Differential expression and enzymatic properties of the Na^+, K^+ -ATPase $\alpha 3$ isoenzyme in rat pineal glands

(Na^+ and K^+ dependence/membrane hyperpolarization)

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ABSTRACT We have used immunoblotting and biochemical techniques to analyze expression of Na^+, K^+ -ATPase α and β subunits in rat pineal glands. Western blot analysis of pineal microsomal membrane fractions with antisera specific for each of the three rat α and two rat β subunits revealed similar levels of expression of $\alpha 1$ and $\alpha 3$ subunits in pineal glands of 5-day-old rats. High levels of $\alpha 3$ and $\beta 2$ subunits and low levels of $\alpha 1$ subunits were detected in adult glands. No $\alpha 2$ or $\beta 1$ subunits were detectable at either developmental stage. Examination of the enzymatic properties of the pineal gland $\alpha 3$ isoform suggests that this enzyme is a ouabain-sensitive ATPase whose activity is dependent upon Na^+ and K^+ . This ATPase exhibited a lower apparent K_m for Na^+ than the kidney $\alpha 1$ isoenzyme and did not show positive cooperative Na^+ activation. Our results suggest that the activity of the Na^+, K^+ -ATPase $\alpha 3$ isoenzyme may be adapted to function under conditions of hyperpolarizing transmembrane potentials.

The Na^+, K^+ -ATPase is a membrane-bound enzyme responsible for the active transport of Na^+ and K^+ in most animal cells. The enzyme has been shown to consist of two subunits. The α subunit is a polypeptide of M_r 100,000 that contains the catalytic site for ATP hydrolysis (1). The β subunit is a glycosylated polypeptide of M_r 55,000 whose function has not yet been established.

The α and β subunits of the Na^+, K^+ -ATPase are encoded by multigene families. Three α - and two β -subunit genes have been localized to different chromosomes in the mouse (ref. 2; D. Malo, E. Schurr, R.L., and P. Gros, unpublished data), and cDNA clones encoding three rat α ($\alpha 1$, $\alpha 2$, $\alpha 3$) and two β ($\beta 1$, $\beta 2$) subunit isoforms have been characterized (3–7). The development of specific molecular probes for each of the subunit components of the Na^+, K^+ -ATPase has permitted a systematic evaluation of Na^+, K^+ -ATPase expression in rat tissues. Substantial differences in the tissue and developmental specificity of expression were found for the genes encoding each of the α and β subunits (4, 8–10). Chromosomal dispersion and tissue-specific expression of the α - and β -subunit genes suggest that the enzyme encoded by each gene may have properties selected in response to different physiological demands. However, the functional significance for α - and β -subunit isoform diversity has not been clearly explained.

The existence of multiple α and β isoforms has made it inherently difficult to study the enzymatic parameters of individual Na^+, K^+ -ATPase isoenzymes. Two biochemically distinct forms of the catalytic subunit, α ($\alpha 1$) and α^+ ($\alpha 2$), have been purified from rat kidney and brain axolemma, respectively, and found to differ in their affinities for ouabain and ATP (11). However, it is now known that the α^+ isoform derived from axolemma represents a mixture of $\alpha 2$ and $\alpha 3$

subunits (12). To date, purification of Na^+, K^+ -ATPase $\alpha 2$ or $\alpha 3$ isoenzymes has not been achieved. Characterization of the $\alpha 2$ and $\alpha 3$ subunits is further complicated by the fact that these polypeptides exhibit similar mobilities on SDS/PAGE (13).

Recent studies have demonstrated that $\alpha 3$ subunits are expressed predominantly in rat brain and copurify with ouabain-inhibitable Na^+, K^+ -ATPase activity (10). Expression of $\alpha 3$ subunits in a membrane fraction enriched for Na^+, K^+ -ATPase activity is consistent with the view that the $\alpha 3$ isoform is a functional component of the brain enzyme. However, the ability to characterize the enzymatic properties of the $\alpha 3$ isozyme is limited because $\alpha 2$ subunits are also expressed at high levels in brain (10). In the course of surveying the tissue distribution of α and β subunits, we analyzed the rat pineal gland, a tissue previously shown to express α - and α^+ -like ATPase activities (25, 26). We identified the pineal gland as a major expression site of the $\alpha 3$ subunit. No $\alpha 2$ subunits were detectable in this tissue. The pineal gland thus provides an opportunity to analyze the enzymatic properties of the $\alpha 3$ isoform in the absence of contaminating $\alpha 2$ isoenzymes.

We observed that in the rat there are similar levels of Na^+, K^+ -ATPase $\alpha 1$ and $\alpha 3$ subunits in 5-day-old pineal glands. Expression of $\alpha 3$ subunits increases relative to the level of $\alpha 1$ subunits in adult glands. No $\beta 1$ or $\beta 2$ subunits were detectable in 5-day-old pineals. However, high levels of $\beta 2$ subunits were present in adult glands. The adult pineal gland $\alpha 3$ enzyme appears to be an ATPase whose activity is dependent upon Na^+ and K^+ . Further, the $\alpha 3$ isoenzyme appears to have a lower apparent K_m for Na^+ when compared to the rat kidney $\alpha 1$ isoenzyme. These findings suggest that the activity of Na^+, K^+ -ATPase $\alpha 1$ and $\alpha 3$ isoenzymes may be regulated in response to different physiological demands. Expression of the $\alpha 3$ isoenzyme in pineal gland suggests a potential role for this enzyme in movement of Na^+ and K^+ across the plasma membrane of hyperpolarized cells.

MATERIALS AND METHODS

Rat Tissue Microsomes and Immunoblotting. Tissues were excised from 5-day-old and adult Sprague-Dawley rats. Crude microsomal membrane fractions were prepared by the method of Jorgenson (14), and protein concentrations were determined as described by Bradford (15). Solubilized microsomal proteins were fractionated in SDS-containing 7.5% or 10% polyacrylamide gels (40 μg of protein per lane) and transferred to nitrocellulose sheets essentially as described (16). After incubation for 1 hr in "blotto" (5% dry milk/0.2% Tween 20/150 mM NaCl/30 mM Tris, pH 7.5) to block nonspecific binding sites, the sheets were incubated with Na^+, K^+ -ATPase antiserum for 16 hr. Blots were rinsed four times with wash buffer (0.2% Tween 20/150 mM NaCl/30

mM Tris, pH 7.5), then incubated with ^{125}I -labeled protein A (Amersham; 5 μCi per blot; 1 μCi = 37 kBq) for 2 hr, rinsed four times in wash buffer, and autoradiographed with Kodak XRP film. Generation of antisera specific for each of the three rat α -subunit isoforms and two rat β -subunit isoforms has been described (10, 29).

Na^+, K^+ -ATPase Assay. Microsomal membrane fractions were assayed for Na^+ -dependent ATPase activity by a modification of the dual-cuvette spectrophotometric coupled assay described by Cantley and Josephson (17). One-tenth milliliter of crude rat tissue microsomes (60 μg of protein) at room temperature was added to a cuvette containing 0.6 ml of prewarmed (37°C) distilled water and 0.2 ml of $5\times$ Na^+ -free coupled-assay buffer [$1\times$ assay buffer is 30 mM histidine/3 mM MgATP (vanadate-free)/20 mM KCl/3 mM MgCl_2 , containing 10 units of pyruvate kinase and 10 units of lactate dehydrogenase per ml, 1.0 mM phosphoenolpyruvate (tricyclohexylamine salt), and 0.2 mM NADH, adjusted to pH 7.3 with KOH]. Then 0.1 ml of a solution containing 0–1.3 M NaCl (total salt concentration adjusted to 1.3 M with recrystallized choline chloride) was added to the cuvette. The reference cuvette contained distilled water only. The rate of ATPase-coupled β -NADH oxidation was continuously monitored in a Beckman double-beam spectrophotometer. Na^+ -dependent ATPase activity was determined by subtracting ATPase activity measured in the absence of Na^+ . Ouabain-inhibitable Na^+ -dependent ATPase activity was measured by the coupled assay in the presence of constant Na^+ (130 mM). After ATPase activity had been monitored for 1 min, 10 μl of ouabain (0–500 mM) in dimethyl sulfoxide was added to the cuvette containing rat tissue microsomes and absorbance was recorded for an additional 10 min. K^+ -dependent ATPase activity was measured according to the timed colorimetric procedure of Esmann (18).

Data Analysis. All data were subjected to nonlinear least-squares analysis using a Marquardt least-squares computer program (30). Na^+ dependence of the Na^+, K^+ -ATPase activity was fit by the cooperative model described by Lytton (19): $v = (V_{\text{max}} \times [\text{Na}^+]^n) / (K + [\text{Na}^+]^n)$. V_{max} , K , and n (the Hill coefficient) were allowed to vary to obtain the best fit for the data. The apparent $K_{1/2}$ for Na^+ was obtained from calculated values of K and n with the following relationship: apparent $K_{1/2} = (K)^{1/n}$. K^+ dependence was determined by fitting the equation $v = (V_{\text{max}} \times [\text{K}^+]) / (K_{1/2} + [\text{K}^+])$ to the data. (When the K^+ dependence was fit using the positive cooperative equation above, the Hill coefficient was ≈ 1.0 for all sets of data.) Ouabain inhibition of Na^+, K^+ -ATPase activity was fit by the two-independent-site model described by Emanuel *et al.* (20): % inhibition = $(I_{\text{max1}} \times [\text{ouabain}]) / (K_1 + [\text{ouabain}]) + (I_{\text{max2}} \times [\text{ouabain}]) / (K_2 + [\text{ouabain}])$, where I_{max1} is the maximal inhibition of the high-affinity component, K_1 is the apparent inhibition constant of the high-affinity component, I_{max2} is the maximal inhibition of the low-affinity component, and K_2 is the apparent inhibition constant of the low-affinity component. I_{max1} and I_{max2} were allowed to vary, while K_1 and K_2 were either varied or fixed at the value obtained from fitting rat kidney (K_2) ATPase data to a one-site model as described by Emanuel *et al.* (20). The two-site model fit the data equally well whether K_1 and K_2 were fixed at the predetermined values or allowed to vary.

RESULTS

Differential Expression of Na^+, K^+ -ATPase α - and β -Subunit Isoforms in Rat Pineal Glands. To evaluate expression of Na^+, K^+ -ATPase polypeptides, antisera specific for the rat $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$, and $\beta 2$ subunits were used to probe Western blots containing microsomes prepared from 5-

day-old and adult rat pineal glands. Adult rat kidney and brain microsomes were run in control lanes. The $\alpha 1$ antiserum reacted with a polypeptide of ≈ 100 kDa in kidney and brain, while $\alpha 2$ and $\alpha 3$ antisera reacted with polypeptides of ≈ 105 kDa in brain (Fig. 1). Moderate amounts of the $\alpha 1$ subunit were detected in 5-day-old pineals, with lower levels present in adult glands. No $\alpha 2$ subunits were detectable in 5-day-old or adult pineal glands or in control kidney microsomes. The $\alpha 3$ subunit was present at high abundance in adult pineal glands and moderate abundance in 5-day-old glands.

The expression of Na^+, K^+ -ATPase β subunits is shown in Fig. 2. Antiserum specific for the rat $\beta 1$ subunit reacted with two bands of 50 and 54 kDa in kidney and with a single broad band of 48 kDa in brain. When the immunoblot was exposed for a shorter period, two bands of 48 and 50 kDa were observed in brain (data not shown). No $\beta 1$ polypeptides were detectable in either adult or 5-day-old rat pineal glands. An antiserum specific for the rat $\beta 2$ subunit reacted with a single band of 46 kDa in brain and a single band of 50 kDa in adult pineal glands. No $\beta 2$ subunits were detected in 5-day-old pineals or in control kidney microsomes. These results indicate that in rat pineal glands, Na^+, K^+ -ATPase α - and β -subunit isoforms are expressed in a tissue-specific and developmentally regulated fashion.

Enzymatic Properties of the Rat Pineal Gland Na^+, K^+ -ATPase $\alpha 3$ Isoenzyme. Ouabain-inhibitable Na^+, K^+ -ATPase activity. Cell fractionation studies have revealed that Na^+, K^+ -ATPase $\alpha 3$ subunits are expressed in rat brain and

FIG. 1. Expression of Na^+, K^+ -ATPase α subunits in rat pineal glands. Microsomes were prepared from 5-day-old and adult rat tissues. Solubilized microsomal proteins were size-fractionated by electrophoresis through an SDS-containing 7.5% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the α -subunit isoform-specific antisera indicated at right. The positions of the molecular mass markers are indicated at left.

FIG. 2. Expression of Na^+, K^+ -ATPase β subunits in rat pineal glands. Solubilized microsomal proteins were electrophoresed through an SDS-containing 10% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with antisera specific for the $\beta 1$ and $\beta 2$ subunits. The positions of the molecular mass markers are indicated at left.

are present in a membrane fraction enriched in Na^+, K^+ -ATPase activity (10). However, the biochemical properties of the $\alpha 3$ isoform have not been determined. The high level of expression of the $\alpha 3$ subunit in adult rat pineal glands provided an opportunity to characterize the enzymatic activity of this Na^+, K^+ -ATPase isoform.

We compared ouabain-sensitive Na^+ -dependent ATPase activity in microsomal membrane fractions prepared from adult rat pineal gland, kidney, and brain. Such assays, when conducted in the presence of saturating Na^+ and K^+ , provide a quantitative estimate of both total Na^+, K^+ -ATPase activity and the proportion of the activity contributed by enzymes with high and low ouabain affinity (20). The results, normalized for each membrane fraction and fit by least-squares analysis, are presented in Fig. 3. Microsomes from rat kidney (solid squares) exhibited a single component of ouabain-sensitive Na^+ -dependent ATPase activity that was closely approximated by the one-site model described by Emanuel *et al.* (20). Nonlinear least-squares analysis of the data produced an apparent K_i (ouabain) of 0.108 mM for the ATPase activity of rat kidney microsomes. This compares well to the value reported for the ATPase activity of rat kidney cortical cells (20).

Data obtained from rat brain and pineal microsomes were best fit by a two-site model (*Materials and Methods*), suggesting that these tissues possessed more than one component of ouabain-sensitive Na^+ -dependent ATPase activity. K_2 was fixed at the value derived for rat kidney ATPase, while K_1 was allowed to vary. This analysis produced an optimal fit to the data curves of Fig. 3 that was not improved by allowing the K_2 values to vary (i.e., the assigned value gave the minimum sum of the squares). In rat brain (open squares), the high-affinity component of ouabain-sensitive Na^+ -dependent ATPase activity produced an apparent K_i of 1.1 μM and accounted for $\approx 62\%$ of total Na^+, K^+ -ATPase activity. In rat pineal gland (solid circles), the high-affinity component produced an apparent K_i of 1.6 μM and accounted for $\approx 52\%$ of total Na^+, K^+ -ATPase activity. These results suggest that the rat pineal gland $\alpha 3$ isoenzyme has a ouabain sensitivity similar to that of the high-affinity component of ouabain-sensitive Na^+ -dependent ATPase activity present in rat brain.

FIG. 3. Ouabain-sensitive Na^+, K^+ -ATPase activity in rat pineal glands. Microsomal membrane fractions from rat kidney ($\blacksquare$), rat brain ($\square$), and rat pineal gland ($\bullet$) were assayed for ouabain-inhibitable Na^+, K^+ -ATPase activity. Symbols represent the normalized values from two separate experiments. Curves represent the best fit of the data obtained for a one-site model (kidney) or a two-site model (brain and pineal gland) (*Materials and Methods*) and the constants presented in Table 1.

Na^+ and K^+ dependence. The Na^+ requirement of the pineal gland $\alpha 3$ isoenzyme was determined by measuring ATPase activity at saturating K^+ (20 mM) and varying Na^+ (0–130 mM) concentrations (Fig. 4). ATPase activity contributed by the ouabain-sensitive $\alpha 3$ enzyme was determined by subtracting ouabain-insensitive Na^+ -dependent ATPase activity (contributed by the $\alpha 1$ isoenzyme) from total Na^+ -dependent ATPase activity. The data from these experiments were analyzed by a nonlinear least-squares method using a cooperative model (*Materials and Methods*). Symbols in Fig. 4 represent the actual data points, whereas the curves represent the best fit of the cooperative model to the data.

FIG. 4. Na^+ -dependent ATPase activity in rat pineal glands. Microsomal membrane fractions from rat kidney ($\blacksquare$), rat brain ($\square$), and rat pineal gland ($\bullet$) were assayed for ouabain-inhibitable Na^+ -dependent ATPase activity under conditions of saturating K^+ . Kidney and brain data represent total ouabain (5 mM)-inhibitable ATPase activity, whereas pineal gland data represent ATPase activity sensitive to 10 μM ouabain. Symbols are normalized values from two separate experiments. Curves represent the best fit of the data obtained for the cooperative model (*Materials and Methods*).

Calculated parameters are summarized in Table 1. The Na⁺-dependent ATPase activity of rat kidney and brain (Fig. 4, solid and open squares, respectively; Table 1) exhibited a sigmoidal dependence on Na⁺ (Hill coefficients of 1.98 and 1.36, respectively), with a calculated $K_{1/2}$ (Na⁺) of 20.6 mM for the kidney enzyme and 15.5 mM for brain Na⁺,K⁺-ATPase. These values were similar to those reported for rat kidney and brainstem axolemma (21). In marked contrast, the Na⁺-dependent ATPase activity of rat pineal gland (Fig. 4, solid circles; Table 1) did not show positive cooperative Na⁺ activation (Hill coefficient of 0.94) and produced a calculated $K_{1/2}$ (Na⁺) of 11 mM. These results suggest that the $\alpha 3$ isoenzyme of rat pineal gland has a lower apparent K_m for Na⁺ than does the $\alpha 1$ isoenzyme present in kidney, and lacks the positive cooperativity for Na⁺ characteristic of kidney and brain Na⁺,K⁺-ATPase (19, 21).

The K⁺ requirement of the pineal gland $\alpha 3$ enzyme was determined by measuring ATPase activity at saturating Na⁺ (130 mM) and varying K⁺ (0–20 mM). The data from these experiments were analyzed by nonlinear least-squares treatment. The Na⁺,K⁺-ATPase activities of rat kidney, brain, and pineal gland all exhibited hyperbolic dependence on K⁺ and were best fit by a simple noncooperative model (data not shown). The calculated values (shown in Table 1) produced a $K_{1/2}$ (K⁺) of 1.75 mM for kidney, 1.3 mM for brain, and 2.1 mM for pineal gland. These results suggest that the Na⁺,K⁺-ATPase $\alpha 3$ isoenzyme exhibits an apparent K_m for K⁺ similar to that of the kidney $\alpha 1$ isoenzyme.

DISCUSSION

The Na⁺,K⁺-ATPase genes make up a multigene family. The development of antisera specific for each of the α and β subunits of the Na⁺,K⁺-ATPase has allowed us to analyze expression of these genes in a variety of rat tissues. Identification of the pineal gland as a major expression site of the $\alpha 3$ subunit has permitted an initial characterization of the enzymatic properties of this Na⁺,K⁺-ATPase isoform. We find that the activity of the $\alpha 3$ isoenzyme exhibits an apparent K_m for Na⁺ different from that of the $\alpha 1$ isoenzyme. This observation suggests that the $\alpha 3$ isoenzyme may be adapted to specialized ion movements in particular cell types.

Our data indicate that the $\alpha 3$ isoenzyme is a ouabain-sensitive ATPase whose activity is dependent on Na⁺ and K⁺. We find that the pineal gland $\alpha 3$ isoenzyme exhibits a half-value ($K_{1/2}$) for ouabain inhibition of 1.6 μ M. This value is similar to that reported for the $\alpha 3$ isoform of brainstem axolemma (22) and for the Na⁺,K⁺-ATPase derived by expression of $\alpha 3$ subunit cDNA (23). However, the apparent affinity of the $\alpha 3$ enzyme for ouabain is lower than that reported for the α -like activity ($K_i \approx 10$ nM) of the pineal gland Na⁺,K⁺-ATPase (25). This discrepancy may reflect differences in the conditions used to measure pineal gland Na⁺,K⁺-ATPase activity. We assume that the fraction of Na⁺,K⁺-ATPase with low affinity for ouabain is contributed by the $\alpha 1$ form of the enzyme. However, it is possible that an

enzyme composed of $\alpha 1$ and $\beta 2$ subunits may have a higher affinity for ouabain than an enzyme composed of $\alpha 1$ and $\beta 1$ subunits. While we view this as unlikely, our data do not exclude this possibility.

No apparent enzyme activity was observed in the absence of either Na⁺ or K⁺. The K⁺-dependent activation of the $\alpha 3$ isoenzyme was similar to that observed for the kidney $\alpha 1$ and brain α enzymes. However, Na⁺-dependent activation of the $\alpha 3$ isoform was significantly different from that of the kidney enzyme. The $\alpha 3$ isoenzyme of pineal gland exhibited a lower apparent K_m for Na⁺ ($K_{1/2} = 11$ mM) than did the kidney Na⁺,K⁺-ATPase ($K_{1/2} = 20.6$ mM). The $\alpha 3$ ATPase also lacked the positive cooperativity for Na⁺ activation that is characteristic of kidney and axolemmal Na⁺,K⁺-ATPases (21). Taken together, our results suggest that the $\alpha 1$ and $\alpha 3$ isoenzymes may have properties selected in response to different physiological demands.

Identification of the $\alpha 3$ subunit isoform in rat pineal glands is consistent with previous studies that described the expression of a high-affinity, ouabain-sensitive class of Na⁺,K⁺-ATPase in this tissue (24, 25). Increases in the level of $\alpha 3$ subunits during pineal development are also reflective of increases in ouabain-sensitive Na⁺,K⁺-ATPase activity and high-affinity ouabain-binding sites in rat pineal glands during the first 60 days of life (25). Na⁺,K⁺-ATPase and membrane potential have been shown to play an important role in the norepinephrine/cyclic AMP stimulation of serotonin *N*-acetyltransferase (25, 26). Norepinephrine has been shown to hyperpolarize the pinealocyte plasma membrane (27), while norepinephrine/cyclic AMP stimulation of serotonin *N*-acetyltransferase is blocked by agents such as ouabain and K⁺, which serve to depolarize the plasma membrane (28). Complete inhibition of the adrenergic induction of *N*-acetyltransferase activity occurs at a concentration of ouabain (1 μ M) (28) that would also inhibit $\alpha 3$ isoenzyme activity. The high level of expression of the Na⁺,K⁺-ATPase $\alpha 3$ isoenzyme in pineal glands suggests that this ATPase may play a specific role in maintaining Na⁺ and K⁺ concentration gradients under conditions of hyperpolarizing membrane potential.

Characterization of the enzymatic parameters of the $\alpha 3$ isoenzyme raises a number of intriguing issues. Of particular interest is the lower apparent K_m for Na⁺ exhibited by the $\alpha 3$ enzyme compared to that of the $\alpha 1$ isoenzyme. It is possible that the $\alpha 3$ form of the Na⁺,K⁺-ATPase may be active under conditions of low intracellular Na⁺ concentration. This property could serve to establish and maintain ion gradients under conditions in which the $\alpha 1$ isoenzyme may be less efficient. Previous studies have demonstrated that the $\alpha 1$ form of the Na⁺,K⁺-ATPase from kidney exhibits positive cooperativity for Na⁺ activation and exchanges three Na⁺ ions for two K⁺ ions during one cycle of ATP hydrolysis (1). However, it is interesting that the $\alpha 3$ isoenzyme does not show positive cooperative Na⁺ activation. Several potential explanations could account for this result. One possibility is that the $\alpha 3$ isoenzyme translocates only two Na⁺ ions per pump cycle.

Table 1. Calculated kinetic parameters for ouabain-inhibitable ATPase activity in rat tissue membrane fractions

Tissue	Ouabain				Na ⁺		K ⁺
	V_{max1}	K_1 , μ M	V_{max2}	K_2 , mM	$K_{1/2}$, mM	n	
Kidney	0%		100%	0.108 $\pm$ 0.086	20.6 $\pm$ 2.1	1.98 $\pm$ 0.01	1.75 $\pm$ 0.09
Brain	62%	1.1 $\pm$ 1.3	38%	0.108*	15.5 $\pm$ 3.6	1.36 $\pm$ 0.03	1.3 $\pm$ 0.13
Pineal	52%	1.6 $\pm$ 2.2	48%	0.108*	11 $\pm$ 1.5	0.94 $\pm$ 0.03	2.1 $\pm$ 0.19

Data were analyzed by the nonlinear least-squares procedure described in *Materials and Methods*. K_1 and K_2 are the apparent ouabain dissociation constants for the high- and low-affinity components of ouabain-sensitive ATPase activity. $K_{1/2}$ values are the half-maximal values for Na⁺- and K⁺-dependent ATPase activity. n represents the Hill coefficient for Na⁺. Data represent the mean $\pm$ SD of two independent experiments done in duplicate.

*The low-affinity component of ouabain inhibition was fixed at the value determined for the rat kidney $\alpha 1$ -type Na⁺,K⁺-ATPase. Allowing this constant to vary did not improve the fit to the data.

If this were true, then the $\alpha 3$ enzyme could exchange Na^+ for K^+ with no net charge movement, thus making it less sensitive to the plasma membrane potential. Alternatively, the $\alpha 3$ enzyme may contain several, noninteracting, Na^+ binding sites that could maintain a $3\text{Na}^+/2\text{K}^+$ exchange ratio. It will clearly be of interest to determine whether the stoichiometry of Na^+/K^+ exchange exhibited by the Na^+, K^+ -ATPase $\alpha 3$ isoform is different from that of the $\alpha 1$ enzyme.

The distinct distribution of α and β subunits in rat pineal glands raises two additional points of interest. Although biochemical studies have suggested that the β subunit is an essential component of the Na^+, K^+ -ATPase (1), the relationship of the β subunit to the activity of the enzyme has not been established. The apparent absence of $\beta 1$ -subunit expression in pineal glands suggests that in this tissue, Na^+, K^+ -ATPases are likely to consist of either $\alpha 1/\beta 2$ or $\alpha 3/\beta 2$ complexes. Comparison of the properties of an $\alpha 1/\beta 2$ enzyme to an $\alpha 1/\beta 1$ ATPase, or an $\alpha 3/\beta 2$ pump to an $\alpha 3/\beta 1$ enzyme, could provide important clues regarding β -subunit function. Finally, our data indicate that $\beta 1$ and $\beta 2$ subunits are virtually undetectable in 5-day-old glands. This raises the possibility that early in development, the pineal gland may contain a β -subunit isoform that does not react with either $\beta 1$ or $\beta 2$ antiserum. If an additional form of the β subunit is identified, it will be of interest to determine whether its tissue distribution and functional properties differ from those of the $\beta 1$ and $\beta 2$ subunits.

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1. Cantley, L. C. (1981) *Curr. Top. Bioenerg.* **11**, 201–237.
2. Kent, R. B., Fallows, D. A., Geissler, E., Glaser, T., Emanuel, J. R., Lalley, P. A., Levenson, R. & Housman, D. E. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5369–5372.
3. Shull, G. E., Greeb, J. & Lingrel, J. B. (1986) *Biochemistry* **25**, 8125–8132.
4. Herrera, V. L., Emanuel, J. R., Ruiz-Opazo, N., Levenson, R. & Nadal-Ginard, B. (1987) *J. Cell Biol.* **105**, 1055–1065.
5. Mercer, R. W., Schneider, J. W., Savitz, A., Emanuel, J., Benz, E. J., Jr., & Levenson, R. (1986) *Mol. Cell. Biol.* **6**, 3884–3890.
6. Young, R. M., Shull, G. E. & Lingrel, J. B. (1987) *J. Biol. Chem.* **262**, 4905–4910.
7. Martin-Vasallo, P., Dackowski, W., Emanuel, J. R. & Levenson, R. (1989) *J. Biol. Chem.* **264**, 4613–4618.
8. Emanuel, J. R., Garetz, S., Stone, L. & Levenson, R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9030–9034.
9. Orlowski, J. & Lingrel, J. B. (1988) *J. Biol. Chem.* **263**, 10436–10442.
10. Shyjan, A. W. & Levenson, R. (1989) *Biochemistry* **28**, 4531–4535.
11. Sweadner, K. J. (1979) *J. Biol. Chem.* **254**, 6060–6067.
12. Sweadner, K. J. (1989) *Biochim. Biophys. Acta* **988**, 185–220.
13. Schneider, J. W., Mercer, R. W., Gilmore-Hebert, M., Utset, M., Lai, C., Greene, A. & Benz, E. J., Jr. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 284–288.
14. Jorgenson, P. L. (1974) *Biochim. Biophys. Acta* **356**, 36–52.
15. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
16. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
17. Cantley, L. C. & Josephson, L. (1976) *Biochemistry* **15**, 5280–5287.
18. Esmann, M. (1988) *Methods Enzymol.* **156**, 105–115.
19. Lytton, J. (1985) *J. Biol. Chem.* **260**, 10075–10080.
20. Emanuel, J. R., Schulz, J., Zhou, X.-M., Kent, R. B., Housman, D., Cantley, L. & Levenson, R. (1988) *J. Biol. Chem.* **263**, 7726–7733.
21. Sweadner, K. J. (1985) *J. Biol. Chem.* **260**, 11508–11513.
22. Urayama, O. & Sweadner, K. J. (1988) *Biochem. Biophys. Res. Commun.* **156**, 796–800.
23. Hara, Y., Nikamoto, A., Kojima, T., Matsumoto, A. & Nakao, M. (1988) *FEBS Lett.* **238**, 27–30.
24. Caspers, M. L., Schwartz, R. D., Labarca, R. & Paul, S. M. (1987) *Brain Res.* **409**, 335–342.
25. Ceña, V., Gonzalez-Garcia, C., Svoboda, P., Weller, J. L. & Klein, D. C. (1987) *J. Biol. Chem.* **262**, 14467–14471.
26. Gonzalez-Garcia, C., Ceña, V. & Klein, D. C. (1988) *Mol. Pharmacol.* **32**, 792–797.
27. Sakai, K. K. & Marks, B. H. (1972) *Life Sci.* **11**, 285–291.
28. Parfitt, A., Weller, J. L., Klein, D. C., Sakai, K. K. & Marks, B. H. (1975) *Mol. Pharmacol.* **11**, 241–255.
29. Shyjan, A.W., Gottardi, C. & Levenson, R. (1990) *J. Biol. Chem.*, in press.
30. Smith, R. L., Zinn, K. & Cantley, L. C. (1980) *J. Biol. Chem.* **255**, 9852–9859.