

Sodium Kinetics of Na,K-ATPase α Isoforms in Intact Transfected HeLa Cells

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ABSTRACT By participating in the regulation of ion and voltage gradients, the Na-K pump (i.e., Na,K-ATPase) influences many aspects of cellular physiology. Of the four α isoforms of the pump, $\alpha 1$ is ubiquitous, $\alpha 2$ is predominant in skeletal muscle, and $\alpha 3$ is found in neurons and the cardiac conduction system. To determine whether the isoforms have different intracellular Na^+ affinities, we used the Na^+ -sensitive dye sodium-binding benzofuran isophthalate (SBFI) to measure pump-mediated Na^+ efflux as a function of $[\text{Na}^+]_i$ in human HeLa cells stably transfected with rat Na-K pump isoforms. We Na^+ -loaded the cells, and then monitored the time course of the decrease in $[\text{Na}^+]_i$ after removing external Na^+ . All transfected rat α subunits were highly ouabain resistant: the $\alpha 1$ isoform is naturally resistant, whereas the $\alpha 2$ and $\alpha 3$ isoforms had been mutagenized to render them resistant. Thus, the Na^+ efflux mediated by endogenous and transfected pumps could be separated by studying the cells at low (1 μM) and high (4 mM) ouabain concentrations. We found that the apparent K_m for Na^+ efflux attributable to the native human $\alpha 1$ isoform was 12 mM, which was similar to the K_m of rat $\alpha 1$. The $\alpha 2$ and $\alpha 3$ isoforms had apparent K_m 's of 22 and 33 mM, respectively. The cells expressing $\alpha 3$ had a high resting $[\text{Na}^+]_i$. The maximal activity of native $\alpha 1$ in the $\alpha 3$ -transfected cells was only $\sim 56\%$ of native $\alpha 1$ activity in untransfected HeLa cells, suggesting that transfection with $\alpha 3$ led to a compensatory decrease in endogenous $\alpha 1$ pumps. We conclude that the apparent $K_m(\text{Na}^+)$ for rat Na-K pump isoforms increases in the sequence $\alpha 1 < \alpha 2 < \alpha 3$. The $\alpha 3$ isoform may be suited for handling large Na^+ loads in electrically active cells.

KEY WORDS: sodium-binding benzofuran isophthalate • transfection • ouabain • sodium affinity

INTRODUCTION

The Na-K pump (Na,K-ATPase) has two main protein subunits, α and β . Four isoforms of the α subunit are known, and three of the β . Of the α isoforms, $\alpha 1$ is ubiquitous. It appears to be a "housekeeping" isoform in most tissues, but is expressed at high levels in epithelia with high rates of solute transport. The other isoforms have a more restricted distribution: $\alpha 2$ is found in muscle, adipose tissue, and brain; $\alpha 3$ is expressed in neural tissue and heart; and $\alpha 4$ is present in testis. The β subunit is essential for normal targeting and correct insertion of the α subunit into the cell membrane. In addition, the β subunit appears to have some influence on the catalytic properties of the enzyme, even though it is the α subunit that has the binding sites for Na^+ , K^+ , ATP, and ouabain (Jaisser et al., 1994).

Although little is known about $\alpha 4$, the other α isoforms exhibit marked anatomic, developmental, and pathophysiological diversity, suggesting that the α isoforms have different functional roles. Experiments in which single, defined α isoforms are transfected into cultured cells

show that rat α isoforms differ in their sensitivity to ouabain: rat $\alpha 1$ is ouabain resistant (inhibited only by millimolar ouabain concentrations), whereas rat $\alpha 2$ and $\alpha 3$ are ouabain sensitive (completely inhibited by 1–5 μM ouabain). Nevertheless, work on transfected cells has been limited by techniques available for assessing functional differences (e.g., differences in affinity for Na^+) among α isoforms. First, it is difficult to distinguish unambiguously the $\alpha 2$ and $\alpha 3$ isoforms in experiments on mammalian cells, which invariably express $\alpha 1$. Second, some assay systems consist of broken membranes, and thus are not "sided." Third, even in sided preparations, previous transfection studies have not controlled intracellular and extracellular ion concentrations independently. Such control is important because the Na-K pump rate depends on intracellular sodium concentration ($[\text{Na}^+]_i$) (Garay et al., 1973).

The objective of the present study was to measure the apparent K_m for $[\text{Na}^+]_i$ of the rat $\alpha 1$, $\alpha 2$, and $\alpha 3$ isoforms, transfected into human HeLa cells. One approach for assessing pump function would be to measure Na,K-ATPase activity. Such assays, however, are usually performed in broken (i.e., "unsided") membranes. Other approaches would be to measure isotopic fluxes or pump current. However, $[\text{Na}^+]_i$ usually cannot be measured in such experiments; $[\text{Na}^+]_i$ must be clamped using a patch pipette or ionophores. Our approach was

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to use the Na⁺-sensitive dye SBFI (sodium-binding benzofuran isophthalate)¹ (Minta et al., 1989; Harootunian et al., 1989) to monitor [Na⁺]_i, and from these data compute a measure of the Na⁺ efflux (i.e., d[Na⁺]_i/dt). We exploit a technique proposed by Negulescu and Machen (1990) for Na⁺ loading cells and using SBFI to monitor the subsequent rate of [Na⁺]_i recovery (i.e., decrease).

We studied untransfected HeLa cells, as well as HeLa lines stably transfected with various rat α isoforms. All cells expressed the ouabain-sensitive, native human α 1 subunit, as well as the native human β 1 subunit of the Na-K pump. One line was stably transfected with rat α 1, which is naturally ouabain resistant. Two other lines were transfected with rat α 2 and α 3 that had been mutagenized to render them ouabain resistant (Jewell and Lingrel, 1991). Thus, we could use differential ouabain sensitivity to distinguish the transfected, ouabain-resistant rat α isoforms from the native, ouabain-sensitive human α 1 pumps. Others have approached the question of whether the Na⁺ affinities of the pump isoforms are different using these cell lines (Jewell and Lingrel, 1991; Munzer et al., 1994). We found that rat and human α 1 isoforms have similar apparent K_m values for [Na⁺]_i, but that rat α 2 and α 3 have progressively higher K_m values. Moreover, the cells expressing α 3 had a very high resting [Na⁺]_i.

MATERIALS AND METHODS

Cells Studied

We used four HeLa-based cell lines ("Jewell-Lingrel cell lines"), three of which were transfected with rat α isoforms (Jewell and Lingrel, 1991). All were kind gifts of Dr. Jerry Lingrel. The first was untransfected HeLa cells, which express only the human α 1 Na,K-ATPase isoform. This isoform is ouabain-sensitive (>95% inhibition at 1 μ M ouabain). The second HeLa cell line (α 1-8G) was stably transfected with the unmodified, but naturally ouabain resistant, rat α 1. The third line of HeLa cells (α 2-2G) was transfected with a mutagenized rat α 2 isoform of the Na-K pump, designated α 2*. In this mutant isoform, two amino acid residues were changed so as to confer ouabain resistance (>95% inhibition at 4 mM ouabain). The fourth HeLa cell line (α 3-5G) was stably transfected with a similarly mutagenized ouabain-resistant rat α 3, termed α 3*.

Cells were grown in DMEM plus 10% FCS supplemented with penicillin, streptomycin, and fungizone. The medium for the three cell lines transfected with rat α isoforms also contained 1 μ M ouabain to inhibit the endogenous human α 1 isoform, thereby applying selection pressure.

Solutions

Table I summarizes the composition of our solutions, all of which were made with ultrapure water (PICOpure water systems; Hydro Research, Triangle Park, NC). The pH was adjusted with HCl or

TABLE I
Composition of Working Solutions

Solution number	1	2	3	4	5
Solution name	Standard buffer	Zero-Na ⁺ buffer	Zero-K ⁺ buffer	Calibration buffer 1	Calibration buffer 2
NaCl	150	0	150	0	30
Na gluconate	0	0	0	0	130
KCl	5	5	0	30	0
K gluconate	0	0	0	130	0
NMDG	0	153.6	8.6	3.6	3.6
CaCl ₂	1	1	1	1	1
MgSO ₄ /7H ₂ O	1.2	1.2	1.2	1.2	1.2
PO ₄	0	2	2	2	2
Hepes	32	32	32	32	32
Glucose	10	10	10	10	10
NaH ₂ PO ₄	2	0	0	0	0
Gramicidin	0	0	0	10 μ M	10 μ M
Nigericin	0	0	0	10 μ M	10 μ M
Monensin	0	0	0	14.5 μ M	14.5 μ M
pH at 37°C	7.40	7.40	7.40	7.10	7.10

Composition of solutions used. Entries are in millimolars unless otherwise specified. NMDG, *N*-methyl-D-glutamate.

NMDG (*N*-methyl-D-glutamate). The solutions for calibrating the intracellular dye had [Na⁺]_i values between 0 and 160 mM, and were made by mixing calibration buffers 1 (solution 4) and 2 (solution 5). The acetoxymethyl ester of SBFI was obtained from Molecular Probes, Inc. (Eugene, OR), and DMSO (dimethyl sulfoxide) from J.T. Baker, Inc. (Phillipsburg, NJ). Other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO).

Spectrofluorometry

We used a dual-beam spectrofluorometer (1681; SPEX Inc., Edison, NJ) to measure [Na⁺]_i in a population of cells. Cells were grown on a 8-mm² glass coverslip, which was inserted into a custom-designed ~0.8-ml quartz flow-through cuvette. A thermistor in the cuvette permitted continuous temperature monitoring. The cuvette was continuously superfused with prewarmed medium delivered at 2.2 ml/min through water-jacketed Tygon tubing.

We grew the cells to 80–100% confluence, and then serum-starved them (0.5% FCS) 12 h before study. On the morning of the study, we loaded the cells with the Na-sensitive dye SBFI (Harootunian et al., 1989; Negulescu and Machen, 1990) as follows. The dye-loading solution was made by adding 7 μ l DMSO and 7 μ l of a Pluronic F-127 solution (20% in DMSO) to a vial containing 50 μ g SBFI-AM. The vial was vortexed, and the contents diluted into 3 ml standard buffer (solution 1) at room temperature (RT). Coverslips were incubated in this solution at RT for 3–4 h, shielded from the light, with gentle agitation. Coverslips were then washed in standard buffer for 30 min at RT with agitation. A coverslip of dye-loaded cells was then inserted into the cuvette and an excitation scan obtained (excitation from 320 to 400 nm, emission at 505 nm). The coverslip was rejected if the excitation spectrum was not a smooth, skewed, bell-shaped curve with a peak intensity at ~355 nm (characteristic of SBFI-free acid) (Harootunian et al., 1989), or if the peak intensity was not at least six-fold greater than that obtained from a coverslip of cells not loaded with dye.

We calculated [Na⁺]_i as follows. First, we obtained fluorescence intensities at 505 nm, while exciting at either 340 or 380

¹Abbreviation used in this paper: SBFI, sodium-binding benzofuran isophthalate.

nm. These I_{340} and I_{380} values were acquired continuously and sampled every 1.5–4.0 s. Second, we corrected these I_{340} and I_{380} values for the autofluorescence at the corresponding wavelengths, and computed the corrected I_{340}/I_{380} ratio. Third, we converted these I_{340}/I_{380} ratios to $[Na^+]_i$ values using the data obtained from a two-point calibration performed at the conclusion of each experiment. In this calibration, we determined the I_{340}/I_{380} ratio while exposing the cells to calibration buffers containing, first, 90 mM Na^+ and, then, 0 mM Na^+ . For both buffers, the sum $[Na^+] + [K^+]$ was 160 mM. To equalize $[Na^+]_i$ and $[Na^+]_o$, we also included in the calibration buffers the monovalent cation ionophores gramicidin D (10 μ M), nigericin (10 μ M), and monensin (14.5 μ M).² (We show below that this ionophore combination did in fact equalize $[Na^+]_i$ and $[Na^+]_o$.) As described in RESULTS, we used the I_{340}/I_{380} ratios thus obtained at $[Na^+]_i = 0$ and 90 mM to convert the I_{340}/I_{380} ratios obtained earlier in the experiment to $[Na^+]_i$ values.

Although the majority of our experiments were done with the standard two-point calibration protocol described above, in the early stages of the project we performed some experiments with calibration procedures that differed in two ways. First, in some experiments, the high Na calibration buffer contained 30 instead of 90 mM Na^+ . For these experiments, we converted ratios to $[Na^+]_i$ values using a formula slightly different from our standard one, exploiting data from multipoint calibration experiments (see RESULTS), which included both 30 and 90 mM Na^+ data. This difference in calibration did not lead to appreciable differences in calculated $[Na^+]_i$ values in the range between 0 and 50 mM (see Fig. 2, below), which includes virtually all of our data.

A second difference was that, in some early experiments, the calibration buffers contained gramicidin and nigericin, but not monensin. We later found that, although the two- and three-ionophore calibrations yielded the same I_{340}/I_{380} ratios when $[Na^+]_o$ was 0 mM, the two-ionophore method yielded lower ratios than the three-ionophore method when $[Na^+]_o$ was 30–90 mM. This result suggested to us that gramicidin plus nigericin did not truly equilibrate $[Na^+]_i$ with $[Na^+]_o$. As shown in RESULTS, however, our flame-photometry data confirmed that the three-ionophore method does indeed equilibrate $[Na^+]_i$ with $[Na^+]_o$. Therefore, we performed additional experiments in which we sequentially perfused the cells with calibration buffer containing 90 mM Na^+ and two ionophores, and then calibration buffer containing 90 mM Na^+ and all three ionophores. We found that the addition of the third ionophore caused I_{340} and I_{380} to decrease by 4 and 7%, respectively. These results allowed us to correct I_{340} and I_{380} data in experiments in which we had calibrated with only two ionophores, thereby yielding accurate $[Na^+]_i$ values.

As a confirmation that the above correction procedures were valid, we examined the ouabain-insensitive Na^+ efflux (i.e., Na^+ leak) in the various Jewell-Lingrel cell lines. Indeed, our corrected data were similar, regardless of whether the calibration buffer contained 30 or 90 mM Na^+ , or two or three ionophores.

Determination of $[Na^+]_i$ by Flame Photometry

To independently validate our method for converting SBFI I_{340}/I_{380} ratios to $[Na^+]_i$ values, we used flame photometry to measure $[Na^+]_i$. Untransfected HeLa cells were grown in six-well tissue plates until 80–100% confluent. Three wells of each six-well plate were used for measuring extracellular and intracellular volume (ECFV, ICFV), with the other three wells being used for flame photometry measurements of total Na. All cells were incubated

²Care was taken to thoroughly rinse the perfusion lines with 70% EtOH and distilled water between experiments so as to remove traces of ouabain and ionophores.

in calibration buffers containing gramicidin, nigericin, and monensin, and varying $[Na^+]$ as noted above. For the ECFV and ICFV determinations, the solutions also contained ^{14}C -inulin and 3H - H_2O (Amersham Corp., Arlington Heights, IL).

At the start of each of these experiments, we aspirated the culture medium and rinsed each well twice with 1 ml of the calibration buffer (prewarmed to 37°C) containing the selected $[Na^+]$ (0–90 mM). After aspirating the second of these 1-ml rinses, we added 1 ml of the selected calibration buffer to each well and placed the six-well plates in a sealed vessel containing a tray of buffer to minimize evaporation. We placed this vessel in a 37°C incubator for 1–2 h. We next took samples of the solution (i.e., ECF) in each well for subsequent scintillation counting. Next, we rinsed each well twice with 1 ml ice-cold choline chloride (160 mM) to minimize contamination by the original calibration buffer. Then, we added 0.5 ml lysis solution (Triton X-100, 0.5%) to each well and allowed cell lysis to proceed overnight with gentle shaking. We then scraped up and suspended any material adherent to the well, and aspirated this suspension. We rinsed the well with an additional 0.5 ml lysis solution, which we combined with the aspirate and centrifuged for 5 min at 15,000 rpm, discarding the pellet (Munzer et al., 1994).

We placed aliquots of samples containing isotopes in scintillation vials and counted them in a Tri-Carb 1600 TR (Packard Instrument Co., Inc., Downers Grove, IL). We separated counts for ^{14}C and 3H using a channels-ratio method. We diluted the nonradioactive lysate samples (as needed), and measured $[Na^+]$ in a flame photometer (400; Corning Glass Works, Corning, NY). We also assayed by flame photometry buffer samples taken from each well at the conclusion of the incubation, as well as samples of unused calibration buffer, lysis solution, and ultrapure water in duplicate or triplicate.

In calculating $[Na^+]_i$, we assumed that the cell lysate represents ICF, contaminated with some ECF. Because ^{14}C -inulin distributes only in the ECF, the ECFV of the lysate is equal to the ^{14}C -cpm in the cell extract divided by the ^{14}C -cpm/ μ l of buffer sample. Because 3H - H_2O labels all the water in the sample, the corresponding quotient for 3H -cpm represents ECFV + ICFV. The $[Na^+]$ of the ECF, as well as the Na^+ content (in micromoles) of the cell lysate, are known from flame photometry. Combining this information with the known ECF and ICF volumes allows calculation of the $[Na^+]$ of the ICF.

Immunoblotting

We prepared crude microsomes from cells grown to confluence as previously described (Zahler et al., 1996). The specific activity of Na,K-ATPase in such preparations from untransfected HeLa cells was ~ 24 μ mol Pi/mg protein per h. We loaded equal amounts (10–30 μ g) of microsomal protein on 7% SDS polyacrylamide gels, performed electrophoresis, and transferred the proteins electrically to Immobilon-P membranes (Millipore Corp., Bedford, MA) as previously described (Zahler et al., 1996). We performed immunoblots using a 1:500 dilution of a monoclonal antibody (anti-LEAVE) raised to a pentapeptide (KNCLVKN-LEAVE) common to the $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms (Pressley, 1992) (kind gift of Dr. T. Pressley). We quantitated the results by including titrated amounts of SY5Y microsomal protein on each blot, measuring band intensity using densitometric scanning, and deriving from this a region where band intensity was a linear function of the amount of protein loaded (Lucchesi and Sweadner, 1991).

Data Analysis

Results are displayed as mean \pm SEM. Curve fits were performed using DeltaGraph 4.0 and Systat 5.1 for Macintosh. We used two

approaches for analyzing data for Na^+ dependence of pump flux. First, data were fitted by the highly cooperative model (Garay et al., 1973; Jewell and Lingrel, 1991)

$$v = V_{\max} / (1 + (K' / [\text{Na}^+]_i)^3), \quad (1)$$

where $K_m = (K')^{1/3}$ (Jewell and Lingrel, 1991), and the exponent is three because there are three Na^+ binding sites. This method is has been used by previous investigators studying the Na^+ dependence of pump flux in transfected cells (Munzer et al., 1994).

The second approach, feasible because our data had high resolution on the $[\text{Na}^+]_i$ axis, was to fit to a cooperative model with variable Hill coefficient:

$$v = V_{\max} / (1 + (K / [\text{Na}^+]_i)^b), \quad (2)$$

where b is the Hill coefficient.

We also wished to determine statistically whether the effluxes in the presence of different ouabain concentrations were significantly different. Because the efflux data are heteroscedastic (standard errors are not random, but increase as $[\text{Na}^+]_i$ increases; see Fig. 5), they are not well suited for evaluation by standard regression methods. Accordingly, we logarithmically transformed the data; the log effluxes were close to a linear function of $[\text{Na}^+]_i$, with uniform standard errors. We tested for equality of the slopes and intercepts of these linear regression lines. Finally, we tested for interisofom differences in the affinity constants for $[\text{Na}^+]_i$ derived from the fitted curves using chi-square analysis and the asymptotic standard errors of estimate of the affinity constants.

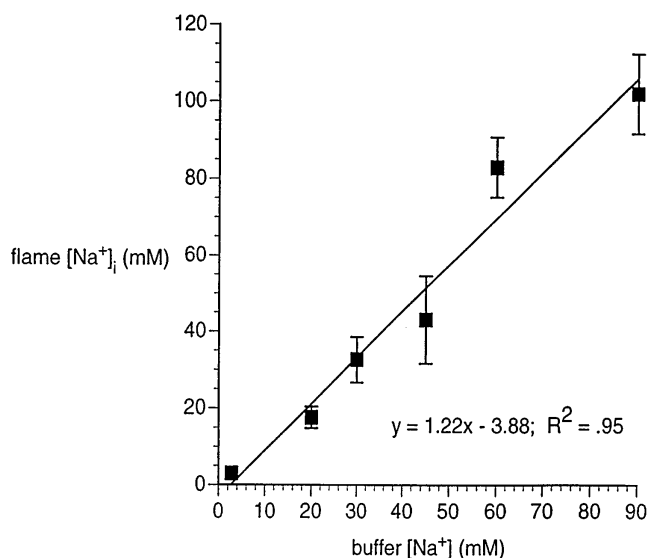


FIGURE 1. Validation of ionophore technique for equalizing intra- and extracellular $[\text{Na}^+]$. Untransfected HeLa cells grown in six-well tissue culture plates were incubated in buffers having a range of $[\text{Na}^+]$ values, and containing gramicidin D (10 μM), nigericin (10 μM), and monensin (14.5 μM). One set of wells contained ^{14}C -inulin and ^3H - H_2O for measuring intra- and extracellular volume. A paired set was subjected to flame photometry for measuring total Na^+ . In five experiments, we sampled solution in each well at the conclusion of the incubation period, measuring $[\text{Na}^+]$ by flame photometry; $[\text{Na}^+]$ in these samples was within 2% of the $[\text{Na}^+]$ of the initial buffer. Each symbol represents the mean of three to seven pairs of wells. Error bars are omitted at the point for which they would have been smaller than the size of the symbol.

RESULTS

Validation of Ionophore Technique Used for Equilibrating Extracellular and Intracellular $[\text{Na}^+]$

As described in METHODS, we incubated untransfected HeLa cells in calibration buffers containing three ionophores (gramicidin, nigericin, and monensin) and various levels of $[\text{Na}^+]$, with and without ^{14}C -inulin and ^3H - H_2O . Scintillation counting showed that ECFV and ICFV were approximately equal, increasing the reliability of the calculation of $[\text{Na}^+]_i$. Combining these data with flame-photometry data for total Na^+ , we computed $[\text{Na}^+]_i$. As shown in Fig. 1, incubation with the three ionophores causes intracellular and extracellular $[\text{Na}^+]_i$ to become essentially equal over a wide range of $[\text{Na}^+]_o$ values.

Multipoint Calibration of SBFI

Alternately exciting at 340 and 380 nm, while monitoring the emission at 505 nm, we determined the response of the I_{340}/I_{380} fluorescence–excitation ratio to defined perturbations of $[\text{Na}^+]_i$ using gramicidin, nigericin, and monensin to equilibrate $[\text{Na}^+]_i$ with $[\text{Na}^+]_o$. A typical multipoint calibration experiment for SBFI in HeLa cells is shown in Fig. 2 A, where the I_{340}/I_{380} ratio appears to closely track $[\text{Na}^+]_o$. Accordingly, we combined such multiple experiments and analyzed the resulting I_{340}/I_{380} vs. $[\text{Na}^+]$ data, as follows.

Let $r([\text{Na}^+]_i)$ be the I_{340}/I_{380} ratio as a function of $[\text{Na}^+]_i$. Then we define the normalized fluorescence–excitation ratio as

$$\bar{r}([\text{Na}^+]_i) = \frac{r([\text{Na}^+]_i) - r(0)}{r(90) - r(0)}, \quad (3)$$

where $r(0)$ and $r(90)$ are the ratios when $[\text{Na}^+]_i$ is 0 and 90 mM, respectively. This transformation forces \bar{r} to be zero when $[\text{Na}^+]_i$ is 0, and 1 when $[\text{Na}^+]_i$ is 90 mM. Combining data from eight similar experiments, we then calculated \bar{r} as a function of $[\text{Na}^+]$. The results, shown by the symbols in Fig. 2 B, indicate that the normalized I_{340}/I_{380} ratio rises monotonically with $[\text{Na}^+]_i$. The tight confidence intervals (average SEM, 2.6 mM Na) indicate that changes in $[\text{Na}^+]_i$ of 3 mM or less can be routinely detected. Finally, we fitted these data with a right rectangular hyperbola, obtaining the curve shown in Fig. 2 B. Rearranging the equation for this hyperbola, we can compute $[\text{Na}^+]_i$ from the normalized ratio:

$$[\text{Na}^+]_i \text{ (mM)} = \frac{119.2\bar{r}}{2.41 - \bar{r}}. \quad (4)$$

Measurement of Basal $[\text{Na}^+]_i$

We determined the basal $[\text{Na}^+]_i$ for each of the cell lines by incubating the cells in standard buffer until the

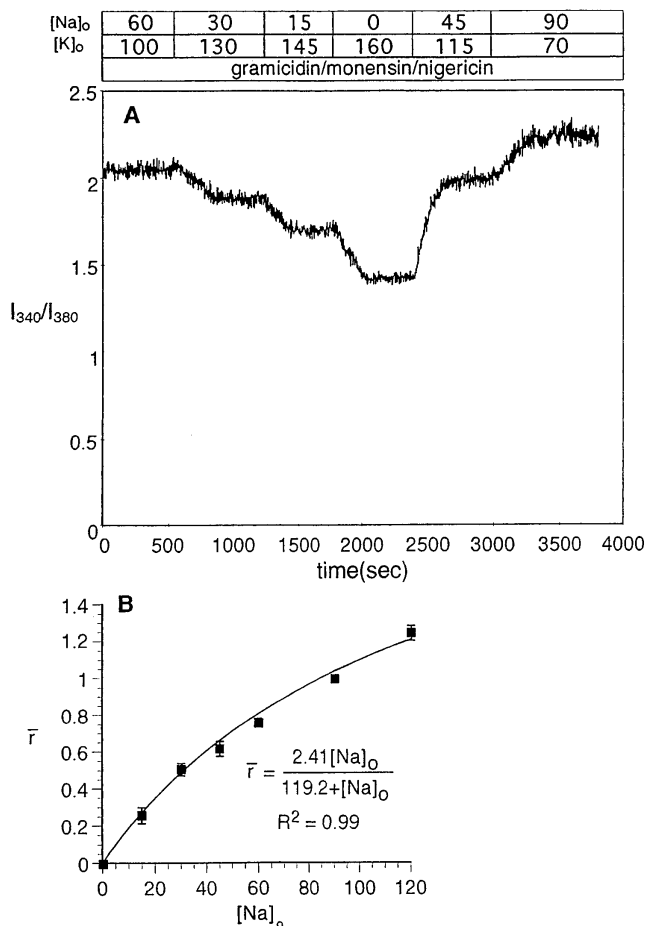


FIGURE 2. Multipoint SBFI calibration in HeLa cells. SBFI-loaded, untransfected HeLa cells on glass coverslips were placed in a dual-beam spectrofluorometer and superfused with calibration buffers of varying $[Na^+]_o$, but a constant $[Na^+] + [K^+]_o$ of 160 mM. pH was 7.10 at 37°C, to approximate intracellular pH. The calibration buffers contained three ionophores, as in Fig. 1, to equilibrate intra- and extracellular Na^+ . (A) Typical experiment showing effect of step changes in extracellular Na^+ on SBFI fluorescence-excitation ratio (I_{340}/I_{380}). (B) Dependence of normalized fluorescence-excitation ratio \bar{r} on $[Na^+]_o$. Ratios from eight experiments similar to the one shown in A were normalized to equal zero at $[Na^+] = 0$ and 1 at $[Na^+] = 90$, and then averaged. Error bars are omitted at the points for which they would have been smaller than the size of the symbol. The curve through the points is the result of a nonlinear least-squares fit for a right-rectangular hyperbola.

I_{340}/I_{380} ratio reached a steady state, and then immediately calibrating. The results are shown in Table II. The basal $[Na^+]_i$ value is 18.6 mM in untransfected HeLa cells, as compared with the value of 13 mM found by Boardman et al. (1974) in HeLa cells (see DISCUSSION). The basal $[Na^+]_i$ for the $\alpha 3$ -transfected cells, 50.3 mM, was significantly greater ($P < 0.05$ by ANOVA, Scheffé multiple comparison test) than the corresponding values for the other Jewell-Lingrel cell lines.

TABLE II
Basal $[Na^+]_i$ Values in Jewell-Lingrel Cell Lines

Isoform transfected	Basal $[Na^+]_i$	n
None	18.6 ± 4.6	5
$\alpha 1$	11.5 ± 5.6	3
$\alpha 2$	13.4 ± 2.7	6
$\alpha 3$	50.3 ± 6.0	7

Basal $[Na^+]_i$ values were determined in untransfected HeLa cells and in HeLa-based Jewell-Lingrel cell lines. Values are in millimolars. The value for the $\alpha 3$ -transfected cells is significantly different ($P < 0.05$) from those for the other cell lines by ANOVA (Scheffé multiple comparison test).

Measuring $[Na^+]_i$ Dependence of Na-K Pump Flux in Untransfected HeLa Cells

We determined the $[Na^+]_i$ dependence of the Na-K pump flux using an approach described by Negulescu and Machen (1990). We obtained the pump flux as a function of $[Na^+]_i$ because the Na-K pump activity is sensitive to $[Na^+]_i$, which it directly modulates. As shown in Fig. 3, we first Na^+ -loaded untransfected HeLa cells (which have only native, human $\alpha 1$ subunits) by incubation in zero- K^+ buffer (segment *ab*). We then removed extracellular Na^+ , and simultaneously raised the $[K^+]_o$ to 5 mM, while monitoring $[Na^+]_i$ as Na^+ left the cells (*bc*). When the I_{340}/I_{380} ratio fell sufficiently, we performed a two-point calibration as described above (*cd* and *de*). In Fig. 3, ouabain was present during *abc* at a concentration (1 μM) high enough to completely inhibit the HeLa cell Na,K-ATPase (Jewell and Lingrel, 1991). Thus, the Na^+ efflux during *bc* represents the Na^+ efflux mediated by “leak” pathways as a function of $[Na^+]_i$. In other experiments (not shown), ouabain was absent during *abc* so that the Na^+ efflux during the equivalent of *bc* represented the Na^+ efflux mediated both by the Na-K pump and leak pathways.

We then numerically differentiated the time course of decrease in $[Na^+]_i$ (Fig. 3, segment *bc*) to yield $d[Na^+]_i/dt$ as a function of $[Na^+]_i$. Fig. 4 A shows the results of this analysis both for experiments conducted in the presence (●) and absence of ouabain (■). As expected, Na^+ efflux was considerably higher in the absence of ouabain than in its presence, and the two curves are significantly different ($P < 0.002$). Moreover, the efflux was similar in low (1 μM) and high (4 mM) ouabain concentrations in this cell type (not shown), as would be expected, given the high ouabain affinity of the native human $\alpha 1$ in untransfected HeLa cells. From Fig. 4 A, we obtained the activation curve of the Na-K pump as a function of $[Na^+]_i$ by subtracting the efflux in the presence of ouabain from the efflux in the absence of ouabain (Fig. 4 B). This activation curve indicates an apparent K_m of 12 mM (fit with variable Hill coefficient, Eq. 2). Others, employing various methods, have obtained similar values: 16 mM in rat brain

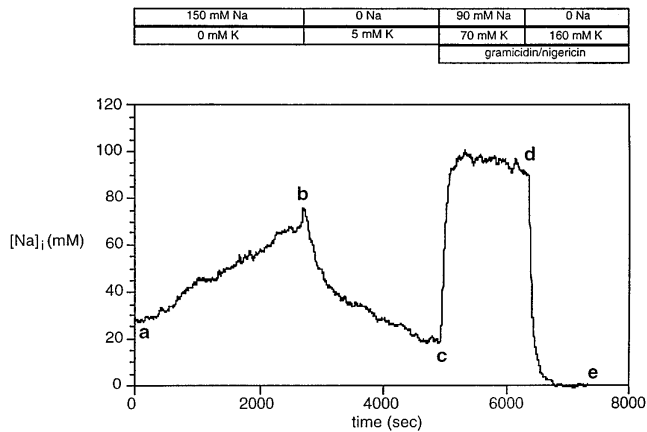


FIGURE 3. Measurement of Na^+ efflux. Untransfected HeLa cells were loaded with SBFI, and then I_{340}/I_{380} data pairs were acquired continuously. Cells were exposed first to zero- K^+ buffer (solution 3; segment *ab*), and then to zero- Na^+ buffer (solution 2; segment *bc*), and finally to two different calibration buffers (mixed from solutions 4 and 5; *cd* and *de*). $[\text{Na}^+]_i$ values were computed from I_{340}/I_{380} ratios, the calibration data from this experiment, and Eq. 4. Because the calibration buffers in this experiment contained two ionophores, we applied a correction (see METHODS) so that the computed $[\text{Na}^+]_i$ values were comparable with those in experiments with three ionophores.

synaptosomes (Brodsky and Guidotti, 1990), and ~ 10 mM in human erythrocytes (Garay and Garrahan, 1973).

Although we obtained $d[\text{Na}^+]_i/dt$ data for $[\text{Na}^+]_i$ values larger than 20 mM in the untransfected HeLa cells and the other Jewell-Lingrel cell lines, we found that values obtained immediately after the switch from zero- K^+ buffer to zero- Na^+ buffer, when $[\text{Na}^+]_i$ was high and falling very rapidly (just after point *b* in Fig. 3), varied widely from experiment to experiment. A major reason for this variability is the inherent difficulty in computing accurate numerical derivatives ($d[\text{Na}^+]_i/dt$) of digital data when $[\text{Na}^+]_i$ is falling rapidly, because the tangent line is fitted to fewer data points. We thus chose to restrict the analysis to $[\text{Na}^+]_i$ ranges in which the $d[\text{Na}^+]_i/dt$ data were reproducible. Therefore, in each experiment, we identified the inflection point in the $d[\text{Na}^+]_i/dt$ versus $[\text{Na}^+]_i$ tracing, and analyzed only data for $[\text{Na}^+]_i$ values lower than the inflection point. This approach had the practical effect of limiting the range of usable data to $[\text{Na}^+]_i$ values from 0 through 30–35 mM.

Measuring $[\text{Na}^+]_i$ Dependence of Flux Attributable to Exogenous Isoforms in Cells Transfected with Ouabain-resistant Pumps

For these experiments, we modified the above protocol to exploit the large difference in ouabain affinities between the native and the transfected Na-K pumps. We loaded cells with Na^+ as above, and then exposed them to a 0- Na^+ /5-mM- K^+ buffer (solution 2). In each case,

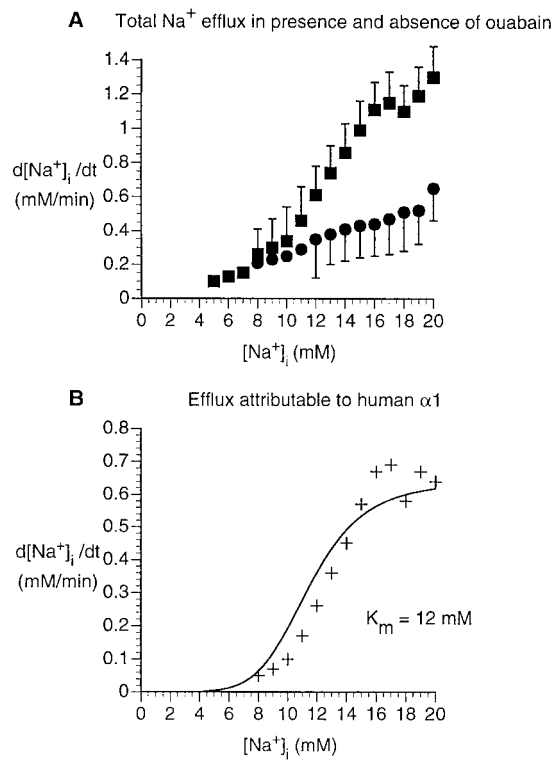


FIGURE 4. Na^+ efflux in untransfected HeLa cells. (A) Dependence of rate of $[\text{Na}^+]_i$ decrease on $[\text{Na}^+]_i$, computed from experiments similar to that shown in Fig. 3. Data were obtained either in the absence of ouabain (\blacksquare , $n = 7$), or with 1 μM or 1 mM ouabain added to the zero- K^+ and zero- Na^+ buffers (\bullet , $n = 9$). $d[\text{Na}^+]_i/dt$ was calculated from the time course of $[\text{Na}^+]_i$ decline in the presence of zero- Na^+ buffer (Fig. 3, segment *bc*). These calculations were performed at $[\text{Na}^+]_i$ intervals of 1 mM. Statistical analysis (see METHODS) shows that the two curves are significantly different ($P < 0.002$). (B) $[\text{Na}^+]_i$ dependence of the Na^+ efflux attributable to the Na-K pump. The $d[\text{Na}^+]_i/dt$ in the presence of ouabain is interpreted as the Na^+ leak, whereas the $d[\text{Na}^+]_i/dt$ in the absence of ouabain is interpreted as sum of the leak and the flux mediated by the Na-K pump. Thus the difference, shown here, represents the pump-mediated efflux. Data were fit with a cooperative model with variable Hill coefficient (Eq. 2).

we performed these two maneuvers either in the absence of ouabain, or at low (1 μM) or high (4 mM) ouabain concentrations. We interpreted the corresponding Na^+ effluxes, calculated as $d[\text{Na}^+]_i/dt$, as follows:

In presence of:	Na efflux represents:
high [ouabain]	Na^+ leak
low [ouabain]	leak + resistant pump rate
no ouabain	leak + total pump rate

Here, “resistant” pumps refers to the transfected rat isoforms, whereas the “sensitive” pumps are the human $\alpha 1$ isoforms endogenously expressed in HeLa cells. In a generalization of the above approach (Figs. 3 and 4), we subtracted high from low ouabain data, obtaining the

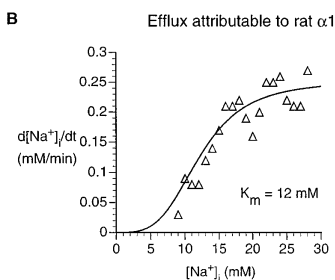
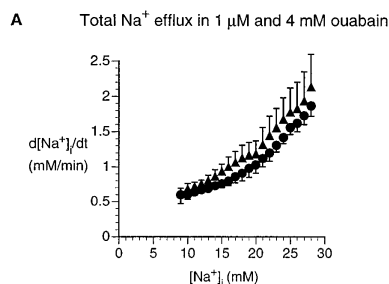


FIGURE 5. Na^+ efflux in HeLa cells transfected with rat $\alpha 1$. (A) Dependence of rate of $[\text{Na}^+]_i$ decrease on $[\text{Na}^+]_i$, computed from experiments similar to that shown in Fig. 3, but for cells transfected with rat $\alpha 1$. Data were obtained in the presence of either 1 μM ouabain (\blacktriangle , $n = 5$) or 4 mM ouabain (\bullet , $n = 3$). $d[\text{Na}^+]_i/dt$ was calculated as in Fig. 4. Statistical analysis (see METHODS) shows that, whereas the slopes of the two curves (when logarithmically transformed) are not significantly different, the in-

tercepts are significantly different ($P < 0.001$). (B) $[\text{Na}^+]_i$ dependence of the Na^+ efflux attributable to the rat $\alpha 1$ pump. The $d[\text{Na}^+]_i/dt$ in the presence of 4 mM ouabain is interpreted as the Na^+ leak, whereas the $d[\text{Na}^+]_i/dt$ in the presence of 1 μM ouabain is interpreted as sum of the leak and the efflux mediated by the ouabain-resistant rat $\alpha 1$ pump (because 1 μM ouabain inhibits the endogenous HeLa-cell human $\alpha 1$ pumps). Thus the difference, shown in B, represents the efflux mediated by the rat $\alpha 1$ pump. The data were fit with a cooperative model with variable Hill coefficient (Eq. 2).

activation curve of the resistant (i.e., rat) Na-K pump isoform as a function of $[\text{Na}^+]_i$. Similarly, subtracting low from zero ouabain data yields the activation curve of the sensitive (i.e., human) Na-K pump isoform.

Rat $\alpha 1$

We studied cells transfected with the rat $\alpha 1$ isoform, which is naturally ouabain resistant, at low and high ouabain concentrations (Fig. 5 A). The difference between these two Na^+ effluxes represents the activity of the transfected rat pumps. As shown in Fig. 5 B, the

maximal Na^+ efflux attributable to the transfected rat $\alpha 1$ pumps was ~ 0.25 mM/min, considerably less than that of the native human $\alpha 1$ pumps in untransfected HeLa cells, ~ 0.64 mM/min (Fig. 4). This difference may be related to a relatively small number of exogenous pumps. The exogenous rat $\alpha 1$ pumps had apparent K_m for $[\text{Na}^+]_i$ of 12 mM, similar to that for the endogenous human $\alpha 1$ pumps in the transfected HeLa cells (Table III).

Rat $\alpha 2^*$

To study the rat $\alpha 2^*$ isoforms, we again compared experiments in $\alpha 2^*$ -transfected cells exposed to low and high ouabain concentrations (Fig. 6 A). The activation curve for Na^+ efflux attributable to this mutated rat isoform had an apparent K_m of 22 mM (Fig. 6 B). The maximal Na^+ efflux attributable to the transfected rat $\alpha 2^*$ isoform was 1.8 mM/min, considerably greater than that for the transfected rat $\alpha 1$ (Table III).

Rat $\alpha 3^*$

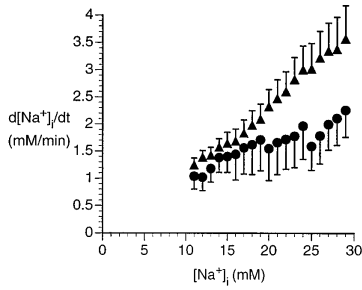
We studied cells transfected with $\alpha 3^*$ (Fig. 7, A and B) in the same manner as for $\alpha 1$ and $\alpha 2^*$. In the case of the $\alpha 3^*$ -transfected cells, the computed V_{max} for the efflux attributable to $\alpha 3^*$ was ~ 2.5 mM/min. More significantly, the activation curve (Fig. 7 B) is substantially shifted to the right, compared with the other isoforms studied. Fitting the $\alpha 3^*$ -flux data in Fig. 7 B using a cooperative model with a Hill coefficient of three (Eq. 1) (Garay et al., 1973) yielded a K_m of ~ 44 mM. This fit, however, is problematic for two reasons. First, the efflux is not above zero until $[\text{Na}^+]_i$ is above ~ 16 mM. Second, although there is a suggestion of saturation at an $[\text{Na}^+]_i$ of ~ 40 mM, we were unable to obtain data at higher $[\text{Na}^+]_i$ values. Studying the same $\alpha 3^*$ -transfected cells with a different approach, Munzer et al. (1994) also noted that the $\alpha 3^*$ flux did not begin to increase until $[\text{Na}^+]_i$ was above ~ 22 mM. Their flux data, plotted as a function of $[\text{Na}^+]_i$, did not appear to saturate.

TABLE III
Curve Fits

	Human $\alpha 1$ in untransfected HeLa cells	Human $\alpha 1$ in HeLa cells transfected with $\alpha 3^*$	Rat $\alpha 1$	Rat $\alpha 2^*$	Rat $\alpha 3^*$
Fit with variable Hill coefficient					
Apparent K_m (mM)	12	13	12	22	33
V_{max} (mM/min)	0.64	0.36	0.25	1.8	2.5
Hill coefficient	6.0	5.9	3.5	4.8	6.8
Fit with fixed Hill coefficient = 3					
Apparent K_m (mM)	17	16	12	33	44
V_{max} (mM/min)	1.2	0.52	0.27	3.7	3.8

(Top) Data were fitted to a highly cooperative model with variable Hill coefficient (Eq. 2). Curve fits in Figs. 4–9 reflect this model. (Bottom) Data were fit to highly cooperative model with fixed Hill coefficient of 3 as explained in text (Eq. 1).

A Total Na⁺ efflux in 1 μM and 4 mM ouabain



B Efflux attributable to rat α2*

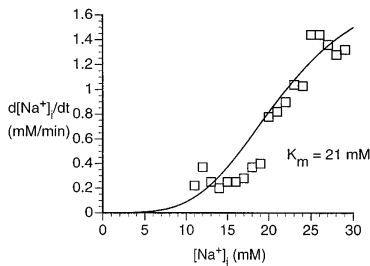


FIGURE 6. Na⁺ efflux in HeLa cells transfected with rat α2*. (A) Dependence of rate of [Na⁺]_i decrease on [Na⁺]_i, as in Fig. 5 (1 μM ouabain, ▲, *n* = 7; 4 mM ouabain, ●, *n* = 7). Statistical analysis shows that the two curves are significantly different (*P* < 0.001). (B) Na⁺ efflux attributable to the rat α2* pump was calculated as in Fig. 5.

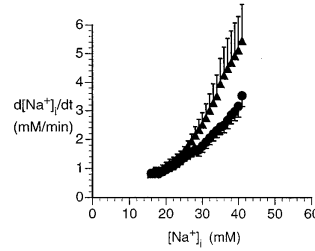
Because our approach generated many more data points than that of Munzer et al. (1994), however, we were able to fit our data using a model with a variable Hill coefficient. The result was a much better fit than the model with a fixed Hill coefficient, and a *K_m* of ~33 mM.

For the sake of comparison, we replotted the activation curves for the three transfected isoforms on the same axes (Fig. 8), normalizing the maximal pump flux for each isoform to unity. The *K_m* values are in the sequence ~12, ~22, and ~33 mM for the rat α1, α2*, and α3* isoforms, respectively. To assess whether these values represent true interisoform differences in affinity for [Na⁺]_i, we tested the Na⁺ efflux curves for human α1, rat α2*, and rat α3* (Figs. 4 B, 6 B, and 7 B) against the corresponding curve for rat α1, using chi-square analysis for heterogeneity. Rat α2* and rat α3* were significantly different from rat α1 (*P* values < 0.001 and 0.005, respectively), but human α1 was not significantly different from rat α1 (*P* > 0.75). We also computed asymptotic standard errors of estimate for the affinity constants, and tested whether they were significantly different. The results were as follows: rat α2* vs. rat α1, *P* < 0.005; rat α3* vs. rat α1, *P* < 0.0005; rat α3* vs. rat α2*, *P* < 0.0005; human α1 vs. rat α1, not significant.

Human α1 in HeLa Cells Transfected with Rat α3*

In the cells transfected with rat α3*, we also measured the efflux attributable to the endogenous human α1 isoform. The apparent *K_m* for the human α1 isoform was 13 mM (Fig. 9 A and Table III), in close agreement with the *K_m* for human α1 measured in untransfected HeLa cells, 12 mM. The maximal Na⁺ efflux attribut-

A Total Na⁺ efflux in 1 μM and 4 mM ouabain



B Efflux attributable to rat α3*

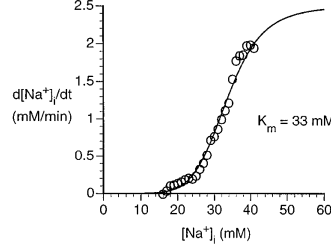


FIGURE 7. Na⁺ efflux in HeLa cells transfected with rat α3*. (A) Dependence of rate of [Na⁺]_i decrease on [Na⁺]_i, as in Fig. 5 (1 μM ouabain, ▲, *n* = 10; 4 mM ouabain, ●, *n* = 10). Statistical analysis shows that the two curves are significantly different (*P* < 0.001). (B) Na⁺ efflux attributable to the rat α3* pump was calculated as in Fig. 5.

able to the α1 isoform in α3*-transfected cells (◇ in Fig. 9 B) was only 56% as great as in the control HeLa cells (+ in Fig. 9 B), suggesting that transfection of (mutated) α3 pumps leads to a compensatory decrease in endogenous α1 pumps. Indeed, in the HeLa cells transfected with rat α2*, the Na efflux attributable to the native α1 was unmeasurably low (not shown). As shown in Fig. 9 C, the *K_m* values were indistinguishable for human α1 in untransfected HeLa cells, for human α1 in α3*-transfected HeLa cells, and for rat α1.

Estimation of Maximal Turnover Rate per Pump Site

Because of differences in transfection efficiency and, perhaps, in maximal intrinsic pumping rate, one must be cautious in comparing the activation curves of the rat α isoforms shown in B of Figs. 6–8. We thus sought to measure the number of pump sites of each isoform type in the transfected cells. Although pump-site quantitation is customarily done using ³H-ouabain binding, this technique is not feasible for rat α1, α2*, and α3* pumps because they are highly ouabain resistant. Instead, we measured the total amount of Na-K α-isoform protein using quantitative immunoblotting with anti-LEAVE (see METHODS), an antibody raised against an epitope common to the three Na-K pump α isoforms (Pressley, 1992).

The immunoblots showed a single major band with an apparent molecular mass of 100 kD, as previously described (Pressley, 1992). Using densitometric scanning referred to a standard curve on each blot, we found that the total Na-K pump immunoreactivity in microsomes from the α2*-transfected HeLa cells was 97% of that in untransfected HeLa cells (*n* = 3 determinations) and that of α3*-transfected cells was 93% of

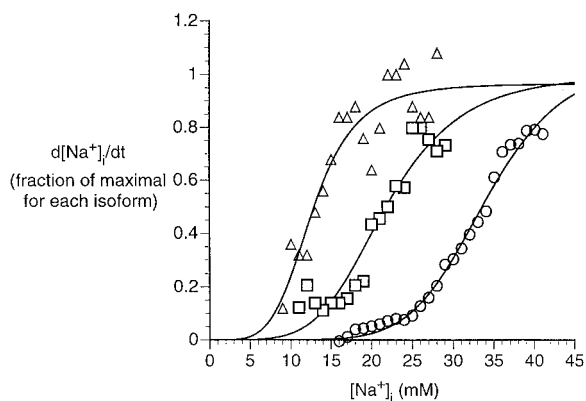


FIGURE 8. Comparison of apparent Na^+ affinities of three rat α isoforms. Because of differences in transfection efficiency and, perhaps, in maximal intrinsic pumping rate, the activation curves of the rat α isoforms shown in *B* of Figs. 5–7 are difficult to compare. Hence we normalized the maximal pump flux for each isoform to 1 and plotted the data for the three isoforms on the same axes. Δ , rat $\alpha 1$; \square , rat $\alpha 2^*$; \circ , rat $\alpha 3^*$.

that in untransfected HeLa cells ($n = 2$). The pump-mediated Na^+ efflux we measured in $\alpha 2^*$ -transfected cells was $\sim 2.8\times$ that in untransfected HeLa cells, and the efflux in $\alpha 3^*$ -transfected cells was $\sim 4.5\times$ that in untransfected HeLa cells. If we assume that the antibody recognizes all pumps equally well, and that an equal fraction of all immunoreactive pumps is at the plasma membrane in all cell lines, then it would appear that the transfected pumps have a higher intrinsic turnover rate than the native pumps. For example, if the activity per human $\alpha 1$ is the same in untransfected and $\alpha 3^*$ -transfected HeLa cells, the maximal activity per rat $\alpha 3^*$ pump would be 8.7-fold greater than that of human $\alpha 1$.

DISCUSSION

Advantages of Spectrofluorometry for Measuring Na^+ Fluxes Mediated by the Na-K Pump

We have demonstrated that the Na^+ -sensitive dye SBFI can be used to measure the Na^+ flux attributable to specific Na-K pump α isoforms, as a function of $[\text{Na}^+]_i$, in intact transfected cells. A special advantage of our spectrofluorometric approach is that we were able to study Na-K pump fluxes in a sided preparation with independent control over $[\text{Na}^+]_i$ and $[\text{Na}^+]_o$. Additional advantages are that $[\text{Na}^+]_i$ and $d[\text{Na}^+]_i/dt$ were obtained simultaneously, and with good resolution for both $[\text{Na}^+]_i$ and time. Measuring the fluxes at specific $[\text{Na}^+]_i$ values is crucial because the Na-K pump flux is steeply $[\text{Na}^+]_i$ dependent, and because many experimental interventions change both pump flux and $[\text{Na}^+]_i$ simultaneously. To our knowledge, this is the first study of specific Na-K-pump isoforms in a sided preparation

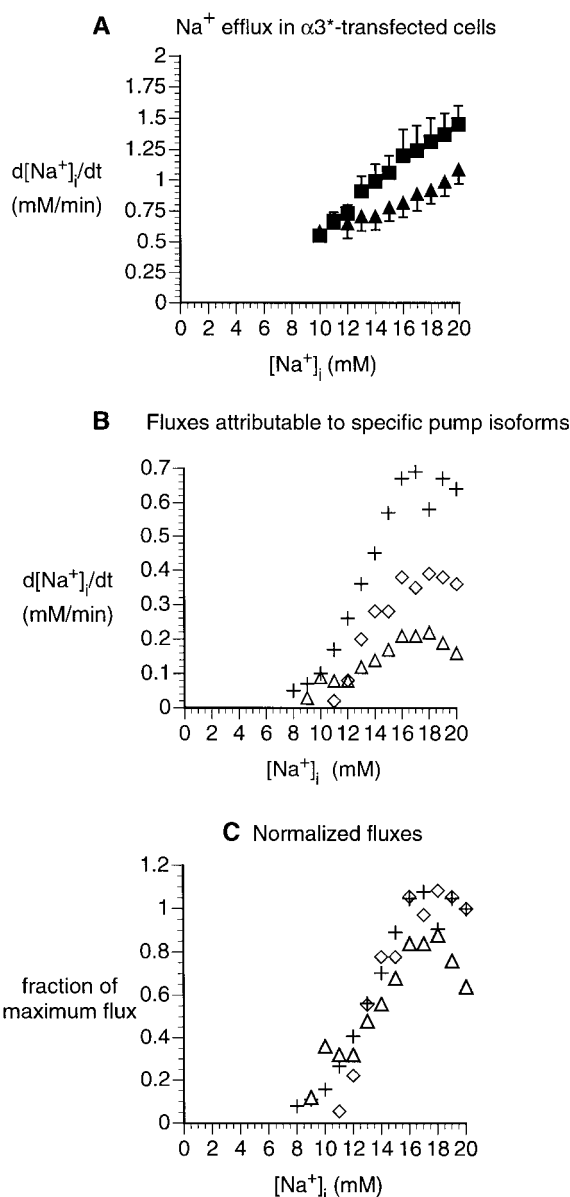


FIGURE 9. Comparison of Na^+ effluxes attributable to human or rat $\alpha 1$ pumps in three cell lines. (A) Dependence of rate of $[\text{Na}^+]_i$ decrease on $[\text{Na}^+]_i$, computed for $\alpha 3^*$ -transfected cells, from experiments similar to that shown in Fig. 7. Data were obtained either in the absence of ouabain (\blacksquare , $n = 7$), or with $1 \mu\text{M}$ ouabain (\blacktriangle , $n = 10$). Statistical analysis (see METHODS) shows that the two curves are significantly different ($P < 0.05$). (B) $[\text{Na}^+]_i$ dependence of the Na^+ efflux attributable to the various $\alpha 1$ pumps. \diamond represents the Na^+ efflux attributable to the ouabain-sensitive pumps (native human $\alpha 1$ pumps) in the $\alpha 3^*$ -transfected HeLa cells. These values are computed from the data in A, assuming that the $d[\text{Na}^+]_i/dt$ in $1 \mu\text{M}$ ouabain is the sum of the leak and the flux mediated by the ouabain-resistant rat $\alpha 3^*$ pump transfected into the cells, and that the $d[\text{Na}^+]_i/dt$ in the absence of ouabain is the sum of the leak and the efflux mediated by both the native and transfected pumps. The $[\text{Na}^+]_i$ dependencies of human $\alpha 1$ in untransfected HeLa cells (+, replotted from Fig. 4) and of rat $\alpha 1$ (Δ , replotted from Fig. 5) are shown for comparison. (C) $[\text{Na}^+]_i$ dependence of the Na^+ efflux attributable to the various $\alpha 1$ pumps, with effluxes for each pump normalized to unity.

permitting independent control of $[Na^+]_i$ and $[Na^+]_o$, the first systematic investigation of pump-mediated Na^+ fluxes by means of spectrofluorometry, the first use of SBFI to study $[Na^+]_i$ in transfected cells, and the first validation of an in vivo SBFI calibration using an independent method for measuring $[Na^+]_i$.

Summary of Results

Implications of K_m values. Our data indicate that the rat $\alpha 3^*$ isoform has a K_m for $[Na^+]_i$ that is more than three-fold greater than that of rat $\alpha 1$. Rat $\alpha 2^*$ has an intermediate affinity. The Na-K pump ought to be most effective at clamping $[Na^+]_i$ when the K_m of the pump is close to $[Na^+]_i$. The existence of an $\alpha 3$ isoform with such a high K_m might help prevent $[Na^+]_i$ from rising excessively in cells subjected to high Na^+ influxes (e.g., excitable cells during electrical activity). This advantage, however, would accrue only if the extra expression of $\alpha 3$ increased the total number of Na-K pumps, or if the $\alpha 3$ pumps had a higher turnover number. Alternatively, expression of $\alpha 3$, with its high K_m , would cause the cell to have a high steady state $[Na^+]_i$, as we indeed observed. Such a high $[Na^+]_i$ might be advantageous because it would reduce ATP use.

Our data also indicate that the K_m values for the endogenous human $\alpha 1$ and transfected rat $\alpha 1$ are almost identical, even though the former is $1,000\times$ more sensitive to ouabain than the latter. This similarity in K_m values is consistent with the notion that the structural elements of the pump that determine ouabain binding (Lingrel and Kuntzweiler, 1994) can be modified independently of those that determine the K_m for internal Na^+ .

Inverse relation between expression of endogenous and transfected pumps. We also found that transfection of rat $\alpha 2^*$ or $\alpha 3^*$ pumps leads to a compensatory decrease in the activity of endogenous human $\alpha 1$. These observations are consistent with previous evidence that the total number of Na-K pump units expressed per cell is tightly regulated (Boardman et al., 1972; Fambrough et al., 1987; Pressley, 1992).

Earlier Work on Na^+ Affinity of Pump Isoforms

To study the K_m of the Na-K pump for intracellular Na^+ ($K_m[Na^+]_i$), it would be best to use a "sided" preparation in which one could control $[Na^+]_i$ and $[Na^+]_o$ independently, and thus vary the parameter of interest (i.e., $[Na^+]_i$) while leaving other relevant parameters (e.g., $[Na^+]_o$) fixed. Such an approach has previously been used only to study the native Na-K pumps of erythrocytes (e.g., Garay et al., 1973) and squid axons (e.g., Brinley and Mullins, 1967).

Pump isoforms in native tissues. Varying $[Na^+]_i$ while equalizing it to $[Na^+]_o$ yields what might be called an "unsided" K_m for Na^+ , $K_m[Na^+]_u$. ^{86}Rb fluxes in rat-brain synaptosomes, obtained in the presence of low

and high ouabain concentrations, yielded apparent $K_m[Na^+]_u$ values of 17 mM for $\alpha 1$ and 49 mM for $\alpha 2$ plus $\alpha 3$ (Brodsky and Guidotti, 1990), consistent with our $K_m[Na^+]_i$ data. In contrast, ATPase assays on rat axolemma, which predominantly have the $\alpha 3$ isoform (Sweadner, 1985) and the pineal gland, which is predominantly $\alpha 3\beta 2$ (Shyjian et al., 1990), yielded $K_m[Na^+]_u$ values that were lower than for kidney, which is predominantly $\alpha 1$.

Pump isoforms in transfected nonmammalian cells. Several groups have studied Na-K pump physiology by transfecting cloned Na-K pump isoforms into mammalian cells. However, measuring $K_m[Na^+]_i$ in such cells is complicated by the presence of the endogenous $\alpha 1$ isoform in mammalian cell lines. One way of circumventing this problem is to express mammalian $\alpha 1$ in nonmammalian cells that have little endogenous Na-K pump activity, such as Sf-9 insect cells (Blanco et al., 1993) or yeast (Horowitz et al., 1990). Thus, in membranes obtained from yeast cells transfected with $\alpha 1\beta 1$, the $K_m[Na^+]_u$ for the Na,K ATPase activity was 9.3 mM, similar to the $K_m[Na^+]_i$ value we obtained for intact, untransfected HeLa cells, which also express $\alpha 1\beta 1$ (Eakle et al., 1995).

Jewell-Lingrel cell lines: $K_m[Na^+]_u$ in membrane preparations. Jewell and Lingrel (1991) circumvented the problem of endogenous Na-K pumps by transfecting ouabain-resistant pumps into cells whose native pumps are ouabain sensitive. Using differential ouabain sensitivity in crude plasma membranes from such cells, those authors found that the $K_m[Na^+]_u$ for the Na,K-ATPase activities of the transfected rat $\alpha 1$, $\alpha 2^*$, and $\alpha 3^*$ isoforms were 3.5, 3.3, and 7.8 mM, respectively.³ Although these values are substantially lower than ours, both studies conclude that the K_m for $\alpha 3$ is greater than that for the other isoforms. The difference in magnitude may reflect the use of an unsided vs. a sided preparation.

Jewell-Lingrel cell lines: immunoblotting and total Na,K-ATPase activity. Putnam et al. (1994) immunoblotted the Jewell-Lingrel cell lines with an antibody that cross-reacts with human and rat $\alpha 1$, and also measured Na,K-ATPase activity. They found that transfecting with rat $\alpha 1$ or $\alpha 2^*$ decreases expression of immunoreactive $\alpha 1$, and that transfecting with rat $\alpha 1$, $\alpha 2^*$, or $\alpha 3^*$ reduces ATPase activity of endogenous $\alpha 1$. These results are consistent with our efflux measurements. Putnam et al. also found that all three transfected lines had about half the total Na,K ATPase activity of untransfected HeLa cells. This result is at odds with our data, which show that the total Na-K pump flux was higher in the $\alpha 2^*$ - and $\alpha 3^*$ -transfected lines. The discrepancy could reflect either differences in assay methods (i.e., ATPase activity vs. flux), or in pump localization (total pumps vs. those in the plasma membrane).

³These results came from a three-site cooperative model.

TABLE IV

Comparison between Pump-mediated Na^+ Efflux Data in Jewell-Lingrel Cell Lines, as Measured in This Study and in Previous Publications

Isoform transfected	K_m for $[\text{Na}^+]_i$			Relative V_{\max}		Basal $[\text{Na}^+]_i$	
	Munzer et al., 1994	Therien et al., 1996	Present study	Munzer et al., 1994	Present study	Munzer et al. 1994	Present study
None	Not done	Not done	12	Not done	—	9.3	18.6
$\alpha 1$	17.6	7.1	12	—	Not done	15.7	11.5
$\alpha 2^*$	19.7	Not done	22	1.0	2.8	19.7	13.4
$\alpha 3^*$	63.5	30.3	33	2.7	4.5	27.5	50.3

Values for basal $[\text{Na}^+]_i$ and K_m are in millimolar Na^+ . “Relative V_{\max} ” is defined as the ratio (V_{\max} in the given cell line)/(V_{\max} of $\alpha 1$ -transfected HeLa cells) for Munzer et al. (1994) data, or as (V_{\max} in the given cell line)/ (V_{\max} of untransfected HeLa cells) for present study. V_{\max} values were not normalized for pump number or transfection efficiency. Munzer et al. (1994) derived K_m and V_{\max} values from a fit with fixed Hill coefficient of 3, whereas we performed fitting with a variable Hill coefficient (Eq. 2 and Table III). Munzer et al. (1994) measured basal $[\text{Na}^+]_i$ of the Jewell-Lingrel cell lines under normal culture conditions, using flame photometry; we used SBFI.

Jewell-Lingrel cell lines: $K_m[\text{Na}^+]_u$ in intact cells in presence of monensin. Munzer et al. (1994) studied the $[\text{Na}^+]_i$ dependence of the pump-mediated $^{86}\text{Rb}^+$ influx, using monensin as a tool to change $[\text{Na}^+]_i$ in response to changes in $[\text{Na}^+]_o$. $K_m[\text{Na}^+]_u$ values derived by Munzer et al. (1994) from fitting their data to a cooperative three-site model are shown in Table IV (column 2). More recently, considering the competition between intracellular Na^+ and K^+ for cytoplasmic Na^+ binding sites of the Na,K-ATPase in these transfected cells (Therien et al., 1996), this group revised its estimates for $K_m[\text{Na}^+]_u$ (column 3). Our $K_m[\text{Na}^+]_i$ values, obtained from the same Jewell-Lingrel cell lines, are similar to the $K_m[\text{Na}^+]_u$ values published by Therien et al. (1996). They concluded that the $K_m[\text{Na}^+]_u$ values are in the sequence $\alpha 1 \cong \alpha 2^* < \alpha 3^*$, whereas our data indicate that the sequence is $\alpha 1 < \alpha 2^* < \alpha 3^*$.

It is reassuring that our data and those of Munzer et al. (1994) are so similar, given the differences in methods: (a) Munzer et al. (1994) measured fluxes with ^{86}Rb , whereas we used SBFI. (b) Munzer et al. (1994) made their flux measurements in the presence of monensin, whereas we made ours in the absence of ionophores.⁴ (c) Munzer et al. (1994) did not measure $[\text{Na}^+]_i$ directly, but assumed it to be equal to $[\text{Na}^+]_o$ in their monensin-treated cells. We measured $[\text{Na}^+]_i$ directly, in real time. (d) Our sampling technique allowed us to generate many more ($[\text{Na}^+]_i$, flux) data pairs along the $[\text{Na}^+]_i$ activation curve, so that we were justified in fitting our data to a model with an additional parameter (i.e., variable Hill coefficient, as in Table IV).

Previous Work with SBFI

SBFI has been used to measure $[\text{Na}^+]_i$ in many cell types (Harootunian et al., 1989; Harrison et al., 1992;

Ahlemeyer et al., 1992). Some investigators have incidentally used SBFI to estimate an instantaneous Na^+ flux. For example, Kondo et al. (1993) Na^+ loaded cells of the thin ascending limb of Henle’s loop by removing extracellular K^+ , and then measured the initial rate of $[\text{Na}^+]_i$ decrease after restoring the K^+ . Similar experiments were reported by Harootunian et al. (1989) on fibroblasts and Borin et al. (1993) on vascular smooth-muscle cells. Because the above studies were performed only in the absence of ouabain, it was not possible to separate the Na-K pump flux from Na^+ leak. Moreover, they measured the flux at a single $[\text{Na}^+]_i$, but did not attempt to measure the dependence of the Na^+ efflux on $[\text{Na}^+]_i$.

SBFI Calibration Procedure

To increase the accuracy of our measurements, we calibrated the SBFI at two $[\text{Na}^+]_o$ values in each experiment. Several ionophore combinations have been used to calibrate SBFI in vivo; however, we know of no example in which an independent measurement of $[\text{Na}^+]_i$ was used to verify that any of these combinations truly equalizes $[\text{Na}^+]_i$ and $[\text{Na}^+]_o$. Using untransfected HeLa cells, we systematically investigated the effects of adding gramicidin, nigericin, and monensin, stepwise in all possible sequences, to a calibration buffer containing 30–90 mM Na^+ . We found that the SBFI fluorescence–excitation ratio consistently increased when any one of the ionophores was added, even when the solution already contained one or two other ionophores (not shown). Adding ionophores had little effect, on the other hand, when the calibration buffer was Na-free. These results indicate that no two of the three ionophores were adequate to totally permeabilize the cells to Na^+ . We then demonstrated, using flame photometry, that the combination of gramicidin, nigericin, and monensin (Harootunian et al., 1989; Tepel et al., 1994) does in fact equalize $[\text{Na}^+]_i$ and $[\text{Na}^+]_o$ over a wide range of $[\text{Na}^+]_o$ in HeLa cells.

⁴We used ionophores only during the calibration phase of each experiment, after the efflux measurements were made.

Potential Limitations

Generalizability of conclusions. Kinetic parameters of the Na-K pump α isoforms may depend, in part, on the tissue in which they are expressed (Therien et al., 1996). Cell-specific variations could arise from differences in such parameters as posttranslational modification, intracellular ionic composition, and membrane-lipid composition. Another variable is β -subunit isoforms. HeLa cells contain only the β_1 isoform; it is possible that the kinetic properties of a specific α could be altered by pairing it instead with β_2 or β_3 (Jaisser et al., 1992; Eakle et al., 1992; Cameron et al., 1994; Eakle et al., 1995).

Buffering and compartmentation. In our flux analysis, we assume that Na^+ buffering is negligible, as has been verified in gastric parietal cells (Negulescu and Machen, 1990). Previous studies have sometimes revealed differences between intracellular free Na^+ and total cell Na^+ content (Negulescu and Machen, 1990), or differences in $[\text{Na}^+]_i$ among intracellular compartments. Borin et al. (1993), however, using digital imaging microscopy of SBFI-loaded smooth muscle cells, observed that, although the 340/380 fluorescence ratio was not uniform throughout the cell, the $[\text{Na}^+]_i$ calculated from an in situ (i.e., intracellular) calibration was uniform.

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