Comparison of the Enzymatic Properties of the Na,K-ATPase α3β1 and α3β2 Isozymes†

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ABSTRACT: The coexpression of multiple isoforms of the α and β subunits of the Na,K-ATPase in mammalian tissues gives rise to the complex molecular heterogeneity that characterizes the Na pump. The expression of the different Na,K-ATPase isoforms in insect cells using recombinant baculoviruses represents a useful system for the analysis of Na,K-ATPase isoform function. In the present study, we use this system to direct the expression of the rat Na,K-ATPase α3β1 and α3β2 in *Sf*-9 cells, a cell line derived from the ovary of the fall armyworm, *Spodoptera frugiperda*. The association of α3 with either β1 or β2 results in catalytically competent Na,K-ATPase isozymes. Analysis of the kinetic characteristics of these enzymes demonstrates that the accompanying β subunit isoform does not drastically affect the properties of the α3 polypeptide. This is evidenced by the similar turnover numbers, apparent affinities for K^+ and ATP, and the comparable high sensitivity to ouabain exhibited by both isozymes. The kinetic dependence on Na^+, however, is different for both isozymes, with α3β2 displaying a 1.6-fold higher apparent affinity for the cation than α3β1. Comparison with other Na,K-ATPase isozymes shows that the apparent Na^+ affinity of α3β2 is similar to that of the α1β1 Na pump widely expressed in every tissue; nevertheless, its reactivity toward K^+, ATP, and ouabain are characteristic of the α3 isoform. The most pronounced kinetic differences in Na,K-ATPase function are a result of variations in α isoform composition. In this regard, the most conspicuous kinetic difference corresponds to the reactivity toward ouabain, with α3β1 and α3β2 displaying a high, α2β1 and α2β2 an intermediate, and α1β1 a low sensitivity for the cardiotonic steroid. Overall, the enzymatic differences toward the individual ligands may be important in adapting cellular Na,K-ATPase activity to specific physiological requirements.

The Na,K-ATPase or Na pump is a plasma membrane enzyme that uses the energy from the hydrolysis of ATP to maintain the transmembrane electrochemical gradients of sodium and potassium. The enzyme is a heterodimer composed of equimolar amounts of two noncovalently linked proteins termed α and β (Glynn, 1985, 1993). The α subunit is a 110 kDa polypeptide that spans the membrane multiple times. The β subunit is a type II glycoprotein with a molecular mass of 40−60 kDa. The catalytic properties of the enzyme are associated with the α subunit, which contains the binding sites for ATP, cations, and the specific inhibitor ouabain (Mercer, 1993; Lingrel, 1994). The exact role of the β subunit in the reaction cycle of the Na,K-ATPase is not clear; however, its extracellular domain has been found to participate in the K^+ binding process of the enzyme (Lutsenko & Kaplan, 1993).

Both Na,K-ATPase subunits are encoded by multigene families (Lingrel et al., 1990). At present, several isoforms for the α subunit (α1, α2, and α3) and β subunit (β1 and β2) have been identified in mammals (Sweadner, 1989; Lingrel, 1992), and a third β isoform has been described in amphibians (Good et al., 1990). The high degree of homology of the α isoforms across species suggests that there are stringent constraints on the structure and function of the polypeptides. While the functional significance for this isoform diversity is not well understood, the tissue-specific and developmentally regulated pattern of expression (Orlowski & Lingrel, 1988; Schneider et al., 1988) suggests different roles for the isoforms.

Because of the coexpression of the different α and β isoforms in most tissues, delineating the enzymatic characteristics of the various Na,K-ATPase αβ complexes has been particularly difficult. To analyze the properties of the rodent Na pump isoforms, we have used the baculovirus expression system to direct the synthesis of the different α and β polypeptides in *Sf*-9 insect cells, a cell line derived from the ovary of the fall armyworm, *Spodoptera frugiperda*. This cell line has proven useful for the expression, assembly, and characterization of catalytically competent Na,K-ATPase molecules. Most importantly, this expression system provides the opportunity to study the different combinations of the α and β isoforms in an environment relatively free of endogenous Na,K-ATPase activity (DeTomaso et al., 1993; Blanco et al., 1993). We have previously examined the kinetic behavior of the rodent α2β1 and α2β2 isoforms (Blanco et al., 1995). Here we use the baculovirus induced expression of the rat Na,K-ATPase α3β1 and α3β2 to investigate the kinetic properties of these Na pump isozymes.

MATERIAL AND METHODS

DNA and Viral Constructions. The cDNAs corresponding to the rat Na,K-ATPase α3, β1 (Mercer et al., 1986; Schneider et al., 1988) and β2 (Martin-Vasallo et al., 1989;

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provided by R. Levenson) subunits 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. *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 (complete medium). Infections were performed in serum-free medium for 1 h using a viral multiplicity of infection ranging from 5 to 10. After addition of complete medium, cultures were maintained for 72 h. Cells were then scraped from the plates, centrifuged at 1500*g* for 10 min, and washed three times in 10 mM imidazole hydrochloride (pH 7.5) and 1 mM EGTA. Cells were resuspended in the same solution and used for assays. For the determination of enzymatic activity, the intact cells were used after permeabilization with the ionophore alamethicin (Xie et al., 1989). For ouabain binding experiments, a cell membrane preparation was used (Blanco et al., 1993).

PAGE and Immunoblot Analysis. Proteins were analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblotting. Proteins were separated by SDS−PAGE (Laemmli, 1970), transferred to nitrocellulose (Hybond C$^+$, Amersham Corp.), and immunoblotted as previously described (Blanco et al., 1995). The α3 isoform was identified with a polyclonal antibody to a synthetic peptide derived from the N-terminal region of the α3 subunit. β1 was detected using an anti-β1 antiserum raised against purified β1 subunit from dog kidney (provided by Dr. Amir Askari, Medical College of Ohio, Toledo, OH), and β2 was identified using a polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) that had been affinity purified.

Immunoprecipitations. Uninfected and 48 h infected *Sf*-9 cells grown in six-well tissue culture plates were lysed with 1% 3-[(3-cholamidopropyl)dimethyl ammonio]-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. To precipitate the α3 isoform, 50 μL of a monoclonal antibody hybridoma supernatant that is specific for the α subunit (5α, provided by Dr. Douglas Fambrough, Johns Hopkins University) and 100 μL (1 mg/mL) of goat anti-mouse coated magnetic beads (BioMag; PerSeptive Diagnostics, Inc., Cambridge, MA) were used. 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 three 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, 100 mM DTT) and incubating for 15 min at 65 °C. Eluted proteins were separated by SDS−PAGE (7.5% gel), transferred to nitrocellulose, and probed with the anti-β1 (α3β1) or the anti-β2 (α3β2) specific antiserum.

Biochemical Assays. Protein assays were performed using the bicinchoninic acid/copper sulfate solution as described by the supplier (Pierce Chemical Co, Rockford, IL) after lysis of the cells in 2% CHAPS.

Na,K-ATPase activity was assayed through determination of the initial rate of release of $^{32}P_i$ from [γ -^{32}P]ATP as previously described (Blanco et al., 1995). The maximal Na,K-ATPase activity of samples (50−100 μg of 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 [γ-^{32}P]ATP (3 mM final concentration). Following a 30 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 isobutanol. Radioactivity of 0.15 mL of the organic phase was measured by liquid scintillation counting. The ATP hydrolyzed never exceeded 15% of the total ATP present in the sample, and hydrolysis was linear over the incubation time. Specific Na,K-ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of 1 mM ouabain. For the analysis of activation by Na^+ and K^+, incubation media were the same as above except that for Na^+ dependency, the Na^+ concentration was varied from 2.5 to 122.5 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 preincubated with the indicated concentrations of ouabain for 30 min at 37 °C in the reaction medium; the reaction was started by the addition of ATP.

Ouabain binding assays were performed using [^{3}H]ouabain (Amersham Corp.) with a specific activity of 32 Ci/mmol (1 Ci = 37 GBq) as described (Blanco et al., 1993). The nonspecific binding was defined as the binding of [^{3}H]-ouabain in the presence of 1 mM unlabeled ouabain.

Data Analysis. Curve fitting of the experimental data was performed 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

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

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

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

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 the half-maximal inhibition.

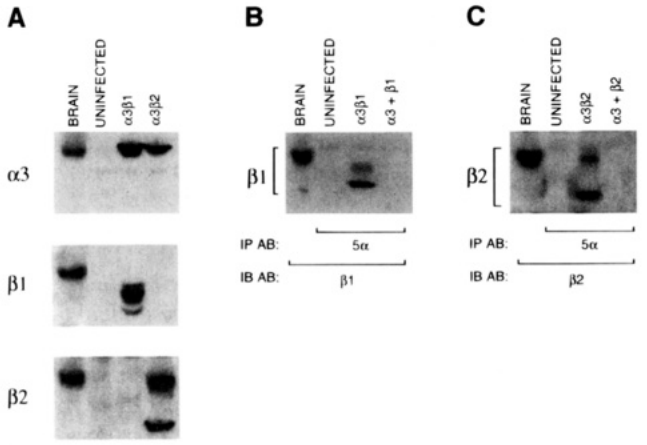

FIGURE 1: Expression of rat Na,K-ATPase α3, β1, and β2 polypeptides in infected *Sf*-9 cells. (A) Immunoblot analysis of *Sf*-9 proteins. Recombinant baculoviruses containing the cDNAs of the rat α3, β1, and β2 Na,K-ATPase subunits were used to infect *Sf*-9 insect cells. After 72 h *Sf*-9 proteins (20 μg) were separated by SDS−PAGE (7.5% gel) and transferred to nitrocellulose. The α3 polypeptide was detected with a polyclonal antibody to a synthetic peptide of the N-terminus of the α3 isoform, β1 was identified with an anti-β1 antiserum raised against dog kidney purified β1 subunit, and β2 was detected using a polyclonal anti-β2 antiserum. (B) Association of α3 and β1 subunits coexpressed in *Sf*-9 cells. Proteins from uninfected, α3β1 coinfected *Sf*-9 cells, and combined proteins from cells individually expressing α3 and β1 (α3 + β1) were immunoprecipitated with the 5α antibody (IP AB). The precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the anti-β1 antiserum (IB AB). The native β1 subunit from brain membranes (15 μg) is shown as standard. (C) Association of α3 and β2 subunits coexpressed in *Sf*-9 cells. Proteins from uninfected, α3β2 coinfected *Sf*-9 cells, and combined proteins from cells individually expressing α3 and β2 (α3 + β2) were immunoprecipitated with the 5α antibody hybridoma supernatant (IP AB). After SDS−PAGE and immunoblotting, the β2 specific antibody was used for detection (IB AB). The β2 subunit from brain membranes (15 μg) is shown as a standard.

Statistical analysis of the concentration-response curves of each isozyme was done applying an *F* test to compare the relative goodness of fit of the curves describing the individual isozymes with the one obtained for the experimental data of both isozymes combined. The *F* value was calculated using the following equation (Waud, 1975; Woodward et al., 1995):

$$F = (SS_c - SS_i/df_c - df_i)/(SS_i/df_i) \quad (3)$$

where SS_c is the total sum of squared deviations for the simultaneous fitting of the data of both isozymes combined, SS_i is the sum of squared deviations for the fits of the individual isozymes, and df_c and df_i, are the degrees of freedom (number of data points − number of parameters) for the simultaneous and both individual fits, respectively. Differences were considered significant when the *F* value exceeded the theoretical F value for the corresponding degrees of freedom ($df_c - df_i$, df_i) with $p = 0.01$.

RESULTS

Recombinant baculoviruses containing cDNAs coding for the rat Na,K-ATPase α3, β1, and β2 isoforms were used to infect *Sf*-9 insect cells. To analyze the expression of the virally induced polypeptides, proteins from cells coinfected with α3β1 or α3β2 viruses were subjected to SDS−PAGE and immunoblotting. As shown in Figure 1A, antibodies specific to the Na,K-ATPase subunits detected, in infected cells, high levels of the Na,K-ATPase α3 and β polypeptides. The virally induced α3 isoform comigrates with the native subunit from rat brain; however, because protein glycosylation in the invertebrate cell is limited, the β polypeptides have a reduced molecular mass compared to their native counterparts (DeTomaso et al., 1993).

To confirm that the expressed α3 polypeptide can form a stable association with either of the β subunits, *Sf*-9 cells were coinfected with the α3 and β1 or β2 viruses. Forty-eight hours after infection, cells were lysed, and the Na,K-ATPase α3 isoform was immunoprecipitated with an anti-α monoclonal antibody. Immunoprecipitated proteins were then separated by SDS−PAGE, transferred to nitrocellulose paper, and probed with the β1 or β2 specific antibodies. As shown in Figure 1B,C, when coexpressed with the α3, the corresponding β subunit coprecipitates with the α3 subunit, indicating that the α3β1 and α3β2 isoform combinations can properly assemble in the insect cells. In contrast, β polypeptides do not coimmunoprecipitate with the α3 subunit when cells separately infected with the β and the α3 baculoviruses are mixed and immunoprecipitated with the α specific antibody. Thus the αβ assembly only occurs in the coinfected cells.

In order to determine whether the expressed polypeptides are catalytically competent, Na,K-ATPase activity assays were performed using optimal ligand concentrations. Specific activity of the *Sf*-9 cells coexpressing α3β1 or α3β2 isozymes was 0.62 ± 0.04 μmol of P_i/mg/h and 0.44 ± 0.05 μmol of P_i/mg/h, respectively, which is approximately 5−7 times higher than the Na,K-ATPase activity observed in uninfected cells. In addition, the baculovirus induced α3β1 and α3β2 isozymes were also capable of binding [^{3}H]-ouabain. Binding of the labeled cardiotonic steroid was measured on membrane fractions of *Sf*-9 cells in medium containing 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM ATP, 40 mM imidazole, pH 7.5, and various concentrations of [^{3}H]ouabain (0.5−5 μCi) ± 1 mM unlabeled ouabain. Scatchard analysis of the data revealed a maximal binding of 1.76 ± 0.06 pmol/mg for α3β1 and 0.78 ± 0.06 pmol/mg for α3β2. Since these values reflect the actual amount of active pump sites of the sample, calculation of the turnover numbers of the enzymes can be performed. Correlation of the values of maximal Na,K-ATPase activity and [^{3}H]ouabain binding gave molar activities of 6500 ± 1400 min^{-1} for α3β1 and 9200 ± 3700 min^{-1} for α3β2. These values correspond with those reported for Na,K-ATPase from other sources (Maixent & Berrebi-Bertrand, 1993). However, as previously reported for the other isozymes of the Na pump (DeTomaso et al., 1993; Blanco et al., 1995), correlation of the levels of Na,K-ATPase activity and amount of protein expressed in the infected *Sf*-9 cells shows that a substantial proportion of the virally directed polypeptides are inactive. This can result from a disparity of expression of the α and β subunits in the individual cells or inefficient folding, assembly, or targeting of the polypeptides in the insect cells.

To determine the affinity of the α3β1 and α3β2 isozymes toward physiological ligands, activation curves of Na,K-ATPase activity by Na^+, K^+, and ATP were performed. The Na^+ dependence of Na,K-ATPase activity was determined at various concentrations of Na^+ and constant saturating K^+ (20 mM). Figure 2 shows the corresponding curves; the

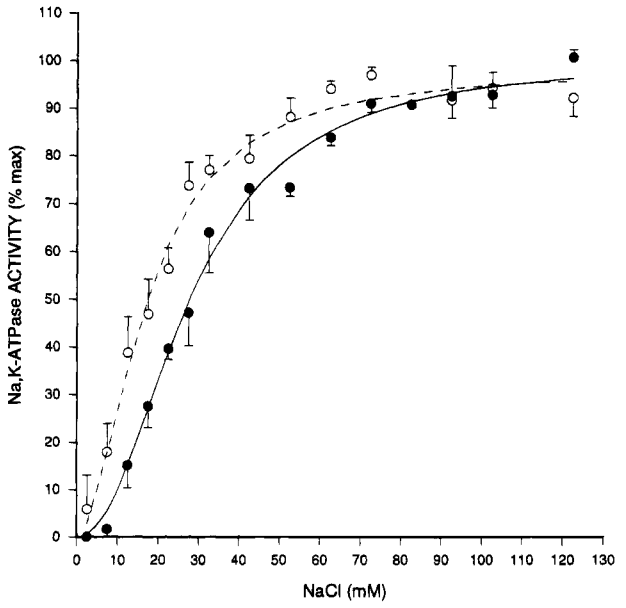

FIGURE 2: Na^+ activation of the rat $\alpha3\beta1$ and $\alpha3\beta2$ Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coexpressing the $\alpha3\beta1$ (●) or $\alpha3\beta2$ (○) isoforms was determined in a reaction medium containing 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP−cold ATP, 30 mM Tris-HCl (pH 7.4), and NaCl as indicated, in the absence or presence of 1 mM ouabain. Ionic strength was kept constant with choline chloride. Data are expressed as percent of the maximal Na,K-ATPase activity obtained. Curves are the best fit of the data to eq 1. Each value is the mean, and error bars represent the standard errors of the mean of seven experiments performed in triplicate on samples obtained from different infections.

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 $\alpha1\beta1$, $\alpha2\beta1$, $\alpha2\beta2$, $\alpha3\beta1$, and $\alpha3\beta2$ Isozymes

iso-zyme	Na^+ activation		K^+ activation		ATP activation
	$K_{0.5}$ (mM)	n_H	$K_{0.5}$ (mM)	n_H	K_m (mM)
$\alpha1\beta1$	16.4 ± 0.7	2.90 ± 0.3	1.9 ± 0.2	1.43 ± 0.2	0.46 ± 0.10
$\alpha2\beta1$	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
$\alpha3\beta1$	27.9 ± 1.3	2.13 ± 0.2	5.3 ± 0.3	2.40 ± 0.3	0.09 ± 0.01
$\alpha3\beta2$	17.1 ± 1.0	1.83 ± 0.2	6.2 ± 0.4	2.40 ± 0.3	0.07 ± 0.03

apparent affinities ($K_{0.5}$ values) and Hill coefficients obtained are tabulated in Table 1. Both isozymes displayed a sigmoidal dependence on the cation, which is reflected by Hill coefficients higher than one (Dixon & Webb, 1979) and is consistent with the existence of multiple interacting Na^+ sites. In addition, the Na,K-ATPase $\alpha3\beta2$ isozyme displayed an apparent affinity for Na^+ that is approximately 1.6-fold higher than that of the $\alpha3\beta1$ enzyme. To confirm the statistical significance of the difference in Na^+ requirement between the isozymes, the relative goodness of fit of the theoretical curve from the combined data versus both individual fits for the isozymes was compared using a *F* test. The obtained values of F = 34.4, with degrees of freedom of 3 ($df_c - df_i$) and 23 (df_i) and $p < 0.01$ indicate that separate curves describing each enzyme population are favored over a single curve representing the $\alpha3\beta1$ and $\alpha3\beta2$ values combined.

To determine the requirements of the Na,K-ATPase $\alpha3\beta1$ and $\alpha3\beta2$ isozymes for K^+, Na,K-ATPase activity was measured at various concentrations of K^+ (0−30 mM) with Na^+ fixed at 120 mM. The obtained curves are presented in Figure 3, and values describing the kinetic parameters are

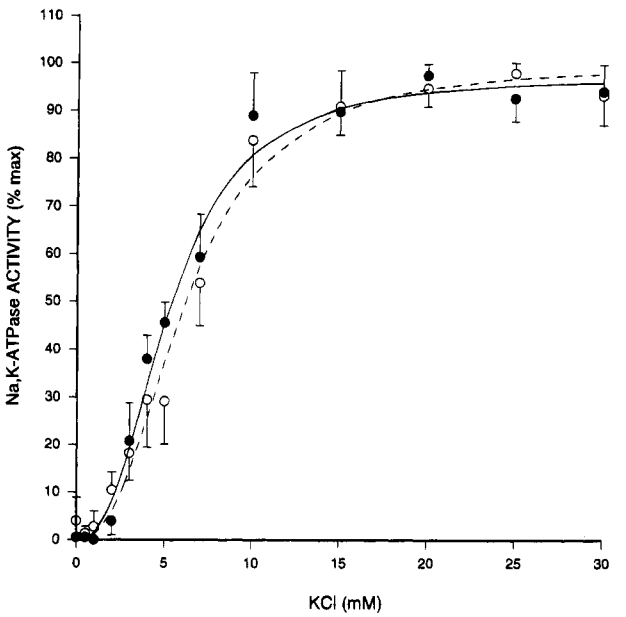

FIGURE 3: K^+ dependence of the $\alpha3\beta1$ and $\alpha3\beta2$ Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coexpressing the $\alpha3\beta1$ (●) or $\alpha3\beta2$ (○) isoforms was determined as described. The reaction medium contained 120 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [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 percent of the maximal Na,K-ATPase activity obtained. Curves are the best fit of the data to eq 1. Each value is the mean, and error bars represent the standard errors of the mean of four ($\alpha3\beta1$) or three ($\alpha3\beta2$) experiments performed in triplicate on samples obtained from different infections.

depicted in Table 1. As shown, the apparent affinity for K^+ is similar for both isozymes. The statistical analysis indicated no significant difference at the 1% level ($F = 1.82$ for degrees of freedom of 3 and 20). The computed Hill coefficients for K^+ reflect positive cooperativity at multiple ligand binding sites for both isozymes.

To characterize the kinetics of the low affinity ATP site, the activation curves of Na,K-ATPase activity at various concentrations of ATP were determined. The ATP dependences of the $\alpha3\beta1$ and $\alpha3\beta2$ isozymes are shown in Figure 4. The calculated kinetic parameters of these experiments are outlined in Table 1 and demonstrate that the K_m for ATP is similar for both isozymes. Also, the kinetic parameters for the cations and substrate activation of the rodent Na,K-ATPase $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha2\beta2$ isozymes, which where determined under the same conditions as the present study (Blanco et al., 1995), are included in Table 1 for comparison.

Na,K-ATPase isozymes differing in the α isoform have been shown to present different sensitivities to cardiotonic steroids (Sweadner, 1989; Blanco et al., 1993; O'Brien et al., 1994). To determine if the β isoforms influence the kinetic properties of the $\alpha3$ subunit toward ouabain, dose−response curves for the ouabain inhibition of Na,K-ATPase activity of the $\alpha3\beta1$ and $\alpha3\beta2$ isozymes were determined under nonlimiting ligand concentrations (130 mM Na^+, 20 mM K^+, and 3 mM Mg^{2+}). The corresponding plots are presented in Figure 5. The baculovirus induced $\alpha3\beta1$ and $\alpha3\beta2$ isozymes display homogeneous curves, indicating the presence of a single population of sites for the inhibitor. Both isozymes are highly sensitive to ouabain with comparable inhibition profiles. The resulting ouabain interaction con-

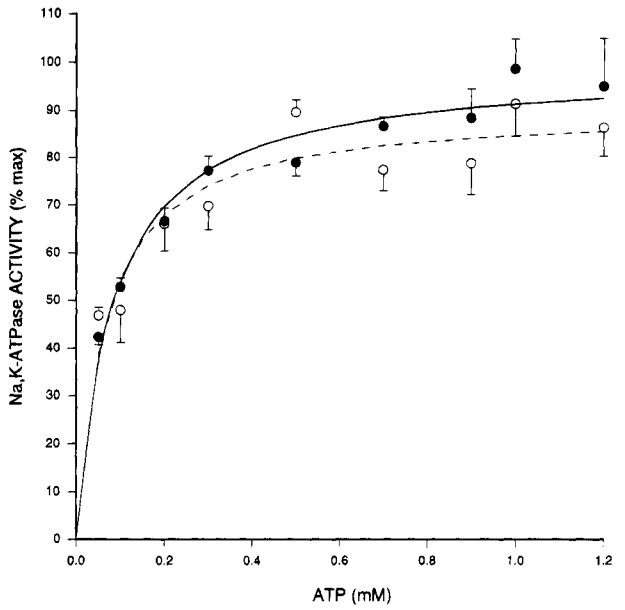

FIGURE 4: ATP stimulation of the α3β1 and α3β2 Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coexpressing the α3β1 (●) or α3β2 (○) isoforms was determined as described, in a reaction medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4), and the indicated ATP concentrations, with or without 1 mM ouabain. Data are expressed as percent of the maximal Na,K-ATPase activity determined from the best fit to the Michaelis–Menten equation. Each value is the mean of six experiments performed in triplicate on samples obtained from different infections. Error bars represent the standard errors of the mean.

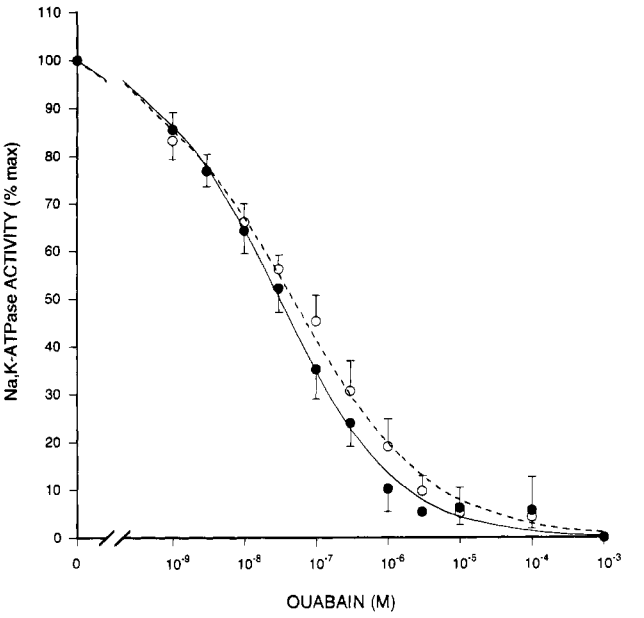

FIGURE 5: Dose–response curves for the ouabain inhibition of α3β1 and α3β2 Na,K-ATPase isozymes. Na,K-ATPase activity of *Sf*-9 cells coexpressing the α3β1 (●) or α3β2 (○) isoforms was determined as described. Samples were preincubated for 30 min at 37 °C in the reaction mixture [120 mM NaCl, 30 mM KCl, 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 percentage of maximal activity in the absence of the inhibitor. 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 four (α3β1) or six (α3β2) experiments performed in triplicate on samples obtained from different infections.

stants are summarized in Table 2, where the corresponding values for the α1β1, α2β1, and α2β2 isozymes have been included for comparison.

Table 2: Apparent Ouabain Affinities (K_i) of the Rat Na,K-ATPase α1β1, α2β1, α2β2, α3β1, and α3β2 Isozymes Expressed in *Sf*-9 Insect Cells

isozyme	K_i (M)
α1β1	$4.3 \times 10^{-5} \pm 1.9 \times 10^{-5}$
α2β1	1.7×10^{-7} ● 0.1×10^{-7}
α2β2	$1.5 \times 10^{-7} \pm 0.2 \times 10^{-7}$
α3β1	$3.1 \times 10^{-8} \pm 0.3 \times 10^{-8}$
α3β2	$4.7 \times 10^{-8} \pm 0.4 \times 10^{-8}$

DISCUSSION

The coexpression of multiple isoforms of the Na,K-ATPase subunits in the same cell and the possibility of association among the various α and β polypeptides allows for the existence of six structurally different variants of the Na pump (Blanco et al., 1994; Lemas et al., 1994). An important goal in understanding the basis for such a complex molecular diversity involves the characterization of the enzymatic properties of the individual isozymes. In the present study, we have used the baculovirus expression system to analyze the enzymatic properties of the α3β1 and α3β2 isozymes. As shown, the α3 isoform is capable of associating with both of the β polypeptides. The assembly of distinct β subunits with the α1 isoform has been previously demonstrated in *Xenopus* oocytes (Ackermann & Geering, 1992; Schmalzing et al., 1992), and stable association of all αβ combinations among the Na,K-ATPase isoforms have been obtained in virally infected HeLa cells (Lemas et al., 1994) and baculovirus-infected insect cells (Blanco et al., 1994). Moreover, the existence of the α3β1 and α3β2 complexes *in vivo* has been suggested from the analysis of the relative levels of expression of the α and β subunit isoforms in rat cerebral microvessels (Zlokovic et al., 1993) and from the identification of α3 in tissues predominantly expressing the β1 or the β2 polypeptides (Schneider & Kraig, 1990; Schneider et al., 1991; Cameron et al., 1994; Levenson, 1994).

Results presented here demonstrate that the baculovirus-induced α3β1 and α3β2 complexes are catalytically competent as defined by their ability to bind ouabain and mediate a ouabain-sensitive ATPase activity. This is consistent with the ability of the β1 and β2 polypeptides to support the activity of the other Na,K-ATPase α isoforms (Blanco et al., 1995). In order to examine the enzymatic characteristics of α3, as well as the role of the β subunits in influencing the catalytic properties of this isoform, the affinities of the α3β1 and α3β2 isozymes for Na^+, K^+, ATP, and the cardiotonic steroid ouabain were determined. The analysis of the kinetic constants showed that the different β subunits do not drastically modify the catalytic behavior of the α3 isoform. The major disparity between the α3 isozymes is their apparent affinity for Na^+, with the α3β2 isozyme showing a slightly higher apparent affinity for the cation than α3β1. These results agree with the observation that the Na^+ dependence of the rat α3 isoform seems to vary depending on its association with β1 or β2 [compare results of Shyjan et al. (1990) with those of Daly et al. (1994)]. Interestingly, the β2 isozyme when associated with the α2 isoform, affects the apparent affinity for Na^+ in a similar fashion (Blanco et al., 1995). This difference in Na^+ affinity between α3β1

and $\alpha 3\beta 2$ most likely depends on the dissimilar amino acid structure of the β isoforms, rather than the sugar composition, because the β isoforms are mainly expressed in their core-glycosylated forms in the insect cells. At present, the role the β subunit plays in the reaction cycle of the Na,K-ATPase is unclear. While its participation in the Na^+ binding process of the Na pump is completely unknown, the subunit has been shown to influence the extracellular K^+ activation of the enzyme (Jaisser et al., 1992, 1994; Eakle et al., 1994) and to be actively involved in forming or stabilizing the K^+-occluding complex of the enzyme (Lutsenko & Kaplan, 1993). A modulatory effect of the β isoforms on K^+ kinetics of the $\alpha 1$ subunit has also been reported for the isozymes expressed in *Xenopus* oocytes (Jaisser et al., 1994). Using this system the $\alpha 1\beta 2$ enzyme has a lower affinity for K^+ than $\alpha 1\beta 1$ (Schmalzing et al., 1992). Similarly, this trend appears to be maintained in *Sf*-9 cells where the $\alpha 2$ and $\alpha 3$ isoforms in combination with $\beta 2$ had a slightly lower affinity for K^+ than the $\beta 1$ counterparts.

A physiological role for the difference between $\alpha 3\beta 1$ and $\alpha 3\beta 2$ is difficult to infer. Although $\beta 2$ confers $\alpha 3$ with the same apparent affinity for Na^+ as the housekeeping $\alpha 1\beta 1$ enzyme, $\alpha 3\beta 2$ still retains the particular K^+, ATP, and ouabain reactivity of the $\alpha 3$ isoform. The importance of the $\beta 2$ polypeptide in Na,K-ATPase function may be related to other properties intrinsic to this subunit. For example, a surprising functional feature of the $\beta 2$ isoform is that, besides its role as a constituent of the Na pump, it serves as a cell adhesion molecule that mediates Ca^{2+} independent neuron–astrocyte interactions (Gloor et al., 1990; Schmalzing et al., 1992). In addition, $\beta 2$ has been shown to be involved in neuronal migration and neurite outgrowth (Antonicek et al., 1987; Gloor et al., 1990; Muller-Husmann et al., 1993). Although the link between cell recognition and ion transport is unknown, it is conceivable that $\beta 2$ might modulate Na pump function to provide the ionic environment required in such specific cellular processes. Recently, the physiological relevance of $\beta 2$ in maintaining ionic homeostasis has been suggested from the study of a mouse deficient in $\beta 2$ expression (Magyar et al., 1994).

Comparison of the different Na,K-ATPase $\alpha\beta$ pairs shows that the major differences in kinetic properties occurs among isozymes that differ in their α subunits. Thus, for the rodent isozymes expressed in *Sf*-9 cells, the apparent affinity for Na^+ varies with a rank of order $\alpha 2\beta 2 > \alpha 2\beta 1 > \alpha 1\beta 1 = \alpha 3\beta 2 > \alpha 3\beta 1$. Also, the apparent affinity for K^+ differs among the isozymes, following the sequence $\alpha 1\beta 1 > \alpha 2\beta 1 = \alpha 2\beta 2 > \alpha 3\beta 1 = \alpha 3\beta 2$. For the activation by ATP, the enzymes composed of the $\alpha 2$ and $\alpha 3$ isoforms display equivalent K_m values, which are approximately four times lower than that of the $\alpha 1\beta 1$. Finally, the most conspicuous kinetic difference is in the response to ouabain. The $\alpha 3\beta 1$ and $\alpha 3\beta 2$ isoforms have a high, while $\alpha 2\beta 1$ and $\alpha 2\beta 2$ an intermediate, and $\alpha 1\beta 1$ a low sensitivity to the cardiotonic steroid.

A comparison of our results with previous work becomes difficult since reports of the enzymatic properties of the Na,K-ATPase isozymes have been fraught with controversy. Discrepancies may result from differences in the source of enzyme, the simultaneous presence of various α and β isoforms, and difficulties in determining the subunit association and composition of the Na pump molecules. However, the properties of certain $\alpha\beta$ isoform combinations have been determined taking advantage of tissues enriched in a particular isoform or by using the differential inhibitory effect of ouabain to discriminate the isozymes. Thus, the Na^+ reactivity of $\alpha 3\beta 2$ expressed in insect cells is in agreement with the native enzyme from the rat pineal gland, which predominantly consists of $\alpha 3\beta 2$ (Shyjan et al., 1990). Moreover, the highly ouabain-sensitive Na,K-ATPase from rat brain or dog heart, ascribed to $\alpha 3\beta 1$, was shown to have a lower apparent affinity for Na^+ than the intermediate and resistant components ($\alpha 1$ and $\alpha 2$) from the same tissues (Gerbi et al., 1993; Berrebi-Bertrand & Maixent, 1994). Another approach examining the properties of Na,K-ATPase isozymes consists of mutating the $\alpha 2$ and $\alpha 3$ isoforms making them ouabain-resistant. By expressing the mutant isoforms in HeLa cells, the ouabain-resistant forms can be separated from the endogenous ouabain-sensitive enzyme pharmacologically. Using this system, the $\alpha 3\beta 1$ isozyme was also found to display a lower affinity for Na^+ compared to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Jewell & Lingrell, 1991; Munzer et al., 1994; Daly et al., 1994). However, the difference in $K_{0.5}$ for Na^+ among the isoforms was greater (2–3-fold) than the one we encountered (1.6-fold). The dissimilarities in affinity for ATP between the $\alpha 3$ and $\alpha 1$ isoforms found using baculovirus-infected insect cells coincide with those reported in HeLa cells (Daly et al., 1994). However, in contrast to the results reported by Daly et al. (1994), we found that the K^+ affinity of the $\alpha 3$ isoform combinations was lower than those of the $\alpha 1$ or $\alpha 2$. The differences in the membrane environment of mammalian and invertebrate cells, the analysis of hybrid Na pump molecules in the HeLa cells, and/or the alteration of the isoforms by mutation of their ouabain binding site may be responsible for the disparity in results.

The lower apparent affinity of $\alpha 3\beta 1$ for Na^+ and K^+ may better suit the proposed hypothesis that, in excitable tissues, this isozyme works as a reserve pump that may be activated only after large changes in the concentration of the cations, as those resulting from the repeated firing of action potentials (Munzer et al., 1994). However, to further speculate about the physiological relevance of the dissimilarity in kinetics of the various Na,K-ATPase $\alpha\beta$ combinations becomes rather difficult. Nevertheless, the presence of properties that are unique to the different Na pump isozymes suggests that this feature may be at least one of the reasons underlying the intriguing complexity of the Na,K-ATPase molecular diversity.

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