosphatase (EC 3.6.1.3); PCR, polymerase chain reaction; T_m, melting temperature.

2. MATERIALS AND METHODS

2.1. *Cloning of the variant Na^+,K^+-ATPase*

The construction and screening of the rat kidney outer medulla plasmid cDNA library has previously been described in detail [6]. Total RNA from the kidneys of three rats (Wistar strain, Møllegaard Breeding Laboratory, Denmark) was used. Approximately 3,500 colonies were screened, out of which 42 gave a positive hybridization signal with an oligonucleotide probe defined by the sequence 5'-AGTCTTG-TCAGAGCAGAT-3' derived from the phosphorylation site region of P-type ATPases. Previously, one of these clones was sequenced throughout, and the deduced amino acid sequence was found to be

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100% identical with that described as the wild-type rat α_1-isoform of Na^+,K^+-ATPase [6]. Further examination by restriction enzyme analysis of the clones giving positive hybridization signal revealed that their sequences were not all identical. A *Hpa*II site present in the coding sequence in the wild-type Na^+,K^+-ATPase was absent in 6 of the clones, and by DNA sequencing it was revealed that this difference from the wild-type was due to a single G-to-C base substitution and that no other base substitutions were present.

2.2. *Expression of the variant cDNA in COS-1 cells*

The full-length cDNAs encoding wild-type or variant Na^+,K^+-ATPases, carrying *Eco*RI/*Not*I adaptors, were cloned into the *Eco*RI site of the expression vector pMT2 [7]. To obtain cell lines with the Na^+,K^+-ATPase cDNA stably integrated into their chromosomes, the cesium chloride gradient-purified plasmids were transfected into COS-1 cells [8] by the calcium phosphate procedure. Following 65 h incubation, ouabain was added to the medium at a final concentration of 5 μM. Individual ouabain resistant colonies appeared after approximately 3 weeks. Two colonies from each of 6 different master dishes were isolated by use of cloning cylinders and expanded into stable cell lines, which were stored in liquid nitrogen.

2.3. *Isolation of genomic DNA and amplification by PCR*

PCR was used to verify that the isolated COS-1 cell line contained the cDNA of the exogenous Na^+,K^+-ATPase stably integrated into the genome. Isolation of genomic DNA from the cells was carried out as described in [9], using an extraction buffer containing 0.1 mg/ml proteinase-K and 0.5% sodium dodecyl sulfate. The primers used in PCR were designed to obtain an efficient amplification of the exogenous stably integrated cDNA encoding the $Glu^{329}\rightarrow Gln$ variant or wild-type Na^+,K^+-ATPase and at the same time avoid amplification of the endogenous COS-1 cell genomic DNA. The sense (5'-CTGTGTTGAAGGAACTGCAC-3') having a T_m of 60°C was designed to hybridize across the boundary between exon no. 6 and exon no. 7 in the cDNA. The antisense primer (5'-TCAGACATACCGTGACGGT-3') having a T_m of 58°C was designed to hybridize across the boundary between exon no. 7 and exon no. 8 in the cDNA. In this way a PCR product of an appropriate size of 290 bp corresponding to exon 7 was expected to be generated from the exogenous Na^+,K^+-ATPase cDNA, whereas the likelihood of obtaining a product from the endogenous genomic DNA (corresponding to exon 7 plus introns 7 and 8) was low. The buffer used in PCR contained 20 mM Tris-HCl (pH 8.3), 50 mM KCl. In addition all amplification reactions included 2.0 mM $MgCl_2$, 200 μM of each dNTP, 50 pmol of each primer, 2 U of Taq DNA polymerase (Perkin-Elmer Cetus), and 400–600 ng genomic DNA. The 60 μl reactions were then amplified for 35 cycles in a DNA thermal cycler (Abacus/Hybaid). To analyze the PCR products 5–8 μl were combined with 1–1.5 μl dye (0.25% Bromophenol blue/0.25% xylene cyanol/30% glycerol in water) and subjected to nondenaturing electrophoresis on a 5% polyacrylamide gel in 1 × TBE (89 mM Tris-borate/89 mM boric acid/2 mM EDTA, pH 8.0). The PCR products were visualized by ethidium bromide fluorescence. The single 290 bp DNA fragment was extracted from the gel, phosphorylated at the 5' blunt-ends by use of bacteriophage T_4 polynucleotide kinase (Pharmacia), and inserted into the *Sma*I site of the Bluescript vector (Stratagene, La Jolla, CA) by use of bacteriophage T_4 DNA ligase. Sequencing was performed using the dideoxynucleotide chain termination method [10] with Sequenase, as described previously [11].

2.4. *Isolation of plasma membranes and assay of Na^+,K^+-ATPase*

A crude plasma membrane fraction was prepared as previously described [6]. Protein concentration was determined by the dye binding method [12] using bovine serum albumin as standard. To open the vesicles, crude plasma membranes at a protein concentration of 0.25–0.45 mg/ml were incubated with 0.65 mg of sodium deoxycholate/ml, in the presence of 2 mM EDTA and 20 mM imidazole for 30 min at 20°C. Na^+,K^+-ATPase activity was measured on 25 μl of the leaky membrane solution essentially as described in [13]. To determine the maximum specific activity the Na^+,K^+-ATPase measurements were carried out at 37°C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, and 3 mM ATP. The rate of ATP hydrolysis was found to be constant over the incubation time (10–20 min). To determine the Na^+,K^+-ATPase activity specifically contributed by the transfected wild-type or variant rat kidney enzymes, assays were carried out as well in the presence of 10 μM ouabain, which inhibits endogenous COS-1 cell Na^+,K^+-ATPase, as in the presence of 20 mM ouabain, which inhibits all Na^+,K^+-ATPase activity. The ouabain-resistant Na^+,K^+-ATPase activity associated with the expressed exogenous enzyme was calculated by subtraction of the background ATPase activity measured at 20 mM ouabain from the ATPase activity measured at 10 μM ouabain. The background ATPase activity resistant to 20 mM ouabain comprised approximately 20–30% of the total ATPase activity in the crude membrane preparation. For the wild-type as well as the variant, the ATPase measurements were carried out in duplicate on each of 4 different clonal isolates.

2.5. *Phosphoenzyme formation*

Phosphorylation was carried out on 25 μl of the leaky membrane suspension (corresponding to a total of 10 μg protein) at 0°C in 100 μl of a reaction mixture containing 0.1–10 μM [γ-^{32}P]ATP, 20 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, and either 150 mM NaCl or 50 mM KCl. In some experiments the ouabain-sensitive endogenous Na^+,K^+-ATPase present in the COS-1 cell was inhibited by formation of a stable ouabain-bound complex. This was achieved by preincubation of 25 μl of the leaky membrane suspension with 5 μM ouabain and 3 mM $MgCl_2$ in the absence of Na^+ and K^+ for 30 min at 20°C, prior to initiation of the phosphorylation reaction under conditions identical to those described above except for the presence of 5 μM ouabain. The phosphorylation reaction was terminated after 10 s by addition of 1 ml of ice-cold 7% (w/v) trichloroacetic acid containing 1 mM phosphate. The denatured protein was washed twice by centrifugation and subjected to SDS-polyacrylamide gel electrophoresis in 7% acrylamide gels under acid conditions [11]. After drying the gel, autoradiographs were produced by exposure at −70°C. Quantitation of the phosphoenzyme was obtained by liquid scintillation counting of slices of the dried gels.

3. RESULTS

I have previously described the isolation of a cDNA encoding the ouabain-resistant rat kidney α_1-isoform of Na^+,K^+-ATPase from a plasmid cDNA library [6]. Further examination of the library revealed, in addition to this wild-type cDNA, a variant Na^+,K^+-ATPase cDNA containing a single G-to-C base substitution, which on amino acid level gave rise to a glutamate-to-glutamine substitution at position 329 within the motif 328-PEGL in the predicted 4th transmembrane helix. As Glu^{329} has recently been attributed a role in cation binding [5], it was pertinent to examine whether the $Glu^{329}\rightarrow Gln$ variant was able to carry out active transport of Na^+ and K^+ and Na^+,K^+-activated ATP hydrolysis. The cDNAs encoding either the $Glu^{329}\rightarrow Gln$ variant or the wild-type rodent Na^+,K^+-ATPase were therefore inserted in the expression vector pMT2 and transfected into COS-1 cells, which were grown in the presence of 5 μM ouabain. This strategy was based on the consideration that 5 μM ouabain preferentially inhibits the ouabain-sensitive Na^+,K^+-ATPase endogenously present in COS-1 cells [6]. Thus, only COS-1 cells containing the exogenous cDNA encoding the ouabain-resistant ro-

dent Na^+,K^+-ATPase stably integrated in their genome would be able to survive under these selective conditions. On the other hand, survival and sustained growth of the transformed COS-1 cells would require the ouabain-resistant Na^+,K^+-ATPase to be functional. Failure of the modified Na^+,K^+-ATPase to confer ouabain resistance would thus indicate that the amino acid substitution was introduced at a critical site in the enzyme, while cell growth would indicate a relatively mild effect or the lack of effect on enzyme function.

Surprisingly, it was found, that the Glu^{329}→Gln variant of the ouabain-resistant rodent Na^+,K^+-ATPase was able to confer ouabain resistance to COS-1 cells, as revealed by the appearance of ouabain resistant colonies after approximately 2–3 weeks of growth in the presence of 5 μM ouabain. By contrast, no ouabain-resistant colonies were formed, when the COS-1 cells were mock-transfected with the expression vector without insert, demonstrating that resistance was not conferred in the absence of the exogenous Na^+,K^+- ATPase cDNA.

Although the growth-rate of the transfectants carrying the Glu^{329}→Gln variant was similar to the growth-rate of the transfectants expressing wild-type enzyme, the number of ouabain resistant colonies appearing on each of the different master dishes was only around 10% that observed on master dishes containing COS-1 cells transfected with wild-type Na^+,K^+-ATPase cDNA. A possible reason is that the Glu^{329}-to-Gln amino acid substitution resulted in partial inactivation of the Na^+,K^+-pump function, so that the viability of COS-1 cells expressing the variant enzyme was critically dependent on the expression level. The latter depends on an optimal cDNA copy number and a favorable location in the chromosome, conditions fulfilled only in a fraction of the cells with stably integrated cDNA.

The relatively small number of stable colonies formed when COS-1 cells were transfected with cDNA encoding the Na^+,K^+-ATPase Glu^{329}→Gln variant raised the question whether the application of a strong selection pressure during months might have induced the formation of functional revertants in which Gln^{329} was resubstituted back to Glu or another residue by natural mutations. It was therefore important to verify that the base substitution encoding the Glu^{329}→Gln replacement was still present in the cDNA stably integrated into the chromosome of the isolated ouabain-resistant COS-1 cells, after these had been expanded into stable cell lines. To this end, genomic DNA was isolated from the stable cell lines and the polymerase chain reaction (PCR) was applied to amplify the genomic 290 bp cDNA fragment spanning the nucleotide region corresponding to residues Cys^{244} to Leu^{339} (exon 7 in the genome), which contains the replaced residue. Information about the location of exon–intron boundaries was used to design primers that permitted selective amplification of the stably integrated cDNA free of contamination with endogenous COS-1 cell Na^+,K^+-ATPase genomic DNA (see section 2). This strategy worked, since the PCR product was detected as a single ethidium bromide-stained band on the electrophoretic gel, and this DNA fragment displayed exactly the size (290 bp) predicted from the target sequence. Not even traces were observed of the larger fragment (introns 7 and 8 plus exon 7) expected if endogenous genomic COS-1 cell DNA had been amplified. Since an endogenous *Hpa*II site was removed by the single G-to-C base substitution in the Glu^{329}→Gln variant, digestion with *Hpa*II could be used to reveal the presence of the base substitution. Fig. 1 shows that the 290 bp-fragment obtained by PCR amplification of genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu^{329}→Gln variant was resistant to digestion with the *Hpa*II enzyme (lane 1), whereas *Hpa*II digestion of the PCR-amplified 290 bp fragment obtained with genomic DNA from cells transfected with cDNA encoding the wild-type led to formation of fragments of size 254 bp and 36 bp (see arrow), in accordance with the wild-type sequence (lane 2). These results tentatively demonstrated that the base substitution encoding the Glu^{329}-to-Gln replacement was retained in the cDNA stably

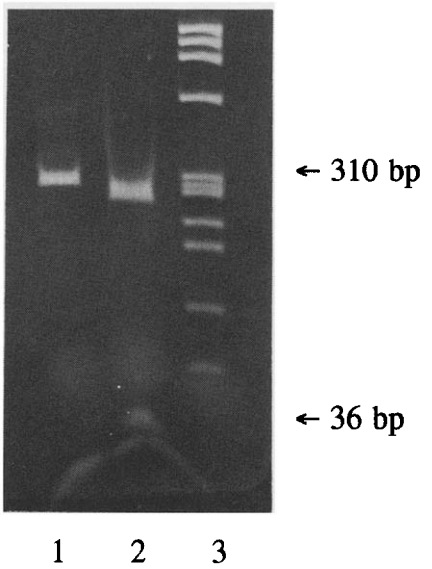


Fig. 1. PCR amplification and restriction enzyme analysis of the 290 bp cDNA fragment corresponding to exon 7 of the exogenous stably integrated Na^+,K^+-ATPase cDNA. The amplification of genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu^{329}→Gln variant or wild-type Na^+,K^+-ATPase was carried out as described in section 2. The PCR products were analyzed on a 5% polyacrylamide gel. Lane 1, *Hpa*II digestion of the PCR product amplified from genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu^{329}→Gln variant Na^+,K^+-ATPase; lane 2, *Hpa*II digestion of the PCR product amplified from genomic DNA isolated from COS-1 cells transfected with cDNA encoding the wild-type Na^+,K^+-ATPase; lane 3, *Hae*III-digested PhiX174 RF DNA molecular size standard. The arrows point at the 310 bp fragment of the standard, and the 36 bp fragment produced by *Hpa*III digestion of the wild-type.

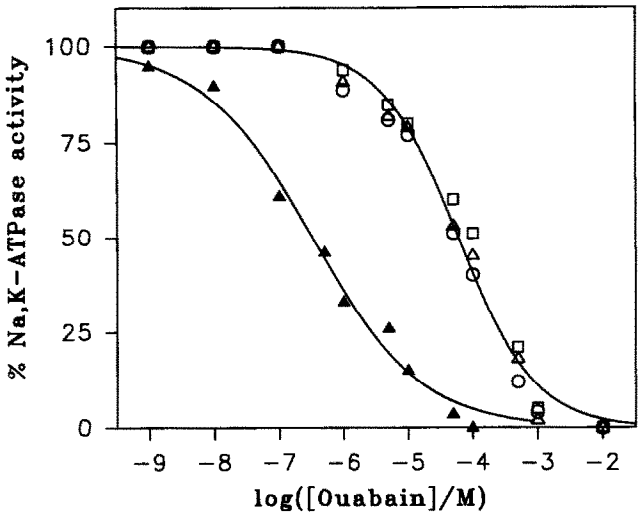


Fig. 2. Ouabain-sensitivity of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells mock-transfected with the PMT2 expression vector without insert and from COS-1 cells transfected with the expression vector containing cDNA encoding the Glu^{329}→Gln variant. The Na^+,K^+-ATPase measurements were carried out in duplicate as described in section 2 in the presence of varying [ouabain]. The average values are presented as percentage of the total Na^+,K^+-ATPase activity measured in the absence of ouabain, as calculated after subtraction of background ATPase activity measured at 20 mM ouabain. The differences between the duplicate measurements were smaller than the size of the symbols. Measurements for 3 different clonal isolates of the Glu^{329}→Gln variant (○, △, □) and one preparation of endogenous COS-1 cell enzyme (▲) are shown. The specific Na^+,K^+-ATPase activities measured in the absence of ouabain (100% values) were as follows. Endogenous COS-1 cell enzyme in plasma membranes from mock-transfected cells grown in the absence of ouabain (▲), 378 nmol/min/mg; plasma membranes from COS-1 cells transfected with the Glu^{329}→Gln variant cDNA and grown in the presence of ouabain, 175 nmol/min/mg (○), 154 nmol/min/mg (△), 91 nmol/min/mg (□).

integrated into the chromosome of the COS-1 cells. The definitive proof was obtained by sequencing the 290 bp PCR-fragment throughout after insertion into the Bluescript vector. The sequence showed the expected single G-to-C base substitution corresponding to replacement of Glu^{329} by Gln.

The Na^+,K^+-ATPase activity was measured in plasma membranes isolated from the stable COS-1 cell lines expressing wild-type rat kidney Na^+,K^+-ATPase or the Glu^{329}→Gln variant, as well as in plasma membranes from cells which had been mock-transfected with the expression vector without insert, so that only the endogenous COS-1 cell Na^+,K^+-ATPase was expressed. Fig. 2 shows titration of the ouabain concentration dependence of the Na^+,K^+-ATPase activity in 3 different clonal isolates of the Glu^{329}→Gln variant and in the membranes containing only endogenous enzyme. The data for the wild-type rat kidney enzyme are not shown, since they have been presented before [6]. The ouabain titration results obtained with the Glu^{329}→Gln variant were almost superposable on those obtained with the wild-type rat kidney enzyme. Both of these rat kidney enzymes displayed an apparent affinity for ouabain which was about 500-fold lower than that of the endogenous COS-1 cell Na^+,K^+-ATPase. The specific activities of four different clonal isolates of the Glu^{329}→Gln variant and the wild-type measured at 10 μM ouabain are compared in Table I. At this ouabain concentration the ATPase activity of the ouabain-insensitive rat Na^+,K^+-ATPase is still near maximal, and under these conditions the relative contribution of the endogenous enzyme to the Na^+,K^+-ATPase activity measured with the plasma membranes of the transfectants could be estimated to be no more than 5% (see [6]). It is seen from Table I that the specific activities corresponding to different clonal isolates of the same enzyme form varied considerably, suggesting variability of the expression level. The difference between the specific activities of clonal isolates of the Glu^{329}→Gln variant and the wild-type rat kidney Na^+,K^+-ATPase fell within this variation range. To determine whether the molecular turnover numbers of the wild-type and the Glu^{329}→Gln variant differed, the concentrations of active Na^+,K^+-ATPase sites present in the membrane preparations containing wild-type and Glu^{329}→Gln variant were measured for the different clonal isolates (Table I). This was accomplished by phosphorylation experiments with radioactive [γ-^{32}P]ATP under conditions where the dephosphorylation rate is low and phosphorylation therefore nearly stoichiometric (0°C, 150 mM Na^+ present, absence of K^+). Since the expressed Na^+,K^+-ATPase constitutes such a minute fraction of the total protein present in the COS-cell plasma membranes, an accurate determination of the amount of phosphorylated Na^+,K^+-ATPase required separation of the phosphorylated Na^+,K^+-ATPase from other phosphorylated proteins. To this end the acid SDS-polyacrylamide gel electrophoresis procedure previously applied to the

Table I

Clonal isolate	Specific activity (nmol ATP hydrolyzed/min/mg crude membrane protein)	Phosphorylation (pmol/mg crude membrane protein)	Turnover number (min^{-1})
Wild-type			
no. 1	137	4.81	28,500
no. 2	107	3.80	28,200
no. 3	52	1.98	26,300
no. 4	42	1.71	24,600
Glu^{329}→Gln			
no. 1	160	5.15	31,100
no. 2	140	4.86	28,800
no. 3	119	4.39	27,100
no. 4	83	3.05	27,200

All measurements were carried out as described in section 2, and the turnover number was calculated as the ratio between the specific activity and the phosphorylation.

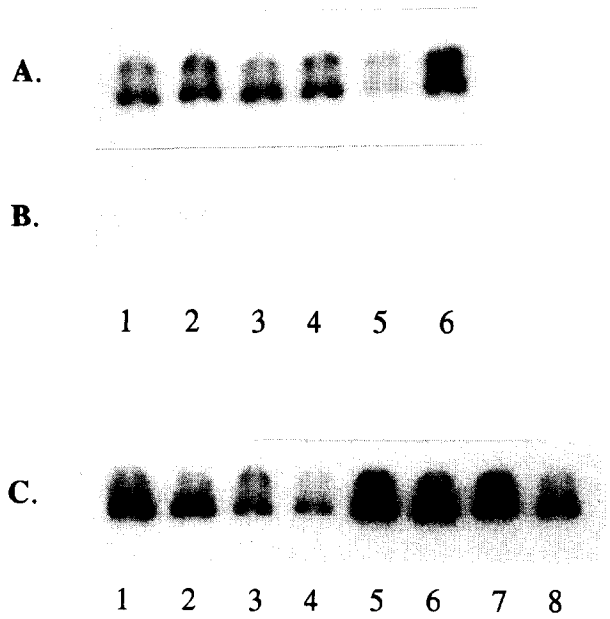


Fig. 3. Phosphorylation from ATP of plasma membranes isolated from COS-1 cells mock-transfected with the PMT2 expression vector without insert and from COS-1 cells transfected with the expression vector containing cDNA encoding either wild-type or the Glu^{329}→Gln variant Na^+,K^+-ATPase. Phosphorylation was carried out at 0°C for 10 s in the presence of 20 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, 4 μM [γ-^{32}P]ATP, and either 150 mM NaCl without K^+ (panels A and C) or 50 mM KCl without Na^+ (panel B). The acid-quenched samples were subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels are shown. Each lane was loaded with 7 μg of plasma membrane protein. (A and B) Lanes 1 and 2, plasma membranes of cells expressing wild-type rat kidney Na^+,K^+-ATPase; lanes 3 and 4, plasma membranes of cells expressing Glu^{329}→Gln variant Na^+,K^+-ATPase; lanes 5 and 6, plasma membranes of mock-transfected COS-1 cells grown in the absence of ouabain. Prior to phosphorylation the samples were preincubated for 30 min at 20°C with (lanes 1, 3 and 5) or without (lanes 2, 4 and 6) 5 μM ouabain in the presence of 3 mM $MgCl_2$ and absence of Na^+, K^+ and ATP. The maximum specific Na^+,K^+-ATPase activities of the samples were the following: wild-type, 133 nmol/min/mg; Glu^{329}→Gln variant Na^+,K^+-ATPase, 137 nmol/min/mg; plasma membranes of mock-transfected COS-1 cells grown in the absence of ouabain, 378 nmol/min/mg. (C) Phosphorylation was carried out with 4 different clonal isolates of the wild type (lanes 1–4) and the Glu^{329}→Gln variant (lanes 5–8) after preincubation with ouabain as described for (A). The quantitation of the phosphorylations in (C) is presented in Table I.

Ca^{2+}-ATPase [11] proved useful. All the phosphoprotein migrating corresponding to 100 kDa on the gel was Na^+,K^+-ATPase as judged from its disappearance upon addition of K^+ (compare Fig. 3A and B). To estimate the fraction of the phosphoenzyme contributed by the ouabain-sensitive endogenous Na^+,K^+-ATPase present in the COS-1 cells, the reaction with radioactive ATP was carried out with and without preincubation with ouabain (Fig. 3A). As expected, phosphorylation was completely prevented by preincubation with ouabain in membranes isolated from cells which had been mock-transfected with the expression vector without insert and maintained in the absence of ouabain, since these cells expressed only the endogenous ouabain-sensitive enzyme. By contrast, the preincubation with ouabain was without significant effect on the phosphoenzyme levels in the membranes isolated from cells expressing the rat kidney wild-type enzyme or Glu^{329}→Gln variant. This indicates that the endogenous enzyme was inactive or not present at all in these membranes. A likely explanation is that the ouabain-sensitive endogenous enzyme had already formed a stable ouabain-bound complex during propagation of the COS-1 cells in the presence of ouabain in the cell culture. Another possible explanation is that only minute amounts of active endogenous Na^+,K^+-ATPase were expressed due to competition with the exogenous α-chains for the assembly with endogenous β-subunits required for functional maturation and transport of the pumps to the plasma membrane [14].

By titration of the ATP concentration dependence it was found that the Glu^{329}→Gln variant displayed an apparent affinity for ATP in the phosphorylation assay which was almost indistinguishable from that of the wild-type (Fig. 4). Both the wild-type and the variant were saturated at 4 μM ATP, and this condition was used for determination of site concentrations.

As seen in Fig. 3C the phosphorylation capacity varied among the differential clonal isolates of the same enzyme form indicating variation of the expression level. Quantitation of the phosphorylation was obtained by liquid scintillation counting of gel slices. The calculated site concentrations varied in parallel with the corresponding specific activities, so that the calculated turnover number was independent of the expression

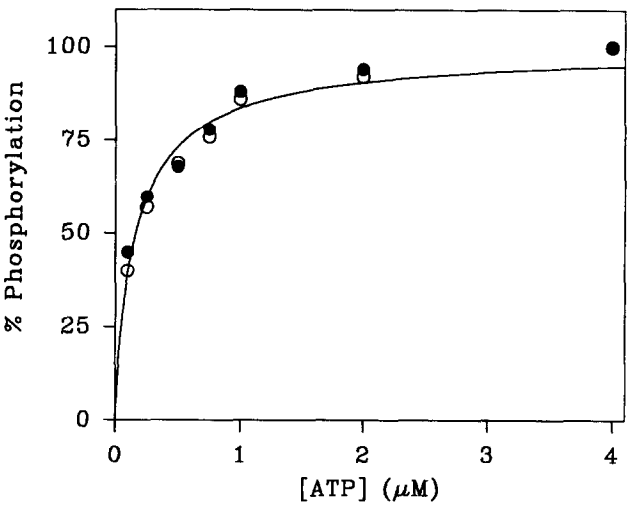


Fig. 4. ATP concentration dependence of phosphorylation from ATP of the wild-type (●) or the Glu^{329}→Gln variant (○) Na^+,K^+-ATPase. Phosphorylation was carried out at 0°C for 10 s in the presence of 20 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, 150 mM NaCl, and varying concentrations of [γ-^{32}P]ATP as indicated on the abscissa. The acid-quenched samples were subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0, and the phosphoenzyme was quantitated by liquid scintillation counting of slices of the dried gels. 100% values correspond to 4.22 pmol/mg crude membrane protein (●) and 4.60 pmol/mg crude membrane protein (○).

level (Table I). Importantly, there was no significant difference between the turnover numbers calculated for the wild-type enzyme and the Glu^{329}→Gln variant.

4. DISCUSSION AND CONCLUSIONS

The establishment of ouabain resistance in COS-1 cells transfected with cDNA encoding the Glu^{329}→Gln variant clearly indicated that the expressed Glu^{329}→Gln modified enzyme was functional in Na^+,K^+-transport. By polymerase chain reaction amplification and DNA sequencing it was unambiguously documented that the Glu^{329}-to-Gln substitution was still intact in the nucleotide sequence of the genome of the transfectants after several months of maintenance in the presence of ouabain. This represents the first time PCR has been used to confirm the stable integration of the Na^+,K^+-ATPase cDNA into the genome of transfectants.

In the presence of saturating substrate concentrations, the plasma membranes harvested from the cell lines expressing the variant displayed a specific Na^+,K^+-ATPase activity very similar to that of the wild-type enzyme, and the measurement of site concentration by phosphorylation with ATP permitted the calculation of similar molecular turnover numbers of the Glu^{329}→Gln variant and the wild-type. Hence it may be concluded that the negative charge of Glu^{329} is non-essential to enzyme function at saturating substrate concentrations.

To identify within the tryptic fragments of Na^+,K^+-ATPase the residues essential for cation occlusion, Karlish and coworkers combined selective tryptic digestion with chemical modification using the hydrophobic carbodiimide DCCD [5,15]. It was observed that Rb^+ and Na^+ occlusion was inactivated by DCCD with identical rates, and the inactivation was equally well protected by Rb^+ with high affinity and Na^+ with lower affinity. Moreover, it was found that each mol of α-subunit bound 2 mol of DCCD. One labeled residue was located in a cyanogen bromide fragment of apparent M_r 4 kDa, and [^{14}C]DCCD incorporation was found to be associated almost exclusively with Glu^{953} [5]. The role of Glu^{953} was, however, tested in a recent mutagenesis study, and it was shown that cation stimulation of Na^+,K^+-ATPase activity was unaffected by single amino acid substitutions of Glu^{953} [16]. It was not possible to determine the exact location of the second DCCD binding site, but peptides corresponding to the transmembrane hairpin loop M_1 plus M_2 or M_3 plus M_4 appeared to be labeled, and Glu^{329} was suggested as the residue most likely labeled by DCCD [5]. This was based on assumption that DCCD labeling occurs at a carboxyl group and on extrapolation to the Na^+,K^+-ATPase of the mutagenesis data obtained with the homologous residue in the Ca^{2+}-ATPase.

While the present study shows that Na^+ and K^+ binding take place in the Glu^{329}→Gln variant, it does not exclude that the residue modified with DCCD in [5] was Glu^{329}. The bulky DCCD molecule might have disturbed ion binding and occlusion, even if the modified residue were located at the periphery of the cation-binding pocket without being an essential component. In the Glu^{329}→Gln variant the apparent affinities for Na^+ and K^+ measured by Na^+ and K^+ titration of ATPase activity were moderately decreased (i.e. 2- to 6-fold), relative to affinities of the wild-type (results to be presented elsewhere).

The present findings indicating that enzyme function is compatible with the Glu^{329}→Gln substitution contrast with the results obtained by mutagenesis experiments with the sarcoplasmic reticulum Ca^{2+}-ATPase, which showed that the residue Glu^{309}, homologous to Glu^{329} in Na^+,K^+-ATPase, is essential to Ca^{2+}-transport. The replacement of Glu^{309} in Ca^{2+}-ATPase with Gln resulted in a more than 1000-fold reduction in apparent Ca^{2+} affinity for one of the two bound Ca^{2+} ions [2–4]. The reason for this major difference between the two closely related cation pumps is not clear, but it may possibly be related to the fact that the Ca^{2+}-pump binds and transports two calcium ions, i.e. four negative charges, while the Na^+,K^+-pump binds and transports only three Na^+.

Finally, one should consider the possibility that the binding site for one of the three sodium ions was disrupted in the Glu^{329}→Gln variant without seriously disturbing the binding and transport of the remaining two sodium ions. That this might be realistic is indicated by experiments demonstrating transport stoichiometries of $2H^+/2K^+$ and $(1Na^++1H^+)/2K^+$ instead of the usual $3Na^+/2K^+$ stoichiometry at very low Na^+ and high H^+ concentrations [17]. These and other data show that the Na^+,K^+-ATPase can adapt to variable cation conditions by different modes of exchange [18]. It is plausible that protonation reduces the number of Na^+ ions bound to the cation sites, and one might speculate whether protonation of the enzyme is imitated by the removal of a negative charge in the Glu^{329}→Gln variant. The data obtained with the Glu^{329}→Gln variant cannot exclude the possibility that the variant catalyzed an electroneutral $2Na^+/2K^+$ exchange or an electrogenic $2Na^+/1K^+$ exchange. Hence, in the future it would be of interest to investigate whether the Glu^{329}→Gln variant mediates an altered charge transfer relative to the wild-type enzyme.

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REFERENCES

[1] Skou, J.C. (1990) FEBS Lett. 268, 314–324.

[2] Clarke, D.M., Loo, T.W., Inesi, G. and MacLennan, D.H. (1989) Nature 339, 476–478.
[3] Vilsen, B. and Andersen, J.P. (1992) FEBS Lett. 306, 247–250.
[4] Andersen, J.P. and Vilsen, B. (1992) J. Biol. Chem. 267, 19383–19387.
[5] Goldshleger, R., Tal, D.M., Moorman, J., Stein, W.D. and Karlish, S.J.D. (1992) Proc. Natl. Acad. Sci. USA 89, 6911–6915.
[6] Vilsen, B. (1992) FEBS Lett. 314, 301–307.
[7] Kaufman, R.J., Davies, M.V., Pathak, V.K. and Hershey, J.W.B. (1989) Mol. Cell. Biol. 9, 946–958.
[8] Gluzman, Y. (1981) Cell 223, 175–182.
[9] Gross-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36, 32–38.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[11] Vilsen, B., Andersen, J.P., Clarke, D.M. and MacLennan, D.H. (1989) J. Biol. Chem. 264, 21024–21030.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Ottolenghi, P. (1975) Biochem. J. 15, 61–66.
[14] Geering, K. (1991) FEBS Lett. 285, 189–193.
[15] Karlish, S.J.D., Goldshleger, R., Tal, D.M., Capasso, J.M., Hoving, S. and Stein, W.D. (1992) Acta Physiol. Scand. 146, 69–76.
[16] Van Huysse, J.W., Jewell, E.A. and Lingrel, J.B. (1993) Biochemistry 32, 819–826.
[17] Blostein, R. and Polvani, C. (1991) in: The Sodium Pump: Structure, Mechanism, and Regulation (Kaplan, J.H. and De Weer, P. eds.) pp. 289–301, The Rockefeller University Press, New York.
[18] Cornelius, F. (1991) Biochim. Biophys. Acta 1071, 19–66.