Kinetic Properties of the α2β1 and α2β2 Isozymes of the Na,K-ATPase†

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ABSTRACT: The presence of multiple isoforms of the α and β subunits of the Na,K-ATPase in most mammalian tissues has hindered the understanding of the roles of the individual isoforms in directing Na,K-ATPase function. Expression of the Na,K-ATPase subunits in insect cells using recombinant baculoviruses has proven to be a useful system for the study of Na,K-ATPase function. Using this system, we have expressed the rat Na,K-ATPase α2β1 and α2β2 isoforms in *Sf*-9 insect cells, a cell line derived from the ovary of the fall armyworm, *Spodoptera frugiperda*. Both β1 and β2 isoforms can efficiently assemble with the α2 subunit to produce catalytically competent Na,K-ATPase molecules. The analysis of the kinetic properties of both isozymes showed that α2β1 and α2β2 have equivalent sensitivities to ouabain, and similar turnover numbers and apparent affinities for K^+ and ATP. The dependence on Na^+, however, differs between the isozymes, with α2β2 displaying a slightly higher apparent affinity for the cation than α2β1. In addition, the even greater kinetic differences between Na,K-ATPase isozymes varying in α isoforms may be important in further differentiating the enzyme. Thus, when compared to the rat α1β1 Na,K-ATPase expressed in *Sf*-9 cells, the α2β1 and α2β2 isozymes have a lower apparent affinity for K^+ and a higher affinity for Na^+ and ATP. Moreover, the α1β1 isozyme is approximately 250 times more resistant to ouabain than α2β1 and α2β2. These different kinetic characteristics of the Na,K-ATPase isozymes may help establish the ionic milieu required by tissues to meet their specific physiological requirements.

The Na^+- and K^+-dependent adenosinetriphosphatase (Na,K-ATPase, Na pump) is a membrane-bound enzyme that uses the energy from the hydrolysis of ATP to maintain the low internal sodium and high internal potassium concentrations characteristic of most animal cells (Glynn, 1985). The minimal functional unit of the enzyme is a heterodimer, composed of two noncovalently linked subunits termed α and β. The α subunit is a 110 kDa polypeptide that spans the membrane multiple times. It contains the binding sites for ATP, cations, and the specific inhibitor ouabain and is responsible for all known catalytic properties of the enzyme. The β subunit is a type II glycoprotein with a molecular mass of 40−60 kDa (Mercer, 1993). Although the exact function of the β subunit is unclear, the subunit is required for normal enzymatic activity (Noguchi et al., 1987; Horowitz et al., 1990; DeTomaso et al., 1993). The β subunit influences extracellular K^+ activation of the Na,K-ATPase (Jaisser et al., 1992) and is essential in stabilizing, or may take part in forming, the cation-occluding complex of the enzyme (Lutsenko & Kaplan, 1992).

Both the α and β subunits are encoded by multiple genes. To date, three isoforms for the α(α1, α2, and α3) and two for the β (β1 and β2) have been identified in mammals [for a review, see Sweadner (1989) and Lingrel et al. (1990, 1992, 1994)], and a third β polypeptide (β3) has been described in amphibians (Good et al., 1990). The high conservation of the α and β isoforms through evolution (Takeyasu et al., 1990), their diverse patterns of expression, and their differential regulation during development and upon hormone stimulation (Sweadner, 1989) suggest that each isoform has a separate physiological role. Because the α2 and α3 isoforms are often coexpressed with the α1 in the same tissues, it has been difficult to investigate their individual kinetic characteristics. Likewise, the role of the multiple β subunit isoforms in directing the enzymatic properties of the enzyme is unknown. Interestingly, the overlap in the distribution of the α and β isoforms permits the existence of multiple αβ combinations and hence the potential for other functional Na,K-ATPase isozymes (Zlokovic et al., 1993). Expression of Na,K-ATPase polypeptides in cultured cells indicates that the three α subunit isoforms are able to assemble with both β1 and β2 into functional enzyme (Blanco et al., 1994). Whether different β polypeptides can distinctly influence the kinetic properties of the α isoforms is not known, since the properties of all plausible αβ complements have not been delineated.

To gain insight into the properties of the Na pump isoforms, we have used the baculovirus expression system to express the Na,K-ATPase polypeptides in *Sf*-9 insect cells, a cell line derived from the ovary of the fall armyworm, *Spodoptera frugiperda*. This cell line contains low levels of endogenous Na,K-ATPase and is capable of expressing high amounts of the baculovirus-induced proteins. When coexpressed with the β subunits, catalytically competent Na pump molecules can be obtained for all α isoforms (DeTomaso et al., 1993; Blanco et al., 1993, 1994). Here we use the baculovirus-induced expression of the rodent Na,K-ATPase α2β1 and α2β2 to investigate some kinetic properties of these isozymes and compare them under a similar

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environment, free of any other major contaminating Na,K-ATPase.

MATERIALS AND METHODS

DNA and Viral Constructions. The rat α1, α2, β1 (Schneider et al., 1985, 1988; Mercer et al., 1986), and β2 (Martin-Vasallo et al., 1989) Na,K-ATPase cDNAs (β2 a gift of Dr. Robert Levenson, Yale University) were subcloned into the baculovirus expression vector pVL1392 (provided by Verne Luckow, Monsanto). Isolation of wild-type *Autographica californica* multiple nuclear polyhedrosis virus (AcMNPV) genomic DNA and recombinant baculovirus preparation and selection were performed following standard procedures (O'Reilly et al., 1992).

Cells and Viral Infections. Uninfected and infected *Sf*-9 cells were grown in 150 mm petri dishes in TNM:FH medium [defined in O'Reilly et al. (1992); JRH Biosciences, Lenexa, KS], supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Fungizone. Viral infections were done at a viral multiplicity of infection ranging from 5 to 10. At 72 h after infection, cells were scraped from the plates in the incubating media, centrifuged at 1500*g* for 10 min, and then washed 3 times in 10 mM imidazole hydrochloride (pH 7.5)/1 mM EGTA. The final pellet was resuspended in the same solution. For the determination of enzymatic activity, the intact cells were used after permeabilization with alamethicin (10 μg/mg of protein) for 10 min at 25 °C as previously described (Xie et al., 1989). Preliminary experiments demonstrated that maximal activation of the enzyme was obtained with this concentration.

PAGE and Immunoblot Analysis. Expressed proteins were analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblotting. Proteins were separated by SDS−PAGE (Laemmli, 1970) and transferred to nitrocellulose (Hybond C+, Amersham Corp.). Nitrocellulose blots were blocked overnight at 4 °C in blotto (5% w/v nonfat dry milk/0.1% sodium azide in 150 mM NaCl/25 mM Hepes, pH 7.4). Primary antibody in 1% blotto was bound for 2 h at 37 °C on a rocking table. After three 10 min washes with 1% Triton X-100 in 150 mM NaCl/25 mM Hepes, pH 7.4, 2 μCi of ^{125}I-labeled goat anti-mouse or goat anti-rabbit secondary antibody in 1% blotto was added. After 1 h at 37 °C, blots were washed as before, dried, and exposed for autoradiography. The α2 isoform was identified with a monoclonal antibody (McB2) provided by Dr. Kathleen Sweadner (Massachusetts General Hospital). β1 was detected using either an anti-αβ antiserum prepared against purified rat kidney Na,K-ATPase [poly(αA)] or an anti-β antiserum raised against purified β1 from dog kidney (provided by Dr. Amir Askari, Medical College of Ohio, Toledo, OH). The β2 subunit was identified using an affinity-purified, polyclonal antibody (Upstate Biotechnology, Lake Placid, NY).

Immunoprecipitations. Uninfected and 48 h infected *Sf*-9 cells grown in 6-well tissue culture plates were lysed with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in 150 mM NaCl/25 mM Hepes (pH 7.4). After removal of the insoluble material (10 min at 15000*g*), samples were subjected to immunoprecipitation. For α2β1, 30 μL of the anti-β1 antiserum and for α2β2 50 μL of a specific monoclonal antibody hybridoma supernatant (5α, provided by Dr. Douglas Fambrough, Johns Hopkins University) were added. In both cases, 100 μL (1 mg/mL) of goat anti-mouse or goat anti-rabbit coated magnetic beads was then added (BioMag; PerSeptive Diagnostics, Inc., Cambridge, MA). After overnight incubation on a rocking table at 4 °C, beads were isolated by holding the microcentrifuge tube to a magnet and aspirating the supernatant. The beads were washed 3 times in the lysis buffer. The precipitated protein was eluted by resuspending the beads in sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 33% glycerol, and 100 mM DTT) and incubating for 15 min at 65 °C. Eluted proteins were separated by SDS−PAGE, transferred to nitrocellulose, and probed with an anti-α2 (McB2) monoclonal antibody (α2β1) or an anti-β2 specific antiserum (α2β2) as indicated.

Biochemical Assays. Protein assays were performed according to the procedure of Smith et al. (1985), using the bicinchoninic acid/copper sulfate solution as described by the supplier (Pierce Chemical Co., Rockford, IL).

Na,K-ATPase activity was assayed at 37 °C through determination of the initial rate of release of [^{32}P]P_i from [γ-^{32}P]ATP (Beaugé & Campos, 1983). The maximal Na,K-ATPase activity of samples (50−100 μg total protein) was measured in a final volume of 0.25 mL of medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 30 mM Tris-HCl (pH 7.4) ±1 mM ouabain. Sodium azide (2.5 mM final concentration) was included in the mixture to inhibit mitochondrial ATPase. The assay was started by the addition of ATP with 0.2 μCi of [γ-^{32}P]ATP (3 mM final concentration). Following a 30−60 min incubation at 37 °C, the tubes were placed on ice, and the reaction was terminated by the addition of 25 μL of 55% trichloroacetic acid. Released [$^{32}P_i$]P_i was converted to phosphomolybdate and extracted with isobutyl alcohol/benzene (Beaugé & Campos, 1983). Benzene can be omitted from the extraction without affecting the results; 0.15 mL of the organic phase containing the phosphomolybdate complex was removed and the radioactivity measured by liquid scintillation counting. The Na,K-ATPase activity, corresponding to 45% of the total ATPase activity, was determined as the difference in ATP hydrolysis in the absence and presence of 1 mM ouabain. The ATP hydrolyzed never exceeded 15% of the total ATP present in the sample, and hydrolysis was linear over the incubation time. For the analysis of activation by Na^+ and K^+, incubation media were the same as above except that for Na^+ dependency, Na^+ concentration was varied from 0 mM (for this point, sodium azide was omitted in the incubation mixture) to 125 mM. For K^+ stimulation, the K^+ concentration was varied from 0 to 30 mM. Choline chloride was added so that the final concentration of Na^+ or K^+ plus choline totaled 150 mM. The ATP dependency was determined under saturating concentrations of all cations (120 mM Na^+, 30 mM K^+, and 3 mM Mg^{2+}). To determine the effect of different ouabain concentrations on Na,K-ATPase activity, samples were incubated in the reaction medium with the indicated concentrations of ouabain for 30 min at 37 °C prior to the addition of ATP.

Phosphorylation of the Na,K-ATPase by ATP was carried out as previously described (Blanco et al., 1990). Phosphorylation of the α subunit was taken as the Na^+-dependent phosphorylation inhibitable by 1 mM ouabain.

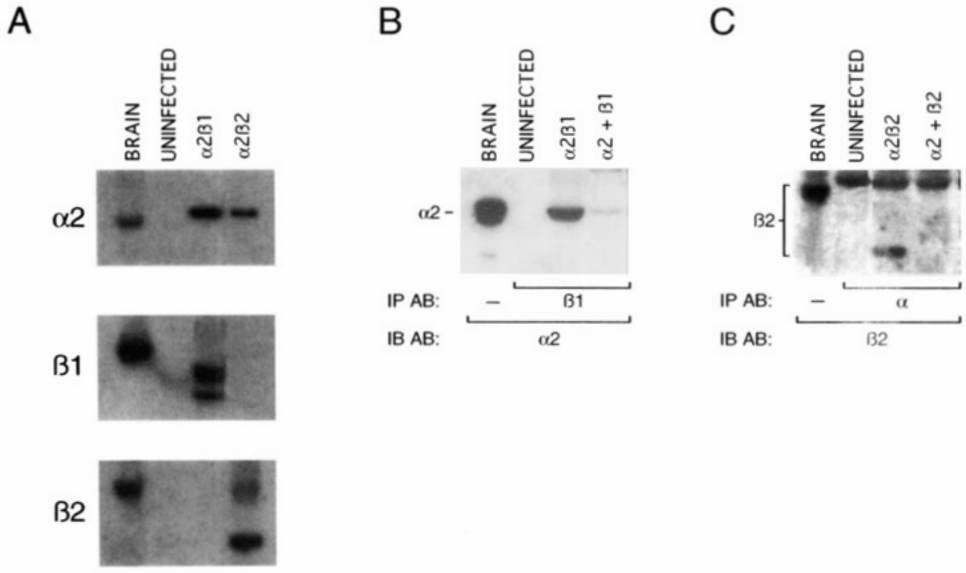

FIGURE 1: Rat Na,K-ATPase α2, β1, and β2 polypeptides in infected *Sf*-9 cells. (A) Immunoblot analysis of *Sf*-9 proteins. *Sf*-9 cells were infected with recombinant baculoviruses containing the cDNAs for the α2, β1, and β2 Na,K-ATPase subunits. 72 hours after infection, *Sf*-9 proteins (20 μg) were separated by SDS−PAGE (7.5% gel) and transferred to nitrocellulose. α2 polypeptide was detected with McB2 monoclonal antibody, β1 was identified with an αβ polyclonal antibody [poly(αA)], and β2 was detected using a polyclonal anti-β2 antiserum. (B) Association of α2 and β1 subunits coexpressed in *Sf*-9 cells. Proteins from uninfected, α2β1-coinfected *Sf*-9 cells, and combined proteins from cells individually expressing α2 and β1 (α2 + β1) were immunoprecipitated with an anti-β1 antiserum (IP AB). The precipitates were separated by SDS−PAGE, transferred to nitrocellulose, and immunoblotted with an α2 specific monoclonal antibody (IB AB). The α2 subunit from brain membranes (15 μg) is shown as a standard. (C) Association of α2 and β2 subunits coexpressed in *Sf*-9 cells. Proteins from uninfected, α2β2-coinfected *Sf*-9 cells, and combined proteins from cells individually expressing α2 and β2 (α2 + β2) were immunoprecipitated with an α specific monoclonal antibody hybridoma supernatant (IP AB). The precipitates were separated by SDS−PAGE, transferred to nitrocellulose, and immunoblotted with a β2 specific monoclonal antibody (IB AB). The β2 subunit from brain membranes (15 μg) is shown as a standard.

Data Analysis. Curve fitting of the experimental data was carried out using a Marquardt least-squares nonlinear regression computing program (Sigma Plot; Jandel Scientific, San Rafael, CA). Na^+ and K^+ activation curves were fitted according to a cooperative model for ligand binding, represented by the equation:

$$v = V_m[S]^n/(K + [S]^n) \quad (1)$$

where [S] is the concentration of the activating cation (Na^+ or K^+) and n is the Hill coefficient. The apparent affinity, $K_{0.5} = K^{1/n}$.

ATP curves were analyzed by the Michaelis−Menten equation. Dose−response relations for the ouabain inhibition of Na,K-ATPase activity showed a single homogeneous population and were fitted by the equation:

$$v = 100[1/(1 + [I]/K_i)] \quad (2)$$

where v is the Na,K-ATPase activity corresponding to a certain concentration of the inhibitor ouabain [I], expressed as a fraction of activity in the absence of ouabain, and K_i is the concentration of ouabain that gives half-maximal inhibition.

RESULTS

Baculoviruses containing the cDNAs for the rodent α2, β1, and β2 isoforms of the Na,K-ATPase were used to infect *Sf*-9 insect cells; 72 h after infection, *Sf*-9 cells express high amounts of the corresponding Na,K-ATPase subunits as determined by immunoblot analysis using antibodies specific to the Na,K-ATPase polypeptides (Figure 1A). As shown, the α2 polypeptide expressed in *Sf*-9 cells displays identical electrophoretic properties with the α2 isoform from rat brain. On the other hand, because protein glycosylation in the invertebrate cell is limited, the β1 and β2 proteins exhibit a smaller molecular mass than their native counterparts (DeTomaso et al., 1993). The different β polypeptides observed reflect the different levels of glycosylation. To determine if the α2 isoform can associate with either the β1 or the β2 subunit immunoprecipitation experiments were performed on proteins from coinfected cells; 72 h after infection with the corresponding recombinant viruses, cells were lysed and subjected to immunoprecipitation using an anti-β1 antibody for α2β1-coinfected cells or an anti-α monoclonal antibody for α2β2-coinfected cells. The immunoprecipitates were electrophoresed, transferred to nitrocellulose paper, and probed with an anti-α2 (McB2) monoclonal antibody (α2β1) or an anti-β2 specific antiserum (α2β2). In this fashion, if the α2 subunit associates with the β1 subunit, it should coimmunoprecipitate with the β1 subunit. Similarly, in the α2β2-coinfected cells, the β2 subunit should be identified in immunoprecipitations using the α specific antibody. To demonstrate the specificity of the association, cells separately infected with the α2 and β1 or the α2 and β2 viruses were combined and immunoprecipitated. As depicted in Figure 1B and Figure 1C, when coexpressed in the same cells, α2 is able to associate with the corresponding β isoform. In order to determine whether the expressed polypeptides are functionally active, assays of Na,K-ATPase activity were performed using optimal concentrations of ligands. *Sf*-9 cells coexpressing α2β1 or α2β2 isozymes displayed Na,K-ATPase activities 4−10-fold higher than the uninfected cells, with α2β1 having an approximately 2-fold higher activity than α2β2. The reason for the difference in activity of the isozymes is unknown, but it may result from different

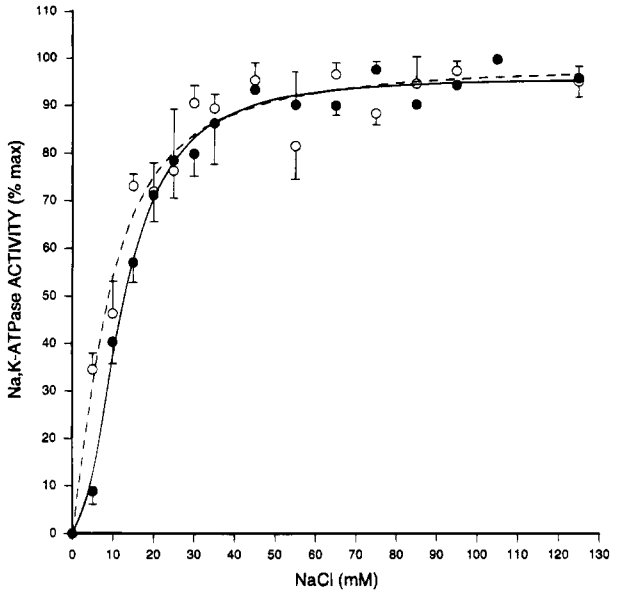

FIGURE 2: Na^+ activation of the $\alpha2\beta1$ and $\alpha2\beta2$ Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coinfected with $\alpha2\beta1$ (●) and $\alpha2\beta2$ (○) isoforms was determined as described. The composition of the reaction medium was 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP−cold ATP, 30 mM Tris-HCl (pH 7.4), and NaCl (from 0 to 125 mM), with or without 1 mM ouabain. Ionic strength was kept constant with choline chloride. Data are expressed as the percent of the maximal Na,K-ATPase activity obtained. Curves are the best fit of the data to eq 1. The average V_{max} values for the $\alpha2\beta1$ preparations were 1.4 ± 0.08 mmol of P_i mg^{-1} h^{-1} and 0.45 ± 0.03 mmol of P_i/mg^{-1} h^{-1} for $\alpha2\beta2$. Each value is the mean, and error bars represent the standard errors of the mean of three experiments performed in triplicate on samples obtained from different infections.

efficiencies of coinfection or a disparate ability of the β isoforms to associate with $\alpha2$. Activity of the baculovirus-induced $\alpha2\beta1$ and $\alpha2\beta2$ can also be demonstrated by the ability of the isozymes to form a Na^+-dependent and ouabain-inhibitable phosphointermediate from ATP. Assuming that the maximal levels of Na^+-dependent and ouabain-sensitive phosphorylation represent the density of active pump sites, the turnover number of the enzyme can be estimated. Correlation of the number of pump sites with the maximal NaK-ATPase activity gave molar activities of 13 300 ± 900 min^{-1} for $\alpha2\beta1$ and 11 900 ± 400 min^{-1} for $\alpha2\beta2$. Both values are in good agreement with those found for the native enzyme from brain or kidney (Glynn, 1985). However, although the infected insect cells express functional Na,K-ATPase polypeptides, it is clear that a substantial proportion of the polypeptides (≈90%) are inactive. This may be a result of the unequal expression of the two subunits in individual cells or the improper folding, assembly, or targeting of the polypeptides. Similar discrepancies between the levels of expression and activity of plasma membrane proteins have been observed using the baculovirus expression system (Klaiber et al., 1992; Li et al., 1992; Smith et al., 1992; Klaassen et al., 1993).

To determine if the β isoforms influence the kinetic properties of the $\alpha2$ subunit, the ligand affinities of the $\alpha2\beta1$ and $\alpha2\beta2$ isozymes were determined. The Na,K-ATPase activity with varying Na^+ concentrations (0−125 mM) was determined with saturating K^+ (20 mM). The Na^+ dependence of the $\alpha2\beta1$ and $\alpha2\beta2$ forms of the Na,K-ATPase is presented in Figure 2, and the apparent affinities ($K_{0.5}$ values)

Table 1: Apparent Affinities ($K_{0.5}$) and Hill Coefficients (n_H) for Na^+ and K^+ Activation and K_m Values for ATP Stimulation of the Na,K-ATPase $\alpha2\beta1$, $\alpha2\beta2$, and $\alpha1\beta1$ Isozymes

	Na^+ activation		K^+ activation		ATP activation
isozyme	$K_{0.5}$ (mM)	n_H	$K_{0.5}$ (mM)	n_H	K_m (mM)
$\alpha2\beta1$	12.4 ± 0.5	2.09 ± 0.2	3.6 ± 0.3	1.22 ± 0.1	0.11 ± 0.01
$\alpha2\beta2$	8.8 ± 1.0	1.52 ± 0.2	4.8 ± 0.4	1.33 ± 0.1	0.11 ± 0.02
$\alpha1\beta1$	16.4 ± 0.7	2.90 ± 0.3	1.9 ± 0.2	1.43 ± 0.2	0.46 ± 0.10

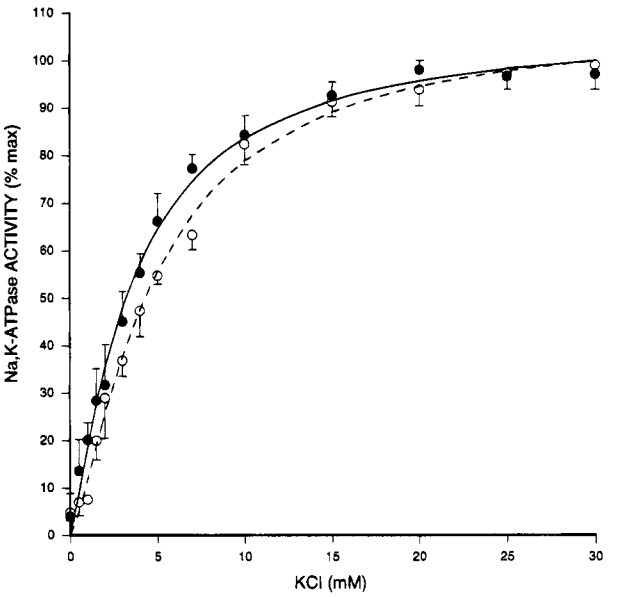

FIGURE 3: K^+ activation of the $\alpha2\beta1$ and $\alpha2\beta2$ Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coinfected with $\alpha2\beta1$ (●) and $\alpha2\beta2$ (○) isoforms was determined as described. The reaction medium contained 120 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, mM [γ-^{32}P]ATP−cold ATP, 30 mM Tris-HCl (pH 7.4), and KCl (from 0 to 30 mM), with or without 1 mM ouabain. Ionic strength was kept constant with choline chloride. Data are expressed as the percent of the maximal Na,K-ATPase activity obtained. Curves are the best fit of the data to eq 1. The average V_{max} values for each preparation were those shown in Figure 2. Each value is the mean, and error bars represent the standard errors of the mean of three experiments performed in triplicate on samples obtained from different infections.

and Hill coefficients are tabulated in Table 1. Both isozymes exhibited a sigmoidal dependence on [Na^+], consistent with the multiple Na^+ sites of the enzyme. In addition, the Na,K-ATPase consisting of the $\alpha2\beta2$ subunits displayed a slightly higher apparent affinity for Na^+ than the $\alpha2\beta1$ enzyme.

To determine the affinity of the Na,K-ATPase isozymes for K^+, the activities of the enzymes in media containing various concentrations of K^+ (0−30 mM) with [Na^+] fixed at 130 mM were measured. The corresponding curves are shown in Figure 3, and values describing the kinetic parameters are given in Table 1. As shown, the apparent affinities for K^+ are similar for both isozymes. As a comparison, the same conditions were used to obtain the Na^+ and K^+ requirements of the $\alpha1\beta1$ Na,K-ATPase expressed in *Sf*-9 insect cells. The $\alpha1\beta1$ Na,K-ATPase has a lower affinity for Na^+ and a higher affinity for K^+ than the $\alpha2\beta1$ and $\alpha2\beta2$ isoforms expressed in the *Sf*-9 cells. The native $\alpha1\beta1$ Na,K-ATPase from rat kidney had identical kinetic properties as the expressed $\alpha1\beta1$ enzyme (data not shown). The resulting $K_{0.5}$ values for both Na^+ and K^+ activation of the Na,K-ATPase $\alpha1\beta1$ are shown in Table 1. The Hill

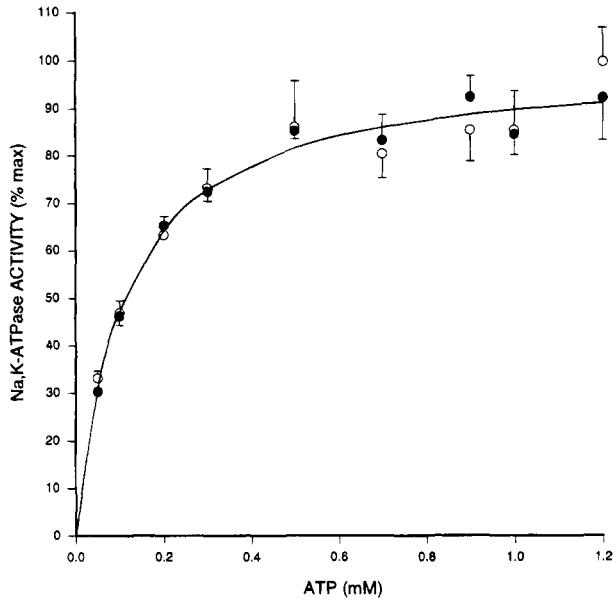

FIGURE 4: ATP stimulation of the α2β1 and α2β2 Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coinfected with α2β1 (●) and α2β2 (○) isoforms was determined as described. The reaction medium contained 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4), and the indicated ATP concentrations, with or without 1 mM ouabain. Data are expressed as the percent of the total Na,K-ATPase activity determined from the best fit to the Michaelis–Menten equation. Each value is the mean of three experiments performed in triplicate on samples obtained from different infections. Error bars represent the standard errors of the mean.

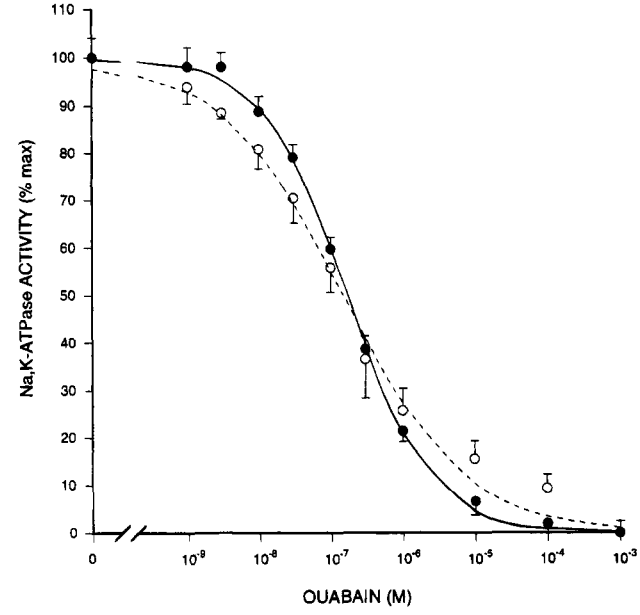

FIGURE 5: Dose–response curves for the ouabain inhibition of α2β1 and α2β2 Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coinfected with α2β1 (●) and α2β2 (○) isoforms was determined as described. Samples were preincubated for 30 min at 37 °C in the reaction mixture [120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4), and the indicated ouabain concentrations]. The reaction was started by the addition of [γ-^{32}P]ATP–cold ATP. Values are expressed as the percentage of maximal activity in the absence of the inhibitor. The average V_{max} values for each preparation were those indicated in Figure 2. Curves represent the best fit of the data using eq 2. Each value is the mean, and error bars represent the standard errors of the mean of three experiments performed in triplicate on samples obtained from different infections.

coefficients for both Na^+ and K^+ are consistent with multiple ligand binding sites. The Hill coefficient has a complex physical significance; however, coefficients greater than 1 generally reflect positive cooperativity at multiple interacting ligand binding sites (Dixon & Webb, 1979).

The Na,K-ATPase has been shown to display a biphasic activation by ATP, with the existence of high- and low-affinity sites for substrate (Glynn, 1985). The high-affinity site was difficult to study in the *Sf*-9 cell preparations because of the nonspecific ATPase activity present. Therefore, we characterized the low-affinity site for ATP by measuring the activation kinetics for ATP in the millimolar range. Figure 4 shows the ATP stimulation curves for the α2β1 and α2β2 forms of the Na,K-ATPase. The kinetic parameters of these experiments are outlined in Table 1 and demonstrate that the K_m for ATP is identical for both isozymes. Moreover, when compared to the α1β1 enzyme, the α2β1 and α2β2 isozymes have K_ms for ATP that are about 4-fold lower (Table 1).

An important property that has been used to identify the Na,K-ATPase isoforms is their differential responses to cardiotonic steroids (Sweadner, 1989; Lingrel et al., 1990). The ouabain sensitivities of the α2β1 and α2β2 isozymes were determined by performing dose–response curves for the ouabain inhibition of Na,K-ATPase activity under nonlimiting ligand concentrations. Figure 5 shows the obtained plots with experimental points expressed as a percentage of the maximal Na,K-ATPase activity in the absence of the inhibitor. The baculovirus-induced α2β1 and α2β2 isozymes display homogeneous curves, indicating the presence of a single population of sites for the inhibitor. The resulting K_i values, which are similar for both isozymes, are summarized in Table 2. Ouabain inhibition curves were also

Table 2: Apparent Ouabain Affinities (K_i) of the Na,K-ATPase α2β1 and α2β2 and the Native and Baculovirus-Induced α1β1 Isozymes

isozyme	K_i (M)
α2β1	$1.7 \times 10^{-7} \pm 0.1 \times 10^{-7}$
α2β2	$1.5 \times 10^{-7} \pm 0.2 \times 10^{-7}$
α1β1	$4.3 \times 10^{-5} \pm 1.9 \times 10^{-5}$
α1β1 (native)	$9.8 \times 10^{-5} \pm 0.9 \times 10^{-5}$

performed for the Na,K-ATPase α1β1 enzyme from rat kidney and α1β1-infected *Sf*-9 cells. These enzymes are more than 250-fold less sensitive to ouabain than the α2 isozymes (Table 2). Interestingly, the α1β1 enzyme expressed in insect cells is over 2-fold more sensitive to ouabain than the native α1β1 from rat kidney. This difference in sensitivity between the native and expressed Na,K-ATPase may be a result of the different lipid environments of enzyme. For example, it has been shown that lipid peroxidation increases the affinity of the Na,K-ATPase toward the cardiotonic steroid strophanthidin (Mishra et al., 1989). This possibility will require further investigation.

DISCUSSION

In the rat, the α2 isoform is expressed in neuronal and glial cells of the brain, in adipocytes, and in skeletal muscle and the heart [see Sweadner (1989)]. In fat and skeletal muscle, insulin stimulates Na,K-ATPase activity, and it appears that this action is accomplished through the α2 subunit (Lytton et al., 1985). In addition, in rodents the α2 isoform, when compared to α1, has a higher affinity for the

cardiotonic steroid ouabain (Sweadner, 1985; Blanco et al., 1993). The expression of multiple isoforms of the α and β subunits in most tissues has made it particularly difficult to characterize the specific properties of the individual isoforms. The baculovirus system provides the opportunity to study the Na,K-ATPase isozymes independent of any other major contaminating Na,K-ATPase polypeptides (DeTomaso et al., 1993; Blanco et al., 1993). Using this system, we characterized the α2β1 and α2β2 isoforms of the Na,K-ATPase in *Sf*-9 insect cells. Results presented here show that the α2 subunit is able to associate with both β isoforms of the Na pump. This is consistent with other results demonstrating that various α and β isoforms can associate and form functional enzyme. For instance, the rodent Na,K-ATPase β2 subunit, the *Xenopus* β3, and the rabbit β subunit of the H,K-ATPase can associate with the α1 subunit from *Xenopus* or *Torpedo*, to support Na,K-ATPase activity (Ackermann & Geering, 1992; Schmalzing et al., 1992). Moreover, in baculovirus-infected insect cells, all αβ combinations among the rodent isoforms result in stable associations (Blanco et al., 1993).

Both the α2β1 and α2β2 isozymes expressed in *Sf*-9 cells are catalytically active as defined by Na,K-ATPase activity and Na^+-dependent phosphorylation from ATP. Although the functional distinction of these Na,K-ATPase isozymes has not been accomplished *in vivo*, the coexpression of α2, β1, and β2 in the same cell strongly suggests their existence (Zlokovic et al., 1993). To examine the enzymatic characteristics of α2, as well as the role of the β isoforms in influencing the catalytic properties of the Na,K-ATPase, we determined the affinity of the α2β1 and α2β2 isozymes for Na^+, K^+, ATP, and the cardiotonic steroid ouabain. α2β1 and α2β2 are similar with respect to their apparent affinities for K^+, ATP stimulation, and ouabain sensitivity. However, the α2β2 enzyme shows a slightly higher apparent affinity for Na^+. These results suggest that the accompanying β subunit does not drastically modify the enzymatic properties of the α2 subunit. Nevertheless, the observed difference in the affinity for Na^+ may represent an advantageous adaptation to cellular needs. There are other reports suggesting a modulatory effect of the β subunit on the catalytic properties of the α subunit. For example, the α1β1 and α1β2 isozymes expressed in *Xenopus* oocytes have different affinities for Rb^+ (Schmalzing et al., 1992; Jaisser et al., 1992). In that study, the Na,K-ATPase molecules were hybrids of the *Torpedo* α1 and the rat β1 and β2 subunits. Moreover, the Na^+ dependence of the rat α3 isoform seems to vary depending on its association with β1 or β2 [compare the results of Shyjan et al. (1990b) with those of Jewell and Lingrel (1991)]. However, in that case, the enzyme consisting of the β2 subunit has a lower affinity for Na^+. The fact that β2 is highly expressed in the nervous system (Shyjan et al., 1990a; Coca-Prados et al., 1990), is present in glial cells at critical stages of neuron development (Pagliusi et al., 1990), and is responsible for specific processes such as cellular migration, neurite outgrowth, and cell adhesion (Antonicek et al., 1987; Gloor et al., 1990; Muller-Husmann et al., 1993) makes it conceivable that this isoform is involved in Na,K-ATPase isozymes with selected properties to serve in these specific cellular events.

Our results also demonstrate that the α2 isozymes have a higher affinity for Na^+ and ATP (1.4−2- and 3-fold, respectively), a higher sensitivity to ouabain (more than 250-fold), and a lower affinity for K^+ (≈2-fold) than the α1β1 Na,K-ATPase (Tables 1 and 2). A considerable amount of effort has been invested in elucidating the enzymatic properties of the Na,K-ATPase isoforms. However, there are vast discrepancies in the reported kinetic properties of the various isoforms [reviewed in Sweadner (1989)]. A comparison with previous work becomes rather difficult since most studies have been complicated by the simultaneous presence of multiple isoforms. Nevertheless, in a few cases, advantage could be taken of enzyme preparations enriched in a particular isoform. Thus, in agreement with our results, similar differences in the requirements for Na^+ (Sweadner, 1985) and K^+ (Sweadner, 1985; Shyjan et al., 1990c) have been detected between Na,K-ATPases containing predominantly the α1 and α2 isoforms. However, our results are in contrast to other studies that suggest that the α1 and α2 isoforms have a similar K_m for ATP (Sweadner, 1985) and that the α1 isoform has a higher Na^+ affinity than α2 (Lytton et al., 1985). In these studies, the β composition of the Na,K-ATPase isozymes was not determined. Other studies have taken advantage of the different sensitivities of the α isoforms to ouabain. To investigate the kinetic properties of the sheep α1, α2, and α3 subunits, the subunits were made ouabain-resistant, and expressed in HeLa cells where they could be functionally separated from the endogenous ouabain-sensitive Na,K-ATPase. Characterization of the α1 and α2 subunits in this system suggests that the isoforms have similar affinities for Na^+, K^+, and ATP (Jewell & Lingrel, 1991; Munzer et al., 1994). The interfering endogenous levels of Na,K-ATPase, differences in the membrane environment of mammalian and invertebrate cells, and/or the alteration of the isoforms by mutation of their ouabain binding site may be responsible for the disparity in results.

A physiological role for the isoforms is difficult to infer from our results. However, the different enzymatic properties found for the α1- and α2-containing isozymes suggest that the existence of Na,K-ATPase isozymes is based, at least in part, on their specific kinetic characteristics. These subtle differences may be essential in adapting cellular Na,K-ATPase activity to specific physiological requirements. For instance, having multiple Na,K-ATPase isozymes with different apparent affinities for Na^+ may be physiologically important in controlling intracellular Na^+ levels. These variations in intracellular Na^+ levels may affect other cellular functions. For example, slight increases in cellular [Na^+] can affect the cytoplasmic levels of free Ca^{2+}, favoring its distribution to intracellular stores. This increase in [Ca^{2+}] available for mobilization is important in the regulation of contraction, secretion, and excitability (Blaustein, 1993). Finally, it is possible that the association of β1 and β2 with the α1 and α3 isoforms results in isozymes with different characteristics, and, if so, delineating the properties of these other Na,K-ATPase isozymes will help in explaining the diversity in Na,K-ATPase structure.

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