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A novel assay of cell rubidium uptake using graphite furnace atomic absorption: Application to rats on a magnesium-deficient diet

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Abstract

The [Na,K]ATPase or sodium pump (SP) is a ubiquitous membrane cation transport system. Because of its potential participation in the pathophysiology of essential hypertension and cataract formation, the SP is under active investigation to detail its function and control. In this paper, we describe a novel, nonradioactive method of measuring SP ion transport activity in intact red blood cells (RBCs) using graphite furnace atomic absorption measurement of rubidium ion (Rb) uptake. This method provided sensitivity comparable to radioactive techniques, as assessed by experiments with human red blood cells (RBC) and ouabain, a known SP inhibitor, but this analytical approach eliminates the use of radioisotopes common to other Rb uptake assay methods. As a demonstration of its broader utility, the assay was used to assess the effects of dietary magnesium intake on SP-mediated ion transport in the RBCs of diet-controlled rats. Rats on 7 weeks of a magnesium-deficient (MgD) diet showed significant reductions in serum magnesium concentration, although levels remained in the lower region of the reference interval for healthy, magnesium replete animals. Red cell Rb uptake was significantly reduced in cells from the magnesium-restricted animals, demonstrating the sensitivity of Rb uptake to reduced magnesium intake, despite serum levels that fell within the reported normal range, and the utility of this Rb uptake assay in measuring physiological changes in SP function.

Keywords: Na^+; K^+ pump; Erythrocytes; Atomic absorption; Dietary magnesium; Rats

1. Introduction

The [Na,K]ATPase or sodium pump (SP) is a ubiquitous and important cell membrane, cation transport system that maintains appropriate intracellular sodium and potassium concentrations and gives rise to the cell membrane potential, which plays a particularly important role in mechanically and electrically active cells. For example, reductions in SP activity, as produced by certain drugs that specifically inhibit the SP, cause increased contractility of heart muscle [1]. Selective reductions in cation transport or inotropic activity, whether as a consequence of reduced SP numbers or accomplished by regulated phosphorylation (and inhibition) of the SP or in response to endogenous inhibitors, may contribute to such diseases as hypertension and cataract formation [2,3]. Indeed, ^{86}Rb uptake has been found to be reduced in the red blood cells (RBCs) of hypertensive individuals and in the arteries of hypertensive dogs [4,5]. The reasons for these reductions are incompletely understood; consequently, identifying changes in SP ion transport activity and the mechanisms underlying those changes is an active field of investigation [6,7]. There are two methods used commonly by researchers to monitor SP activity. One is a [Na,K]ATPase assay that measures the rate of ATP hydrolysis [8] and has primarily been used as an estimate of factors in the circulation that inhibit an isolated form of the SP. It employs a single, partially purified preparation of [Na,K]ATPase that is incubated with a test solution.

The other assay focuses on the inotropy (ion transport) of the SP and most commonly measures the rate of cation transport by the substitution of ^{86}Rb for potassium and monitoring its selective uptake into tissues or isolated cells. It represents a more direct assessment of SP functional activity in vivo [9]. However, it is a more complex and time-consuming assay.

The ^{86}Rb uptake assay is the assay of the two that can monitor the responses of activity to physiological changes, for example, to assess the effects of SP phosphorylation or

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dephosphorylation produced by hormone-receptor-mediated events [10]. It can describe changes in ion transport in tissues as a consequence of a disease or can be used in other approaches [11]. However, it uses radioactive $^{86}Rb^{+}$, a strong beta emitter, as a surrogate for K^{+}. Such radioactivity may be problematic in some settings or simply undesirable. Indeed, the use of radioactivity has become more complex, restricted and costly. In addition, $^{86}Rb^{+}$, because it is relatively short lived ($t_{0.5}$=18.65 days), has a limited shelf life. Consequently, we sought an alternative approach to the measurement of Rb uptake into cells, using nonradioisotopic Rb, and determining Rb uptake by means of a graphite furnace atomic absorption instrument.

In this study, data are provided to validate an assay using this methodology to measure ion transport into human RBCs and to assess the effects of known SP inhibitors. As a demonstration of its broader utility, the assay was than used to answer the important question of whether dietary magnesium (Mg) intake has an influence on SP inotropic activity. Low Mg has been implicated in essential hypertension and part of its influence may be through the SP [12–14]. To carry out the study, rats were placed on either a low or normal Mg intake for 7 weeks and their RBCs harvested and Rb uptake assayed. The results of those experiments are reported here.

2. Materials and methods

2.1. Isolation of human RBCs

Specimens of blood were drawn from healthy volunteers into EDTA containing tubes. The blood was maintained at 0°C until centrifuged (within 30 min) at 3000×*g* (Sorvall RT7, Kendro Laboratory Products, Newtown, CT) for 5 min at 4°C. The plasma and buffy coat were removed by aspiration. The RBCs were washed twice with 2.5 ml of a buffer (A) containing (in mmol/L) KCl 140, NaCl 10, KH_2PO_4 1.43, K_2HPO_4 1.07 and $MgCl_2$ 1.0 with a pH of 7.40 with intervening centrifugation steps to isolate the cells. The washed cells were then stored in 3–4 ml of buffer A at a final concentration of 8×10^6 to 2×10^7 RBCs/5 μl. The cells remained usable in this assay for at least 4 days based on our own and other's experience [15].

2.2. Preparation of RBCs

To individual Eppendorf tubes, 5 μl of the cell solution and 1.0 ml of buffer A were added, vortexed and centrifuged at 2000×*g* (Eppendorf Centrifuge 5401, Brinkmann Instruments, Westbury, NY) for 2 min at room temperature. The supernatant was removed and discarded. An additional 500 μl buffer A was added and the cell solution was vortexed. The tubes then were placed in a shaker at 37°C for 20 min to allow the cells to stabilize at this physiological temperature.

After 20 min, the cells were brought down by centrifugation at 2000×*g* (Eppendorf Centrifuge 5401) for 2 min at RT. The cells were then washed with 0.8 ml of a potassium-free buffer (B) containing (in mmol/L) choline chloride 149, $MgCl_2$ 1.0, MOPS 5.88 and TRIS 2.12 at a pH of 7.40. The cell solution was vortexed and centrifuged at 2000×*g* (Eppendorf Centrifuge 5401) for 4 min at RT. The supernatant was removed and discarded. To the pellet, 800 μl of buffer B was added. Then the cell solution was divided into four equal portions for later quadruplicate determinations (200 μl each). The cell solutions were centrifuged at 2000×*g* (Eppendorf Centrifuge 5401) for 4 min at RT and the supernatant was removed and discarded.

To the pellet of washed RBCs, 500 μl of a Rb solution containing (in mmol/L) NaCl 135, RbCl 6.73, Na_2HPO_4 8.10, NaH_2PO_4 1.27 and $MgCl_2$ 1.0 at a pH of 7.40 was added and the mixture vortexed. The tubes were allowed to incubate in a shaker at 37°C for 30 min. Mixing the cells and Rb solution well was critical for consistent results.

After the incubation, the RBCs were centrifuged at 2000×*g* (Eppendorf Centrifuge 5401) for 4 min at RT and the supernatant removed and discarded. Then, a 1.0-ml aliquot of ice-cold buffer B was used to wash each aliquot of cells. After this wash, the mixture was centrifuged as before to pellet the cells. The wash was repeated quickly four times and the supernatants discarded. Finally, 1.0 ml of ultrapure water was added to lyse the cells at RT. The suspension of cells was allowed to lyse for at least 30 min.

2.3. Dietary magnesium study in rats

Twenty-seven female Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed a rodent stock diet until they were 8–10 months old. Then they were divided into two diet groups, a magnesium-deficient (MgD, N=14) or a magnesium-adequate diet (MgA, N=13), and fed their respective diets and distilled water ad libitum. The diets were based on the AIN-93 purified diets for rodents [16]. The MgD and MgA diets contained 125 and 1000 ppm magnesium, respectively. Rats were housed in hanging wire cages in a temperature-controlled room with 12-h periods of light and dark. Animal procedures were approved by the Brigham Young University Animal Care and Use Committee.

After 7 weeks, rats were anesthetized (43.2 mg of sodium pentobarbital per 1000 g of body weight) and blood was drawn from the abdominal aorta and transferred to EDTA tubes and serum tubes. The EDTA tubes were immediately cooled to 4°C and the plasma quickly separated from the cells, the plasma was frozen and the cells used for the ion transport studies immediately. Serum tubes were kept at room temperature for 30 min and then centrifuged, the serum separated and was frozen immediately. For these dietary studies, because the SP is sensitive to the external Rb (K) concentration, the Rb concentration was kept constant for all assays as described above.

2.4. Measurement of serum magnesium

Serum was stored at −80°C until analyzed for magnesium by flame atomic absorption spectrophotometry (Model 306, Perkin-Elmer, Norwalk, CT).

2.5. Measurement of the Rb concentration taken up by human or rat RBCs

A graphite furnace atomic absorption instrument (5100ZL Zeeman Furnace Module, Perkin-Elmer, Norwalk, CT), equipped with a Rb lamp, was used to measure the concentration of intracellular Rb. The instrument was calibrated at the beginning of each day using a commercially available Rb atomic absorption standard solution (Aldrich, Milwaukee, WI). The uptake of Rb into RBCs was generally linear over a period of 4 h ($y=0.66x+0.53$, $R=.994$). The limit of detection for the determination of Rb was assessed by measuring progressively lower concentrations of Rb. A Rb concentration that could no longer be distinguished from background (signal to noise of 3) was considered to be the limiting concentration. A dilution curve with Rb concentrations ranging from 1 to 100 ppb was developed to assess assay linearity over the useful concentration interval.

The reproducibility of the assay was also studied by repeated measurement of a single standard as part of one run or from run to run. For these assays and the linearity study, each data point was the summation of four replicates.

For the rat magnesium diet studies, both normal and low magnesium rat red cells were included in each assay. Because of the time involved in specimen preparation, rats were sacrificed, blood collected and cells assayed on four separate days. There was some modest variation evident from assay to assay. To correct for this so as to allow for data from different days to be combined, the controls present in a single assay were averaged and then individual values for both Mg diets were referenced to that mean. The ratios were expressed as a percent of control. The results of the four assays were then combined and the statistical analysis was performed.

2.6. Measurement of the Rb concentration taken up by RBCs in the presence of a SP inhibitor

In these experiments, the initial isolation and wash steps were identical to those described above and were done without inhibitor present. Beginning with the warming and stabilization step, ouabain, a known, specific SP inhibitor, was introduced. Analogous to the experiments described above for the preparation of RBCs, the RBCs (8×10^6~2×10^7 cells) were treated in 500 μl of solution A containing a single, graded concentration of ouabain. Concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol/L were used. The cells were incubated in a shaker at 37°C for 20 min with the inhibitor present. The cells were brought down by centrifugation and washed with the choline chloride solution to remove the potassium. During these wash steps, the cells were not exposed to ouabain. Then the Rb solution was applied and the cells incubated for 30 min at 37°C. The Rb solution applied to human RBCs had ouabain present at the same concentration (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol/L) as initially applied. Some preparations of rat RBCs were also studied by ouabain inhibition. The Rb solution was then removed and the cells washed and lysed in a manner identical to that described above. During these steps, there was no ouabain present. As before, the final solution was divided for quadruplicate Rb determination by graphite furnace atomic absorption.

2.7. Statistical analyses

The data are presented as the mean±1 S.D. In general, each data point represents the average of four replicates. The linearity study was analyzed by linear regression analysis. For the reproducibility studies, the relative standard deviation (RSD) was calculated as 1 S.D./mean and expressed as a percentage. The ouabain inhibition curves were analyzed by a curve fitting program (Kaleidograph 3.5, Synergy Software, Reading, PA), which calculated the concentration of ouabain producing 50% inhibition of the SP (IC_{50}). This program also provided an estimation of error in the IC_{50} based on a single set of data points. Using this program, we determined both the 17 individual IC_{50}'s as well as combining the 17 sets of data and calculating a single IC_{50}. While this last approach provided an estimation in error based on the fitting program, we determined the mean of the 17 different determinations of IC_{50} and the RSD for the 17 individually determined IC_{50}'s and considered this a better estimate of the variability of the assay. Several of the earlier experiments were carried out with high cell number and hence higher final Rb concentrations. We compared these results to those of the later experiments using Student's *t* test as the data were normally distributed. Comparisons of serum Mg and red cell Rb uptake in blood specimens from rats on the MgA and MgD diets were made by GLM ANOVA using NCSS 7.0 (Kaysville, UT). A *P* value less than .05 was considered statistically significant.

3. Results

3.1. Atomic adsorption Rb assay linearity and sensitivity

Over the concentration range of 1 to 100 ppb, the measurement of Rb was highly linear (see Fig. 1). The square of the correlation coefficient (R^2) was .998 with a corresponding *P* value of 1.4×10^{-9}. The baseline Rb concentration for human RBC after 30 min of incubation with the Rb solution was typically 50 ppb, indicating that the common response was within the linear and established range of the assay.

3.2. Variability of the Rb measurement

Using a RbCl standard of known concentration (40.4 μg/L), the RSD within run ($N=20$) was 1.46%. Using the same Rb standard, the RSD run-to-run was 2.67% based on results from experiments done on three different days.

3.3. Assay of Rb uptake into RBCs in the presence of a SP inhibitor

Ouabain is a known and specific inhibitor of the SP. Reductions of SP activity lead to a reduced uptake of Rb into

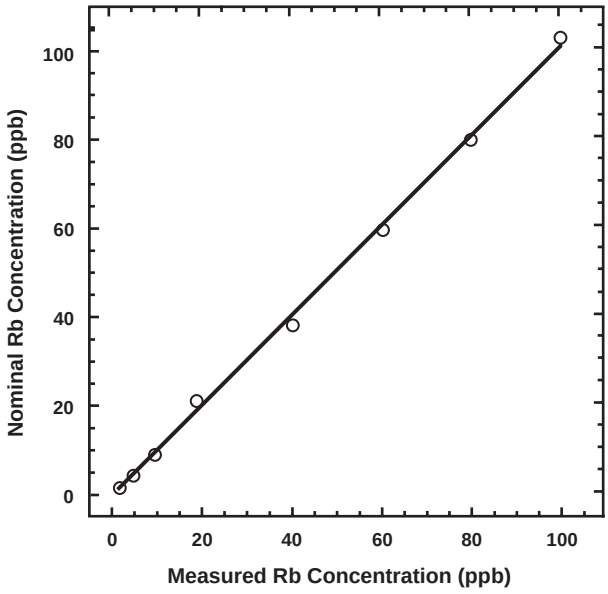

Fig. 1. Linearity of Rb measurement by graphite furnace atomic absorption. Solutions of differing nominal Rb concentrations were prepared by serial dilution of a single commercial RbCl standard as described in Materials and Methods. The performance of the assay was linear (R^2=.998, P=1.4×10^{-9}). Each data point represents four replicate determinations of the same standard by graphite furnace atomic absorbance.

the cell during the incubation period and lower intracellular concentrations of Rb. As a validation of the Rb uptake assay, RBCs were incubated with graded concentrations of ouabain prior to and during their being incubated with Rb. A plot of the ouabain concentration-dependent inhibition of Rb uptake into intact red cells is provided in Fig. 2. The uninhibited Rb uptake activity was calculated to be 1.33±0.11 pmol Rb/min/10^6 cells but fell to 0.53±0.08 pmol Rb/min/10^6 cells, with a saturating concentration of ouabain causing 60.6% inhibition of the Rb uptake. As can be seen in Fig. 2, increasing concentrations of ouabain resulted in a typical dose-inhibition profile with a calculated IC_{50} of 5.4±0.3×10^{-9} mol/L, which is in good agreement with its determination by the radioactive method [17–19]. The standard error bars are based on 17 experiments, with each data point being the mean of four determinations per single assay. When the individual IC_{50}'s were determined, their mean was 6.1±3.4×10^{-9} mol/L. The RSD of the individual IC_{50} values then was 55% indicating that the variability in this assay is primarily due to the difficulty of working with cells and is large relative to the

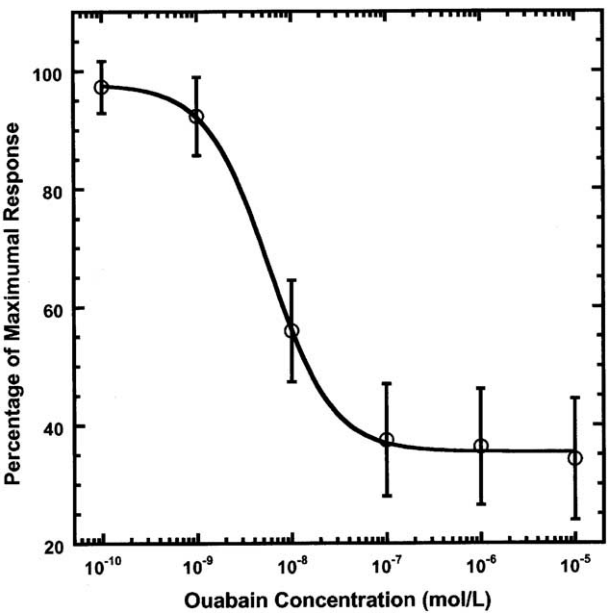

Fig. 2. Effects of ouabain on Rb uptake into RBCs. Rubidium uptake into human RBCs was determined in the presence of graded concentrations of a known SP inhibitor ouabain. Each data point represents the mean of 17 sets of experiments, each done in quadruplicate. Because the actual Rb concentrations varied somewhat from experiment to experiment due to differences in cell number, the maximal Rb concentration for each set of data was assigned the value of 100, and all the other concentrations were related to it by means of the formula C/C_{max}*100.

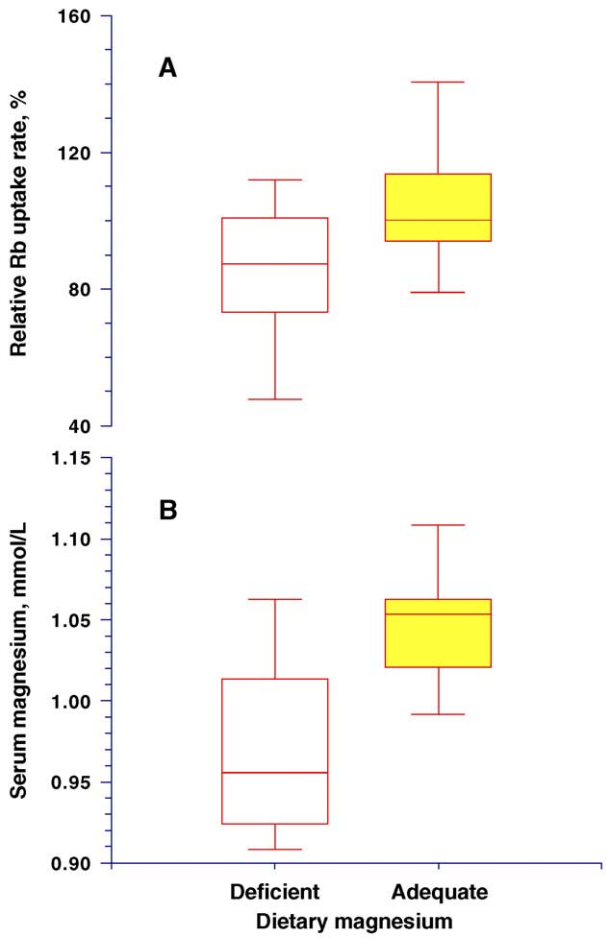

Fig. 3. Box plots of the effect of magnesium deficiency on the normalized rat red cell Rb uptake rate (A) and rat serum magnesium concentration (B). The bottom and top of each box represent the 25th and 75th percentiles. The median is the horizontal line within each box. The adjacent lines demonstrate the variance of the data. The left boxes represent data for Mg-deficient dams and the right boxes represent data for Mg-adequate dams. The normalization of the Rb uptake data and the statistical analyses are described in Materials and Methods. The actual means and S.E. values are provided in the Results.

variability of the instrumental determination of the Rb. To put this into perspective, we have carried the [Na,K]ATPase assay and measured ouabain inhibition of ATP hydrolysis using a purified preparation of [Na,K]ATPase (data not shown). The RSD of this assay for 27 sets of data was 48%. While these RSD values are high, they are probably indicative of the variability inherent in functional bioassays in general. Note that not all Rb entry into the cell is by means of the SP, in this assay using human RBCs ~40% of Rb uptake could not be inhibited by ouabain.

The number of cells used for the assay was also considered. When higher cell numbers (4.5×10^7 to 6.8×10^7 cells) were used in the ouabain inhibition experiments, the IC_{50} was $7.3\pm3.1\times10^{-9}$ mol/L ($N=8$), whereas the same parameter determined with lower cell number (8×10^6 to 2×10^7 cells) was $5.1\pm3.4\times10^{-9}$ mol/L, which was not significantly different, even though the RSD was somewhat lower for the high cell number experiments.

Even for the highest ouabain concentrations where the intracellular Rb was most reduced, the Rb concentrations still fell within the linear range of the assay and indicate that the assay is adequately sensitive for these determinations based on the numbers of cells used.

3.4. Influence of dietary Mg on rat RBC SP activity

There were no differences in the beginning or sacrifice weights of the two groups of animals participating in the Mg diet study. Serum Mg significantly declined 7.6% from 1.05 ± 0.03 mmol/L in the MgA group to 0.97 ± 0.05 in the MgD group ($F=21.35$, $P=.0001$). The uninhibited rat red cells had an average Rb uptake of 3.2 pmol Rb/min/10^6 cells, which is more than 2.5 times the rate found for human red cells. The ouabain inhibitable, SP-mediated Rb uptake was determined to be 44.8%, somewhat lower than that found for human red cells. The normalized Rb uptake of rat red cells was significantly reduced by 18.2% from $104.7\pm15.5\%$ in the MgA group to $85.6\pm17.4\%$ in the MgD group ($F=9.04$, $P=.0057$). See Fig. 3.

4. Discussion

These results demonstrate that we have developed a red cell Rb uptake assay that does not require radioactive Rb. While the ^{86}Rb uptake assay has proven to be a valuable tool in the characterization of the SP and its modifiers, the use of high-energy radioactive isotopes may be undesirable, restricted or expensive. Moreover, the "Rb" used in this assay is stable, which may be important if the assay is not carried out frequently. Radiolabeled Rb 86 has a half-life of 18.6 days. These experiments establish that the nonradioactive assay is sufficiently sensitive to allow for the same types of experiments to be conducted as with the radioisotope method with as good results. Sensitivity could be easily increased by using either increased cell number or by longer incubation times with the Rb or both. This new assay provides an alternative and attractive research approach to the study of the SP in physiological and pathologic conditions. As a demonstration of the assay's utility, we studied two groups of rats on two diets, one with normal amounts of dietary magnesium and one with substantially reduced amounts of magnesium present. After 7 weeks on the diet, the animals on the low magnesium diet had a reduction in the serum magnesium concentration. Nevertheless, their magnesium concentrations were still in the low normal range. Despite this, the transport of potassium (as measured by Rb uptake) into their RBCs was significantly reduced. The mechanisms resulting in this decrease are unknown, but include the possibility of a reduction in SP number or its activity in these animals. It cannot be explained directly by differences in ion concentration in the serum because the red cell assay was carried out in a physiological buffer solution and the assay conditions were the same for cells from both sets of animals. It is worth noting that reduced magnesium and reduced SP transport have both been associated with hypertension [2,4,5,12,13] and have been proposed as having a mechanistic role in the hypertensive process. It would be of interest and potential importance to determine whether dietary differences in humans give rise to similar differences in red cell Rb uptake.

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