Tissue-specific Versus Isoform-specific Differences in Cation Activation Kinetics of the Na,K-ATPase*

Alex G. Therien‡, Nestor B. Nestor‡, William J. Ball§, and Rhoda Blostein‡

From the Department of Biochemistry, McGill University, Montreal, Canada and the Department of Pharmacology, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267-0575

The sodium potassium adenosine triphosphatase (Na,K-ATPase) or sodium pump is responsible for maintaining the electrochemical gradient of Na⁺ and K⁺ across the plasma membrane of animal cells. It normally couples the hydrolysis of one molecule of ATP to the transport of three Na⁺ ions out and two K⁺ ions into the cell (for reviews, seeRefs. 1–4). This cation pump is a heterodimer comprised of a catalytic α subunit (≈105 kDa) and a highly glycosylated β subunit (45–55 kDa), and may (5, 6) or may not (7, 8) form larger oligomers. The α subunit contains the binding sites for Na⁺, K⁺, ATP, and the highly specific cardiac glycoside inhibitors such as ouabain, as well as the site of phosphorylation (1). The function of the β subunit is not completely understood; it appears to be essential for the normal delivery and correct insertion of α into the plasma membrane (9) and to have some influence on the catalytic activity of α (10–14). A third peptide subunit known as the γ subunit (6.5 kDa) appears to exist in association with α and β, at least in certain tissues, although its role is yet to be determined (15).

In mammals, three isoforms of the α subunit (α1, α2, and α3) and two of the β subunit (β1 and β2) are known to exist (4). Isoforms of the α subunit are expressed in a tissue-specific manner: α1 is present ubiquitously; α2 is detected mainly in skeletal muscle, heart, and certain neuronal cells (neurons and astrocytes); and α3 is mainly in neurons (4, 16).

Earlier studies on cation activation of the Na,K-ATPase by Sweadner (17) using rat kidney and axolemma and later studies by Shyjan et al. (18) using kidney, brain, and pineal gland indicated a higher affinity for Na⁺ in preparations now known to be predominantly α3. Thus, the order of apparent affinities for Na⁺ in the former study was axolemma (predominantly α3) > kidney (α1 only) and in the latter, pineal gland (predominantly α3) > brain (a mixture of α1, α2, and α3) > kidney. In contrast, Jewell and Lingrel (19), using membranes isolated from HeLa cells transfected with the individual α isoforms, reported that the order of apparent affinities for Na⁺ is α1 ≈ α2 > α3, and for K⁺, α3 > α2 > α1, where α2* and α3* denote ouabain-resistant mutants of α2 and α3, respectively. Moreover, studies of pump-mediated K⁺ (Rb⁺) influx into these individual isoform-transfected cells confirmed the general conclusions drawn from the aforementioned work, except that considerably larger kinetic differences among the isoforms were observed (20). Interestingly, in experiments carried out with kidney and axolemma microsomal membranes delivered by membrane fusion into red cells, the order of apparent affinities for cytoplasmic Na⁺ and K⁺ resembled those of α1- and α3*-transfected HeLa cells, respectively (20).

The aim of the experiments described in this study was to reconcile the discordant results obtained in the foregoing studies, as well as numerous earlier reports, regarding the order of apparent affinities for Na⁺ and K⁺ of different tissues and/or isoforms (for review, see Ref. 4). In particular, the question of isoform-specific versus tissue-specific properties of the rat Na,K-ATPase has been addressed by studying the same isoform, either α1 or α3, in the membranes of various cells. Thus, the properties of the α1 isoform were examined in kidney, axolemma and rat α1-transfected HeLa cells, and those of the α3 isoform, in axolemma, pineal gland, and α3*-transfected...
HelA cells. The results provide evidence for isorm-independent, tissue-specific modulation of the kinetic behavior of the Na,K-ATPase, the most striking being the differences in the effects of intracellular K⁺ as a competitive inhibitor of Na⁺ at cytoplasmic Na⁺ activation sites.

**Experimental Procedures**

**Antibodies**—Antibodies used include M7-PB-E9, a rat α3-specific monoclonal antibody, and polyclonal antiserum 757 and 50946 which recognize rat β1 and β2, respectively (21, 22), and 754 which was raised against the NH₂-terminal (amin acids 1-13) sequence of lamb α1. A polyclonal antiserum specific for rat α3 and monosialic antibodies specific for α1 (6H) were generous gifts from Dr. Michael Caplan, Yale University. Goat anti-mouse antibodies used for immunoprecipitations were purchased from Tago Immunologicals, and horseradish peroxidase-labeled secondary antibodies (donkey anti-rabbit) from Bio/Can Scientific.

**Cell Culture and Membrane Preparations**—Rat kidney microsomes were prepared as described by Jorgensen (23) and stored in a sucrose-histidine-EDTA buffer (SHE buffer: 0.25 mM sucrose, 0.03 M histidine, 1.0 mM Tris-EDTA, pH 7.5) at −70 °C. Rat axolome membranes were prepared as described by Sweadner (24) and stored at −70 °C in a solution comprising 0.315 M Sucrose, 10 mM Tris, and 1 mM EDTA, at pH 7.4. Rat pineal gland membranes were prepared as described by Cerkovec et al. (25), with the following modifications. After centrifugation (Braun-Sonic 1510 sonicator) four times at low setting for 30 min at 4 °C using a TLA100 rotor in a Beckman TL-100 centrifuge, resuspended in SHE buffer (−500 μl/10 mg of original tissue), and stored at −70 °C. Protein concentrations of the tissue preparations were determined using the Lowry assay as modified by Markwell et al. (27). Specific activities are indicated in the legends to Figs. 1 and 2.

**Enzyme Assays**—Membranes were permeabilized as described by Forbush (28). Briefly, they were diluted to 0.06–0.5 mg/ml and treated for 10 min at 22 °C with 1% BSA, 0.65 mg/ml SDS and 25 mM imidazole, after which they were diluted 6-fold with 0.3% bovine serum albumin, 25 mM imidazole. ATP hydrolysis was measured as described previously (29). In a final volume of 100 μl containing 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 3 mM MgCl₂, and, unless indicated otherwise, concentrations of NaCl varying from 0.5 to 100 mM with KCl kept constant at 10 mM, or KCl concentrations varying from 0.2 to 50 mM with NaCl kept constant at 100 mM, with choline chloride added so that ([NaCl] + [KCl] + [ChCl]) was constant at 150 mM. Prior to the assay, membranes were preincubated in the reaction medium without or with 10 μM or 5 mM ouabain for 10 min at 37 °C. The reaction was initiated by adding γ³²P-ATP (final concentration of 1 mM) and NaCl, KCl, and choline chloride to the concentrations listed above.

Immunoprecipitation and Immunoblotting—Axolomem membranes (0.4 mg/ml) were solubilized for 20 min at room temperature in solubilizing buffer comprising 1% Triton X-100 or 1% CHAPS, 0.32% bovine serum albumin, 5 mM EDTA, dissolved in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄). The insoluble material was removed by centrifugation for 1 min at 500 × g, and the supernatant was then incubated for 1 h at 4 °C with monoclonal mouse antibodies specific for α1 or α3 (6 μg/150 μl solubilized axolome). Protein G covalently linked to agarose beads (Pharmacia Biotech Inc.), pretreated for 3 h at 4 °C with goat anti-mouse antibody (100 μl of antibody added to 125 μl of dried beads), were added to the antibody-treated solubilized axolome (25 μl of the original dry beads) and incubated for 1 h at 4 °C. After several washes of the beads (suspension in 200 μl of solubilizing buffer, centrifugation for 1 min at 500 × g), the protein was eluted with 60 μl of sample buffer (0.06 m Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.00125% bromphenol blue), incubated at 37 °C for 5 min, and then separated on a 12% SDS-polyacrylamide gel using a Bio-Rad Mini gel apparatus as described by Laemmli (30). Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) which was then blocked for 1 h at 37 °C in blocking buffer (PBS containing 5% milk powder and 0.1% Tween 20) and probed overnight at 4 °C with polyclonal rabbit anti-rat α1, α3, β1, or β2 antisera diluted in blocking buffer. After several washes with 0.1% Tween 20 antisera diluted in blocking buffer (PBS containing 5% milk powder and 0.1% Tween 20) and probed overnight at 4 °C with polyclonal rabbit anti-rat α1, α3, β1, or β2 antisera diluted in blocking buffer. After several washes with 0.1% Tween 20.

**RESULTS**

Na⁺ and K⁺ Activation Profiles of Distinct α Isoforms—To gain insight into the basis for the discrepancies in apparent cation affinities, a series of experiments were carried out in which the cation activation profiles of pumps of the same α isoform but from different tissues were compared. This comparison was confined to α1 and α3 of the rat. It was not technically feasible to include α2 in the analysis since there are virtually no suitable tissues with predominantly this isoform. (Although α2 may predominate in adult skeletal muscle, a high background Mg-ATPase activity precludes meaningful kinetic analysis of Na,K-ATPase.) For α1, the tissues compared were kidney, α1-transfected HeLa cells, and axolome. The activity of α1 in axolome was determined by taking advantage of the low sensitivity of the rodent α1 isoform to cardiac glycosides. Thus, axolome α1 was assayed in the presence of 10 μM ouabain which effectively inhibits α2 and α3 (33). The α3-rich tissues compared were pineal gland, α3-transfected HeLa cells, and axolome. The difference in activity observed in the absence and presence of 10 μM ouabain was ascribed mainly to α3 since the proportion of α2 in axolome is relatively low (20). In the case of the pineal gland, we have confirmed the report by Shyjan et al. (18) showing that the predominant α isoform detected in immunoblot of the adult rat pineal gland is α3 (results not shown). In addition, α1 is also detected, but the immunoblots do not provide information regarding the relative activities of the two isoforms. Therefore, assays to quantify ATPase activity sensitive to low (10 μM) versus high (5 mM) ouabain concentrations were carried out, and the results indicated that the activity of α1 is less than 5% that of α3 in pineal gland (experiment not shown).

The results of kinetic experiments carried out with α1-containing membranes isolated from rat kidney, axolome, and rat α1-transfected HeLa cells are shown in Fig. 1. The results for membranes rich in α3, in Fig. 2. The data are expressed as percentages of V_max and the curves are best fits to Equation 2. The insets in Figs. 1A and 2A represent the same data fitted to Equation 1. As shown in Fig. 1A, the apparent Na⁺ affinity of α1 from kidney, with the K⁺ concentration held constant at 10 mM, appears somewhat lower than that of α1 from either axolome or HeLa; K_{0.5(Na)} values were 6.6 ± 0.6, 4.7 ± 0.9, and 5.0 ± 0.3 mM for the three tissues, respectively. At a higher K⁺ concentration (20 mM; cf. Ref. 18), the difference in the K_{0.5(Na)} value for α1 of kidney became greater as indicated below (see Fig. 4). In the case of K⁺ activation (Fig. 1B), the order of apparent affinities (assayed at 100 mM Na⁺) are as follows: axolome <
kidney, axolemma, and HeLa cells. Membranes were prepared and assayed as described under "Experimental Procedures." Ouabain-sensitive activities are the difference between hydrolysis measured in the presence of 10 μM and 5 mM ouabain, and are the means ± S.D. (triplicate determinations) expressed as percentages of $V_{\text{max}}$. The curves were fitted to Equation 2. Representative experiments are shown and values of $K_{\text{a}}$ and $n$ for replicate experiments are shown in Table I. A, activation by Na$^+$ at 10 mM KCl ($V_{\text{max}}$ values are 3.7 ± 0.07, 0.260 ± 0.004, and 1.07 ± 0.003 μmol/(mg min) for kidney, axolemma, and HeLa cells, respectively); B, activation by Na$^+$ at 100 mM KCl; $V_{\text{max}}$ values are 4.3 ± 0.14, 0.330 ± 0.030, and 1.08 ± 0.003 μmol/(mg min) for kidney, axolemma, and HeLa cells, respectively). ○, kidney; □, axolemma; △, transfected HeLa cells. In the insets the data were fitted to Equation 1.

FIG. 2. Activation by Na$^+$ and K$^+$ of rat α3 Na,K-ATPase from pineal glands, axolemma, and transfected HeLa cells. Assays were carried out and analyzed as described in Fig. 1, except that ouabain-sensitive activities attributed mainly to α3 are the differences between hydrolysis measured in the absence and presence of 5 mM ouabain (pineal gland) or the absence and presence of 10 μM ouabain (axolemma) or in the presence of 10 μM and 5 mM ouabain (α3*-transfected HeLa cells). A, activation by Na$^+$ at 10 mM KCl ($V_{\text{max}}$ values are 0.353 ± 0.011, 3.34 ± 0.07, and 0.078 ± 0.002 μmol/(mg min) for pineal gland, axolemma, and HeLa cells, respectively); B, activation by K$^+$ at 100 mM NaCl ($V_{\text{max}}$ values are 0.465 ± 0.008, 5.19 ± 0.07, and 0.075 ± 0.003 μmol/(mg min) for pineal gland, axolemma, and HeLa cells, respectively). ○, pineal gland; □, axolemma; △, transfected HeLa cells. In the insets the data were fitted to Equation 1.
ouabain in the absence of K\(^+\) (see "Experimental Procedures"), and that the enzyme activity measured thereafter remained constant as a function of time.

K\(^+\) Interactions at Cytoplasmic Na\(^+\) Binding Sites—One of the inherent problems in kinetic studies of Na\(_{\text{K}}\)-ATPase in membrane fragments is the lack of control of the composition of cations at the cytoplasmic versus extracellular milieu. Specifically, it has been shown that K\(^+\) binding and inhibition at the cytoplasmic Na\(^+\) activation sites alters the enzyme's apparent affinity for Na\(^+\) (31). To determine whether Na\(^+\)/K\(^+\) interactions are, indeed, distinct for the sodium pumps of different tissues, a series of activity measurements were carried out at varying K\(^+\) concentration and Na\(^+\) maintained constant at a low 5 mM rather than 100 mM concentration. The results shown in Fig. 3, A and B, indicate that the extent of K\(^+\) inhibition at the presumably cytoplasmic Na\(^+\) binding site is at least partly affected by the nature of the tissue. As shown in Fig. 3A, the kidney a1 enzyme is significantly more sensitive to K\(^+\) inhibition than a1 from either HeLa cells or axolemma; at 20 mM KCl, a concentration at which the [K\(^+\)]/[Na\(^+\)] ratio of 4 is still lower than the normal physiological value (>10), the activity of kidney a1 is reduced by 40%, whereas that of the other tissues is minimally affected. The results for a3 (Fig. 3B) show that the transfected HeLa enzyme is much more sensitive to inhibition by K\(^+\) than either the axolemma or pineal gland enzymes.

It has been observed that the antagonistic effect of vanadate, a potent inhibitor of the sodium pump, is facilitated by the presence of K\(^+\) ions (35, 36). To ensure that the K\(^+\)-mediated inhibition observed in this study is not the result of vanadate present in the kidney preparation, two control experiments were performed: (i) in one, assays were carried out in the presence of 2.5 mM norepinephrine, which reverses the effect of vanadate (36), and (ii) in the other, the assay time was reduced 10-fold and the amount of kidney microsome sample increased 10-fold; if vanadate were present in the microsome suspension, such an increase in endogenous vanadate concentration should result in greater inhibition of activity. Neither of these conditions altered the K\(^+\)-inhibition profiles, which argues against an apparent K\(^+\) inhibition secondary to the presence of vanadate in the kidney preparation.

Based on the Albers-Past model of the Na\(_{\text{K}}\)-ATPase reaction mechanism and, more specifically, on the model which assumes random binding of Na\(^+\) and K\(^+\) to (the same) three equivalent sites on the cytoplasmic side of the enzyme, Garay and Garrahan (31), in their studies on Na\(^+\) efflux in red cells, and Sachs (37), in studies of ouabain-sensitive ATPase activity in broken red cell ghosts, showed that activity adhered closely to the following relationship:

\[
v = \frac{V_{\text{max}} [\text{K}_{\text{Na}}]}{[\text{Na}]^{1+} + [\text{K}]^{1+}},
\]

(Eq. 3)
Are the Tissue-specific Kinetic Differences the Result of \( \alpha \) Associations with Different \( \beta \) Isoforms?—The question as to whether differences in cation activation are due, at least to some extent, to differences in the \( \beta \) isoform which associates with \( \alpha \) was approached by carrying out a series of experiments involving immunoprecipitation of \( \alpha 1 \) and \( \alpha 3 \) from axolemma, followed by immunoblotting with \( \alpha \) and \( \beta \) isoform-specific antibodies to determine the nature of the associated \( \beta \) subunits. The results of these experiments are shown in Fig. 5 and summarized below. This question is relevant only to axolemma membranes since only \( \beta 1 \) is present in kidney and only \( \beta 2 \), in the adult pineal gland. In addition, HeLa cells contain \( \beta 1 \) message (38, 39) and \( \beta 1 \) protein has been detected by Western blotting\(^2\); neither \( \beta 2 \) message nor protein were detected by polymerase chain reaction, Northern analysis, or Western blotting.\(^3\)

\(^2\) W. J. Ball, unpublished results.

\(^3\) R. Levenson, personal communication.

---

**Fig. 3.** Potassium inhibition of Na,K-ATPase of kidney, axolemma, pineal gland, and transfected HeLa cells at low Na\(^+\) concentration. Membranes were prepared and ATPase activity assays performed as described in Figs. 1 and 2, but in the presence of 5 mM NaCl. The representative experiments show the mean \pm S.D. of triplicate determinations expressed as percentages of the activity measured at 2 mM KCl. A, \( \alpha 1 \) pumps: ○, kidney; ○, axolemma; Δ, transfected HeLa cells. B, \( \alpha 3 \) pumps: ○, pineal gland; ○, axolemma; Δ, transfected HeLa cells.

**Fig. 4.** Dependence of \( K_{Na}^* \) on K\(^+\) concentration for rat pumps from kidney, axolemma, pineal gland, and transfected HeLa cells. Assays were carried out as described in Fig. 1, but at varying concentrations of KCl (5, 10, 20, 35, and 50 mM). \( K_{Na}^* \) were first determined by fitting the data obtained for each Na\(^+\)-activation curve to Equation 1 and were then plotted as a function of KCl concentration. Each point represents an average \pm S.D. of at least three separate experiments, and the values of \( K_{Na} \) and \( K_Na^* \) obtained are shown in Table II. A, \( \alpha 1 \) pumps: ○, kidney; ○, axolemma; Δ, transfected HeLa cells. B, \( \alpha 3 \) pumps: ○, pineal gland; ○, axolemma; Δ, transfected HeLa cells.

When a mouse monoclonal antibody specific for \( \alpha 1 \) was used to immunoprecipitate the enzyme of Triton X-100-solubilized axolemma membranes, the only subunit isoforms detected on Western blots using rabbit polyclonal antisera specific for \( \alpha 1 \), \( \alpha 3 \), \( \beta 1 \), and \( \beta 2 \) as primary antibodies, were \( \alpha 1 \) and \( \beta 1 \) (Fig. 5, A-D, lanes 3). Further, when a mouse monoclonal antibody specific for \( \alpha 3 \) was used, \( \alpha 3 \) and \( \beta 1 \) were detected along with a barely visible band corresponding to \( \beta 2 \) (Figs. 5, A-D, lanes 4). Control experiments carried out omitting the precipitating antibody showed minimal amounts of nonspecific binding of the axolemma Na,K-ATPase subunits to the protein G-linked agarose beads (Fig. 5, A-D, lanes 5). The fact that the appearance of two bands, one of slightly higher mobility than \( \alpha 1 \) and \( \alpha 3 \) (Fig. 5, A and B, lane 3), the other at \( \approx 50 \text{ kDa} \) (Fig. 5C, lanes 3–5), reflect nonspecific reactions was evidenced in the following controls (not shown). (i) The first band was present even when the primary detecting antibody was omitted, thus indicating that it is the result of nonspecific binding of the secondary blotting antibody to the primary immunoprecipitating antibodies. (ii) The second nonspecific band appeared even when...
the primary antibody (mouse anti-α1 or α3) was omitted (Fig. 5C) or when the procedure was carried out in the absence of solubilized axolemma (not shown), indicating that it probably represents a nonspecific reaction involving the primary blotting antibody to β1 and goat anti-mouse antibodies.

α-β Stoichiometries in Axolemma—The stoichiometry of the α-β associations was then evaluated in order to assess whether these associations may have been disrupted as a result of the membrane solubilization procedure. These analyses were done utilizing different exposures of the Western blots from several replicate experiments which were quantified as described under "Experimental Procedures." In this work, two assumptions were made: first, that the α3β1 subunit stoichiometry of the kidney enzyme, as based on studies of the purified enzyme, is 1:1 (for example, see Ref. 40) and second, that heterodimers comprising β1 are not preferentially immunoprecipitated compared to those comprising β2.

The estimate of α1β1 stoichiometry in axolemma was based on a comparison of the densities of the α and β bands of axolemma immunoprecipitated with anti-α1 monoclonal antibody with those of unprecipitated kidney microsomes, following exposures to anti-α1 and anti-β1 antisera as shown in Fig. 5, A and C (lanes 2 and 3). The ratio of α1 to β1 in precipitated axolemma was found to be 1.08 ± 0.17 (S.E. for five independent experiments).

Because there is no tissue in which α3 and β1 have been shown to be expressed in a 1:1 ratio, the question of possible α3β1 versus α3β2 associations was evaluated as follows. The α3β1 ratio as detected in anti-α3 immunoprecipitated axolemma samples was compared to that found in unprecipitated axolemma membranes, after correcting the ratio for the proportion of β1 presumed to associate with α1. The ratio in the immunoprecipitate of axolemma sample was found to be reasonably close to the "corrected" ratio observed in the unprecipitated membranes. Thus, if the corrected α3β1 ratio of unprecipitated axolemma is normalized at 1.00, the ratio of the precipitate is 1.08 ± 0.09 (S.E. for four independent experiments).

It should also be mentioned that in other experiments (not shown) aimed to determine whether the detergent Triton X-100 interfered with subunit interactions, immunoprecipitations were also carried out with a 4-fold lower concentration of Triton X-100 (0.25%) and with 1% CHAPS. Under both conditions, the α:β ratios obtained were not significantly different from those observed with 1% Triton X-100 (data not shown).

### Table II

| Tissue/cells      | K<sub>n</sub> | K<sub>k</sub> | K<sub>n</sub>/K<sub>k</sub> |
|-------------------|--------------|--------------|--------------------------|
| α1                |              |              |                          |
| Kidney            | 1.02         | 10.0         | 0.102                    |
| Axolemma          | 0.78         | 18.7         | 0.042                    |
| HeLa              | 0.92         | 19.9         | 0.046                    |
| α3                |              |              |                          |
| Pineal gland      | 0.79         | 15.5         | 0.051                    |
| Axolemma          | 0.72         | 8.20         | 0.088                    |
| HeLa              | 1.35         | 6.30         | 0.214                    |

### DISCUSSION

In this study we show that the divergent results regarding the relative affinities of the Na,K-ATPase of the different rat isoforms as reported in different laboratories are not simply accounted for by differences in the experimental conditions used. Thus, we have reproduced the relative cation affinities for α1 versus α3 as reported by Jewell and Lingrel (19) and Munzer et al. (20) on the one hand, and those of Sweadner (17) and Shyjan et al. (18), on the other. To gain insight into the basis for this dichotomy, we have assessed the apparent cation affinities of pumps of the same catalytic isoform, either α1 or α3, but from different tissues and, therefore, membrane environments. Marked differences in the apparent affinities for both Na<sup>+</sup> and K<sup>+</sup> were observed in pumps of the same α isoforms isolated from different cellular sources. These data and previous work in lamb (22) and dog (41) tissues are consistent with the conclusion that factors other than the type of

---

**Fig. 5.** Coimmunoprecipitation of the β subunit with α1 and α3 from rat axolemma. Membranes were prepared, solubilized in 1% Triton X-100 and immunoprecipitated with either an α1- or α3-specific monoclonal antibody as described under "Experimental Procedures." Following SDS-polyacrylamide gel electrophoresis, the proteins were analyzed by Western blotting using polyclonal antisera specific for: A, α1; B, α3; C, β1; and D, β2. Lanes are: 1, axolemma; 2, kidney; 3, immunoprecipitate from axolemma using α1-specific monoclonal antibody 6H; 4, immunoprecipitate from axolemma using α3-specific monoclonal antibody M7-PB-E9; and 5, control: immunoprecipitation performed in the absence of the primary antibody. Molecular masses are given in kilodaltons.
catalytic isoform influence interactions of the pump with Na\(^+\) and K\(^-\).

The most obvious tissue-specific protein component which interacts with the pump is the \(\beta\) subunit. In fact, effects of different \(\beta\) subunits on both K\(^-\) (10–12) and Na\(^+\) (13, 14) affinities have been described. Accordingly, one question is whether the kidney enzyme’s lower apparent affinity for Na\(^+\) and higher apparent affinity for K\(^-\) as compared to other \(\alpha_1\) pumps are the result of interactions of \(\alpha_1\) with different \(\beta\) subunits. To address this question, particularly in axolemma, in which both \(\beta_1\) and \(\beta_2\) have been identified, the nature of the \(\beta\) subunit which communoprecipitates with the distinct \(\alpha\) subunits was assessed. The results of these experiments indicate that \(\beta_1\) associates with \(\alpha_1\) in axolemma and that the stoichiometry of the association is close to 1.0. The determination of \(\alpha/\beta\) stoichiometries imply that little, if any, \(\beta_2\) associates with either \(\alpha_1\) or \(\alpha_3\). Whether \(\beta_2\) associates preferentially with \(\alpha_2\) in axolemma remains to be determined. Although HeLa cells contain human \(\beta_1\), while kidney cells contain rat \(\beta_1\), these subunits are 95% identical (39). A difference in both type and amount of glycosylation has been observed between kidney and brain \(\beta_1\) (42) and is presumably the basis for the differences in mobilities in immunoblots of kidney and axolemma as shown in Fig. 5C. The possibility remains that these differences are at least partly responsible for the distinct kinetics, even though there is evidence that the oligosaccharides are not essential for primary function (reviewed in Ref. 43).

It is unlikely that the presence of distinct \(\beta\) isoforms account for differences of \(\alpha_1\) of kidney, axolemma, and HeLa, and of \(\alpha_3\) of axolemma and HeLa; in both instances, \(\beta_1\) is the predominant \(\beta\) isoform present or associated with \(\alpha_1\) or \(\alpha_3\). However, tissue-specific differences in cation affinities, despite similar \(\alpha/\beta\) pairing, do not imply that the \(\beta\) subunit has no effect on function. In fact, a kinetic difference due to the distinct \(\beta\) subunits is observed in the case of \(\alpha_3\). Thus, as shown in Table II, the ratio K\(_{Na}/K_{K}\) is 1.7-fold lower in pineal gland compared to axolemma, reflecting the 1.9-fold difference in K\(_{K}\). That this difference is a result of \(\beta_2\) association with \(\alpha_3\) in the pineal gland and of \(\beta_1\) with \(\alpha_3\) in axolemma is supported by a recent report showing a 1.6-fold higher apparent affinity for Na\(^+\) of \(\alpha_3/\beta_2\) compared to \(\alpha_3/\beta_1\) in Sf-9 cells transfected with these isoform pairs (14). In this study, the Na\(^+\) activation kinetics from which the kinetic constants were obtained were carried out in the presence of 30 \(\mu\)M K\(^-\) so that the difference in apparent affinity for Na\(^+\) may also reflect a difference in K\(_{K}\). Other kinetic differences were not detected. Taken together, these results are consistent with a role for the distinct \(\beta\)s in modulating K\(^+\) interactions at cytoplasmic Na\(^+\) sites.

An important observation regarding the communoprecipitation studies presented here is the isoform specificity of the reactions as evidenced in the communoprecipitation of \(\alpha\) with \(\beta\), but not of \(\alpha_1\) with \(\alpha_3\). This lack of communoprecipitation between the different \(\alpha\) isoforms is in contradiction with reports that pumps communoprecipitate as \(\alpha/\beta\) heterodimers in rat brain and in baculovirus infected Sf-9 cells (5). Although it is possible that the detergent (1% CHAPS) used in that study (5) did not fully solubilize the membranes, or that the Triton X-100 used in this study disrupted \(\alpha/\beta\) interactions, these are unlikely explanations, since we confirmed our results using 1% CHAPS.

It can be argued that certain methodological procedures may be responsible for some of the differences observed for the \(\alpha_3\) enzyme of axolemma as compared to that of other tissues. As described above, the activity ascribed to \(\alpha_3\) in axolemma is that which is sensitive to 10 \(\mu\)M ouabain. Unfortunately, the ouabain-activities of \(\alpha_2\) and \(\alpha_3\), both present in axolemma, are quite similar (4), and it was technically difficult to distinguish the two on that basis. However, the amount of \(\alpha_2\) in axolemma is relatively low (~25%) (20), so that its effect on the kinetic behavior cannot account for the magnitude of the differences in the observed kinetic constants as discussed below. As well, the similar fold difference in apparent affinity for external K\(^-\) ascribed to \(\alpha_1\) versus \(\alpha_3\) in two separate systems (transfected HeLa cells compared to axolemma- and kidney-fused red blood cells; see Ref. 20) argues against a substantial contribution of \(\alpha_2\) to the behavior of the ouabain-sensitive pumps of axolemma. For the same reasons, it is unlikely that the mutation of \(\alpha_3\) to render it ouabain-resistant in HeLa cells alters its behavior.

There has been some evidence that detergents, such as SDS used here to increase the permeability of membrane vesicles to substrates, can have an effect on cation activation kinetics of the Na,K, pump (44). Although such an effect was not observed in the case of the Na\(^+\)-activation profile of axolemma enzyme, or in the case of the Na\(^+\) and K\(^-\) activation profiles of transfected HeLa cells (data not shown), it is entirely possible that other pumps might react differently to SDS treatment. However, the SDS concentration and the SDS:protein concentration ratio were identical in all of our experiments. Therefore, if SDS affects the different enzymes to varying extents, it should be the result of differential interactions with the surrounding environment, which would be consistent with the notion that the catalytic behavior of the pump does not depend solely on the isoform of the \(\alpha\) subunit.

The most intriguing results of this study concern tissue-specific K\(^-\)/Na\(^+\) antagonism. Differences in K\(_{Na}\), the apparent affinity for K\(^+\) at (cytoplasmic) Na\(^+\) activation sites, underlie tissue-specific differences in the sodium-activation profiles noted in both the present and earlier studies (17–20). There is evidence also of differences in apparent Na\(^+\) affinity, independent of K\(^+\) concentration, between pumps of the same catalytic isoform, although this difference was slight in the case of the kidney enzyme compared to other \(\alpha_1\) pumps (see Table II). In general, the ability of K\(^+\) to act as a competitive inhibitor of Na\(^+\) binding is reflected in the ratio of K\(_{Na}\) to K\(_{K}\), the cytosolic
binding constants for Na" and K", respectively. It is apparent from Table II that, of the membrane systems examined, α1 in the kidney and α3 transfected into the HeLa cell have the largest KNa/KK ratios when compared to other pumps with the same α isoforms. It is these two enzyme preparations which exhibit the greatest sensitivity to inhibition by K+ as depicted in Fig. 3. Specifically, it seems that in the case of kidney α1, this inhibition is due to its relatively lower KNa compared to the other α1 pumps, whereas with HeLa α3 pumps, it is due mainly to a higher KNa (Table II). Whether these characteristics are intrinsic to the enzyme, for example due to tissue-specific co- or post-translational modification(s), or rather, the result of modulation of the enzyme by another associated protein remains unresolved.

The kinetic analyses of the sigmoid activation kinetics described in this and previous studies (17–20) are based on conventional cooperative or noncooperative models. Using the noncooperative model, the apparent affinities of α1 and α3 for intracellular Na" and K+ of the same tissue (HeLa) derived in the present study can account for the low affinities of α3 compared to α1 observed in studies of Munzer et al. (20) using intact cells, with the following provisos. Those authors pointed out that their data points for Na" activation of α3 pumps could be obtained only in the region of the curve well below saturation due to the technical difficulty of raising intracellular Na" above ~45 mM. This precluded a reliable estimate of the kinetic constants for α3 when using the noncooperative model. However, the data fitted well to a cooperative model (Equation 2 in Ref. 20) giving K0.5 values for intracellular Na" of 17.6 mM for α1 and and 63.5 for α3. In the present study, the KNa and KK values for rat α1 and α3 in HeLa membranes (Table II) were used to obtain the observed apparent affinity, KNa, at 135 mM intracellular K+. A concentration approximating that of the intact cells used by Munzer et al. (20). Using these values of KNa (7.1 mM for α1 and 30.3 mM for α3) we derived curves of pump activation as a function of varying intracellular Na" using the cooperative model (Fig. 6). The curves (solid lines) and the values of K0.5 thus obtained (19.2 mM for α1 and 75.2 mM for α3) are similar to those derived from the data of previous studies (20) with intact cells (Fig. 6, dashed lines). Therefore, these results indicate that the physiologically significant extremely low apparent affinity of α3 reflects its much greater sensitivity to inhibition by intracellular K+.

Modulation of pump behavior by the membrane environment is most likely the explanation for the discrepancies among previous reports concerned with isoform-specific behavior. A slightly higher Na" affinity in axolemma compared to kidney was reported first by Sweadner (17), and later by others including Shyjan et al. (18) who compared brain and kidney. This observation was replicated in the present study of kidney and axolemma Na,K-ATPase, with values very close to those reported by Sweadner, i.e. KNa values of 0.72 mM and 1.02 mM, respectively (Table II) corresponding to K0.5 values of 4.0 mM and 4.3 mM for axolemma and kidney, respectively (not shown).

Tissue-specific as well as isoform-specific behavior is evident not only in apparent affinities for cyttoplasmic Na" and K+ but also for K+ at extracellular sites (Table I). Thus, it was only in the same (red cell or HeLa cell) environment that a higher affinity for extracellular K+ of α3 or axolemma compared to kidney (α1) was observed (19, 20). It may be relevant that exogenous kidney pumps fused into red cells and endogenous red cell pumps behave identically with respect to the apparent affinity for extracellular K+ [KNa (text)], (45). The greater difference between α1 and α3 observed in studies with intact cells may reflect the limitation of kinetic studies with unsided preparations.

The foregoing considerations argue in favor of the conclusion that the primary structure of the α isoform is not the sole determinant of the magnitude of cation affinity/selectivity. Our observations are consistent with the existence of some pump modulator, for example one which interacts and effects a greater sensitivity of α1 to K+ inhibition in the kidney compared, for example, to α1 of axolemma; in the microsome-fused red cell system, association of the α Subunit with the putative regulator may be interrupted following its association with other components of the new (red cell) environment. This kind of regulation is reminiscent of the effects of an intrinsic red cell membrane (blood group) antigen, Lp, found in genetically low potassium sheep red blood cells. This protein or glycoprotein interacts with the pump and effects K+ inhibition (for review, see Ref. 46). Interestingly, when kidney pumps are delivered from microsomes into low potassium sheep red cells, the Lp antigen effects susceptibility to K+ inhibition (47), which supports the idea that fusion into red blood cells confers a new membrane environment for the pump.

The study described in this report provides evidence in support of the conclusion that factors in addition to the primary structure of the α isoforms dictate the kinetic behavior of the Na,K-ATPase. Likely candidates include other membrane-bound components or modulation by co- or post-translational modifications of either subunit.

Acknowledgments—We thank Dr. Michael Caplan, Yale University School of Medicine, for his generous gifts of the polyclonal anti-α3 and monoclonal anti-α1 antibodies and Drs. E. A. J. Sweadner and J. B. Lingrel, University of Cincinnati, for the gifts of the isoform-transfected HeLa cells. We are grateful to Dr. K. J. Sweadner, Massachusetts General Hospital, for helpful suggestions, Dr. R. Levenson, Pennsylvania State University, for communicating his unpublished results, and Dr. Stewart Daly for technical assistance.

REFERENCES

1. Vasilets, L. A., and Schwarz, W. (1993) Biochim. Biophys. Acta 12154, 201–222
2. Blöstein, R. (1989) Curr. Opin. Cell Biol. 1, 746–752
3. Glynn, I. M. (1993) J. Physiol. 462, 1–30
4. Sweadner, K. J. (1989) Biochim. Biophys. Acta 988, 185–220
5. Blanco, G., Kastner, J. C., and Mercor, R. W. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 8542–8546
6. Periyasamy, S. M., Huang, W.-H., and Askari, A. (1983) J. Biol. Chem. 258, 9878–9885
7. Martin, D. W., and Sachs, J. R. (1992) J. Biol. Chem. 267, 23922–23929
8. Sachs, J. R. (1994) Biochim. Biophys. Acta 1193, 199–211
9. Geering, K. (1990) J. Membr. Biol. 115, 109–121
10. Schmalzing, G., Krömer, S., Schadnich, M., and Gloor, S. (1992) J. Biol. Chem. 267, 20212–20216
11. Jässer, F., Canessa, C. M., Horisberger, J.-D., and Rossler, B. C. (1992) J. Biol. Chem. 267, 16895–16903
12. Jässer, F., J. J. Aunin, P., Geering, K., Rossler, B. C., and Horisberger, J.-D. (1994) J. Gen. Physiol. 103, 605–623
13. Blanco, G., Kastor, J. C., Sánchez, G., and Mercor, R. W. (1995) Biochimie 34, 319–325
14. Blanco, G., Sánchez, G., and Mercor, R. W. (1995) Biochimie 34, 9897–9903
15. Mercer, R. W., Briesemiderer, D., Bliss, D. P., Collins, J. H., and Forbusch, B., III (1993) J. Cell Biol. 121, 579–586
16. Sweadner, K. J. (1992) Can. J. Physiol. Pharmacol. 70, 5255–5259
17. Sweadner, K. J. (1985) J. Biol. Chem. 260, 11508–11513
18. Shyjan, A. W., Cea, V., Klein, D. C., and Levenson, R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1178–1182
19. Jowell, E. A., and Lingrel, J. B. (1991) J. Biol. Chem. 266, 16925–16930
20. Munzer, J. S., Daly, S. E., et al. (1994) J. Biol. Chem. 269, 16668–16676
21. Abbott, A., and Ball, W. J. (1993) Biochemistry 32, 3511–3518
22. Sun, Y., and Ball, W. J. (1992) Am. J. Physiol. 262, C1491–C1499
23. Järresäter, P. L., and Skou, J. C. (1971) Biochim. Biophys. Acta 236, 366–380
24. Sweadner, K. J. (1988) Methods Enzymol. 156, 65–71
25. Cea, V., González-García, C., Svoboda, P., Weller, J. L., and Klein, J. D. (1987) J. Biol. Chem. 262, 14467–14471
26. Lane, L., Feldmann, J. M., Farschheim, C. E., and Rybczynski, C. L. (1993) J. Biol. Chem. 268, 17930–17934
27. Markwell, M. A. K., Haas, T., Tobbert, N. E., and Bierer, L. L. (1983) Methods Enzymol. 72, 296–303
28. Forbusch, B., III (1983) Anal. Biochem. 128, 159–163
29. Wierzbicki, W., and Blöstein, R. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 70–74
30. Laemmli, U. K. (1970) Science 170, 591–595
31. Caracta, L. A., and Schatz, W. (1993) J. Cell Biol. 121, 579–586
32. Sachs, J. R. (1977) J. Biol. Chem. 252, 489–514
33. Lytton, J., Lin, C. J., and Guidotti, G. (1985) J. Biol. Chem. 260, 1177–1184

Downloaded from http://www.jbc.org/ on July 26, 2018
34. Akera, T., Ng, Y.-C., Shieh, I.-S., Bero, E., Brody, T. M., and Braselton, W. E. (1985) Eur. J. Pharmacol. 111, 147-157
35. Cantley, L. C., Jr., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C., and Guidotti, G. (1979) J. Biol. Chem. 252, 7421-7423
36. Josephson, L., and Cantley, L. C., Jr. (1977) Biochemistry 16, 4572-4578
37. Sachs, J. R. (1986) J. Physiol. 374, 221-244
38. Kawakami, K., Nojima, H., Onta, T., and Nagano, K. (1986) Nucleic Acids Res. 14, 2833-2844
39. Mercer, R. W., Schneider, J. W., Savitz, A., Emanuel, J. E., and Levenson, R. (1986) Mol. Cell. Biol. 6, 3884-3890
40. Jorgensen, P. L. (1980) Physiol. Rev. 60, 864-913
41. Berrebi-Bertrand, I., and Maixent, J. M. (1994) FEBS Lett. 348, 55-60
42. Sweadner, K. J., and Gilkeson, R. C. (1985) J. Biol. Chem. 260, 9016-9022
43. Chow, D. C., and Forte, J. G. (1995) J. Exp. Biol. 198, 1-17
44. Foussard-Guilbert, F., Ermias, A., Laget, P., Tanguy, G., Girault, M., and Jallet, P. (1982) Biochim. Biophys. Acta 692, 296-304
45. Munzer, J. S., and Blostein, R. (1994) in The Sodium Pump: Structure, Mechanism, Hormonal Control, and Its Role in Disease (Schoner, W., and Bamberg, E., eds) pp. 464-467, Springer-Verlag New York Inc., New York
46. Dunham, P. B. (1992) Comp. Biochem. Physiol. 102A, 625-630
47. Xu, Z.-C., Dunham, P. B., Munzer, J. S., Silvius, J. R., and Blostein, R. (1992) Am. J. Physiol. 263, C1007-C1014
Tissue-specific Versus Isoform-specific Differences in Cation Activation Kinetics of the Na,K-ATPase
Alex G. Therien, Nestor B. Nestor, William J. Ball and Rhoda Blostein

J. Biol. Chem. 1996, 271:7104-7112.
doi: 10.1074/jbc.271.12.7104

Access the most updated version of this article at http://www.jbc.org/content/271/12/7104

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 19 of which can be accessed free at http://www.jbc.org/content/271/12/7104.full.html#ref-list-1