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Development of an HTS Assay for Na^+, K^+-ATPase Using Nonradioactive Rubidium Ion Uptake

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Abstract: A high-throughput screening (HTS) assay was developed for the Na^+,K^+-ATPase channel in order to study rubidium uptake as a measure of the functional activity and modulation of this exchanger. The assay uses elemental rubidium as a tracer for K^+ ions. Three cell lines were used to study the exchanger, and the assay was performed in a 96-well microtiter plate format. Rb^+ uptake was carried by the CHO-K1 cells at 37°C; the maximum ion influx was at 80 min of incubation of the cell line in the medium containing 5.4 m*M* RbCl. The cells were incubated in Rb^+ uptake buffer (5.4 m*M*) and with the pump blocker ouabain for 1, 2, and 3 h, respectively. A complete block of the Rb^+ uptake was observed with a 5 m*M* concentration of ouabain for all the three time intervals. The ouabain 50% inhibitory concentration (IC_{50}) value for CHO-K1 cell line ATPase was observed to be 298 μM after 3 h of incubation. In addition, IC_{50} values of 94 and 89 μM were observed at 30 min of incubation, indicating that the protocol shows reproducible results. A Z' factor higher than 0.7 was observed in the assays. These studies extend the profile of Na^+,K^+-ATPases and demonstrate the feasibility of this HTS assay system to screen for compounds that pharmacologically modulate the function of Na^+,K^+-ATPase.

Introduction

HUMAN NA^+,K^+-ATPASE, also known as the Na^+,K^+ pump, is emerging as an important drug target ever since cardioglycosides such as digoxin and digitoxin began to be used for the treatment of congestive heart failure and related conditions.[1–3] Similarly, C-induced phosphorylation of cardiac α-1 Na^+,K^+-ATPase is a likely target for treatment with antihypertensive compounds that have protein kinase inhibitory activity.[4] Additionally, other types of ATPases like gastric H^+,K^+-ATPase, the sarcoplasmic reticulum Ca^{2+}-ATPase, the plasma membrane Ca^{2+}-ATPase, and the *Neurospora* H^+-ATPase are attractive pharmaceutical targets.[5–8]

The electrogenic nature of the Na^+,K^+-ATPase has been described by the hydrolysis of one ATP molecule; it actively exports three Na^+ molecules with an import of two K^+ ions. In this way, Na^+,K^+-ATPase regulates ion and voltage gradients, and it has a fixed Na^+:K^+ stoichiometry of 3:2. This particular pump maintains electrochemical gradients responsible for driving the transport of many ions and nutrients across the plasma membrane. It is also responsible for other cellular functions, such as transmembrane electric potential of the cell maintenance, cell volume, and transepithelial movement of salt.[9]

The Na^+,K^+-ATPase is a member of a large family of enzymes known as the P2-type ATPases.[10] This class of proteins couples the hydrolysis of ATP to the translocation of cations across the membrane and is spread throughout both plant and animal kingdoms. The catalytic subunit of the Na^+,K^+-ATPase is expressed in various isoforms (α_1, α_2, α_3) that are detectable by specific antibodies. The functional enzyme is composed of an α and β subunits; families of isoforms for both subunits exist.

Activity of Na^+,K^+-ATPase has been determined by both non–cell-based and cell-based assays. In the former category, the activity of Na^+,K^+-ATPase has been determined by ATP hydrolysis using purified preparations

Aurora Biomed, Inc., Vancouver, BC, Canada.

ABBREVIATIONS: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; HTS, high-throughput screening; IC_{50}, 50% inhibitory concentration; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate.

of the enzyme; the activity was calculated by measuring the difference between the ATP hydrolysis observed in the presence and absence of ouabain.[8,11–13]

The cell-based assays are based on several distinct techniques such as electrophysiology, fluorescence, radioligand binding, and radiotracer flux assays. The electrogenic characteristics of the ATPase have been measured by voltage-clamp techniques in several cell types.[14–18] In smooth muscle cells, the physiological roles of Na^+,K^+-ATPase have been examined mainly by measuring ion flux, isometric tension, and membrane potential with blockers for Na^+,K^+-ATPase such as ouabain, and incubation with K^+-free solution.[19] The patch-clamp technique would be of advantage as intra- and extracellular ionic conditions can be controlled and undesired ionic currents could be eliminated by various blockers during the experiment.

With the fluorescence method, the Na^+,K^+-ATPase flow measurements have been carried using the Na^+-sensitive and electrochromic dye named fluorescence labeled 5-iodoacetamidofluorescein and the styryl dye RH 421.[20–29]

Radiotracer-based Rb^{86} uptake is another tool for determining the functional characteristics of Na^+,K^+-ATPase.[11,30,31] Radioligand binding assays using [^{3}H]-ouabain binding has also been employed to study Na^+,K^+-ATPase.[8,32,33] Similarly, Rb^+ influxes via Na^+,K^+-ATPase and ouabain-resistant pathways have been studied in human blood lymphocytes.[34–36] The radiotracer determination of Na^+,K^+-ATPase activity by Rb^{86} has advantages in throughput, miniaturization, and the signal-to-noise ratio. The radiotracer assays, however, suffer from safety and disposal issues associated with radioactivity. These issues can be resolved by using non-radioactive Rb^+ that can be substituted for K^+ as a tracer in bioassays. Based on this tracer, K^+-channel activity has been reliably determined with Rb^+ using atomic absorption spectrometry in different cell types.[37,38] Therefore, in the present study, we developed an assay for examining Na^+,K^+-ATPase in cultured cells of CHO-K1, HEK-293, and L(t)K cell lines.

Materials and Methods

Procurement of chemicals and drugs

Molecular biology-grade chemicals, antibiotics, tissue culture media, and anion transport inhibitors including ouabain, glibenclamide, NPPB, and tolbutamide were obtained from Sigma-Aldrich (St. Louis, MO). Fetal calf serum was obtained from Cansera, Inc., Rexdale, ON, Canada.

Cell culture

CHO-K1 cell line CCL-61, HEK-293 cell line CRL-1573, and L(t)K cell line CRL-10422 were procured from American Type Culture Collection (Manassas, VA). The cell lines were grown by standard procedures. The CHO-K1 cells were in HAM's F-12 supplemented with 2 m*M* glutamine, 10% fetal calf serum, and pencillin/streptomycin. HEK-293 cells were grown in DMEM with 10% fetal calf serum and pencillin/streptomycin. The L(t)K cells were similarly grown in DMEM supplemented with 1 m*M* sodium pyruvate and pencillin/streptomycin. All cell cultures were cultured at 37°C with 5% CO_2. A confluent monolayer of the cells was grown in a flask and split twice a week; the trypsinized cell suspension was grown in 96-well polystyrene microplates by loading 50,000 cells/well in 200 μl of growth medium for 24 h in 5% CO_2 in a 37°C incubator.

Rb uptake

The culture medium in the multiwell plates was removed by rapidly washing the cell monolayer once with Rb^+ uptake buffer containing 15 m*M* HEPES, 140 m*M* NaCl, 5.4 m*M* RbCl, 1 m*M* $MgCl_2$, 0.8 m*M* NaH_2PO_4, and 2 m*M* $CaCl_2$, pH 7.4 set with NaOH. It was followed by incubation of the monolayer in the uptake buffer for different time intervals. The cells were then washed three times with Rb-free buffer. The monolayer was lysed by addition of lysis solution containing 0.15% sodium dodecyl sulfate. Ouabain was also added to the uptake buffer as an antagonist to study its effect on Na^+,K^+-ATPase.

Measurements

The quantification of Rb^+ concentration in intracellular samples was carried with flame atomic absorption-based technology (model ICR8000) from Aurora Biomed, Inc. (Vancouver, BC, Canada). One hundred microliters of each sample was processed automatically with an autosampler from 96-well microplates by dilution with an equal volume of ICR analysis solution (Aurora Biomed, Inc.) and injection into an air–acetylene flame. The automated system was capable of reading two 96-well plates in 90 min. The amount of free Rb^+ in the samples was measured by absorbance at 780 nm using an Rb hollow cathode lamp as a light source and a photomultiplier tube detector (PMI). A calibration curve covering the range 0–5 mg/L Rb^+ in sample analysis solution was generated with each set of samples.

Analysis

The amount of Rb^+ present in each sample was calculated with the ICR8000 instrument. All the experiments were carried in three replicates ($n = 3$). The data were presented as means, standard error, and number of replicates. Best curve fits were drawn, and IC_{50} values were

calculated with Xlfit3 (IDBS, Inc., Emeryville, CA) software. The Z' factor, which measures the overall assay quality, was determined by the method of Zhang *et al*.[39]

Results and Discussion

Rubidium uptake

In order to develop the assay using Rb^+ as a tracer, Rb^+ uptake was studied with a CHO-K1 cell monolayer in a 96-well plate using Rb^+ uptake buffer containing RbCl (5.4 m*M*) over a period of 3 h. It was observed that Rb^+ uptake followed a linear order up to 80 min, beyond which it turned into a plateau (Fig. 1). Under these conditions, in addition to the Na^+,K^+-ATPase uptake, the intracellular content of Rb^+ may be resulted by passive diffusion, and other channels or transporters. In order to verify Rb^+ entry through Na^+,K^+-ATPase, ouabain, a known blocker of the channel, was used to abolish the uptake in the CHO-K1 cells. Since Na^+,K^+-ATPase activity is dependent on the concentration of intracellular ATP, blockade of the Na^+,K^+-ATPase by cardiac glycosides was attempted. Ouabain is a cardiac glycoside that blocks the active efflux of Na^+ and reuptake of K^+ by binding to the H5 and H6 transmembrane domains of Na^+,K^+-ATPases.[8,11–13] Blocking of Rb^+ uptake was tested in both the absence and presence of increasing concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 m*M*) of ouabain for 1-, 2-, and 3-h periods, respectively. The maximum block of Rb^+ uptake was observed with a 5 m*M* concentration of ouabain at all the three time intervals (Fig. 2).

For the development of the assay, Rb^+ uptake was subsequently assessed for a period of 30-min time intervals as it represented a midpoint in the linear phase of the uptake behavior of Na^+,K^+-ATPase. An IC_{50} of 94 μM was observed at 30 min of incubation (Fig. 3). Similarly, an IC_{50} of 89 μM was observed in another experiment. These observations also suggest that KCl (5.4 m*M*) can be replaced by RbCl (5.4 m*M*) for developing the assay.

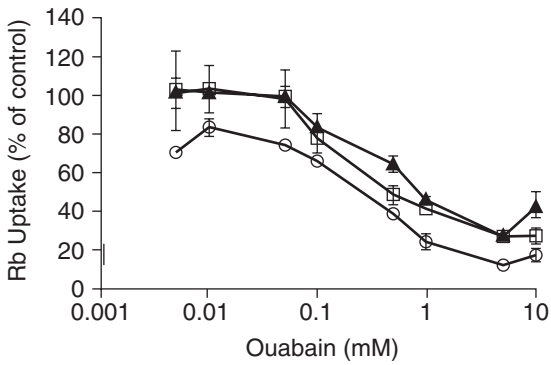

FIG. 2. Effect of ouabain on Na^+,K^+-ATPase in the assay. The Rb^+ uptake by CHO-K1 cell monolayers in the 96-well plate plotted as a percentage of control shows a maximum block of the Rb^+ uptake by the 5 m*M* dose of ouabain at 1 (▲), 2 (□), and 3 (○) h of incubation.

Effect of K^+ on Rb^+ uptake

Extracellular Na^+ and K^+ concentrations have been reported to affect the shape of the Na^+,K^+-ATPase *I–V* relationship.[40] The effect of K^+ in the assay buffers was investigated, and it was observed that the cells treated with 60 m*M* KCl had a significant change in the total Rb^+ content and relative uptake rates (Fig. 4a and b). However, the IC_{50} values did not alter appreciably from 79 μM (resting membrane potential, 5 m*M* KCl) to 88 μM (slightly hyperpolarized membrane potential, 0 m*M* KCl) and 99 μM (depolarized membrane potential, 60 m*M* KCl). These results also reflected the lack of significant changes in the blocking behavior of Na^+,K^+-ATPase by ouabain shown in normalized Rb^+ uptake graphs (Fig. 4c). Further, the voltage dependence of the

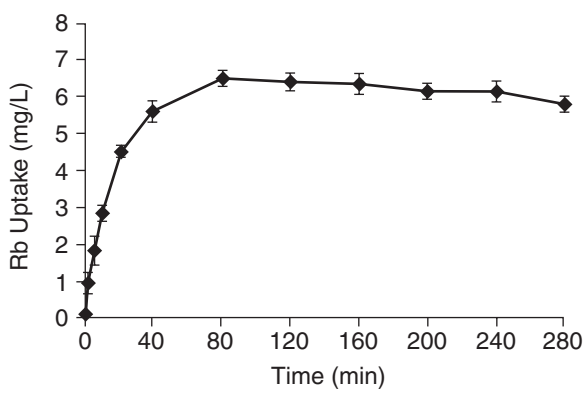

FIG. 1. Rb^+ uptake by CHO-K1 cells over a period of 3 h shows a linear order up to 80 min, beyond which it turned into a plateau.

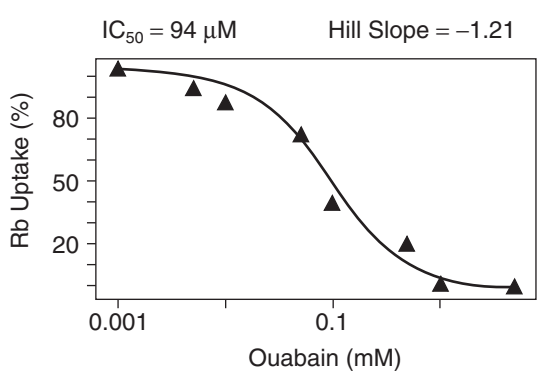

FIG. 3. Effect of ouabain on the midpoint of the linear phase of Rb^+ uptake in the assay. An IC_{50} of 94 μM for CHO-K1 cells occurs at 30 min of incubation.

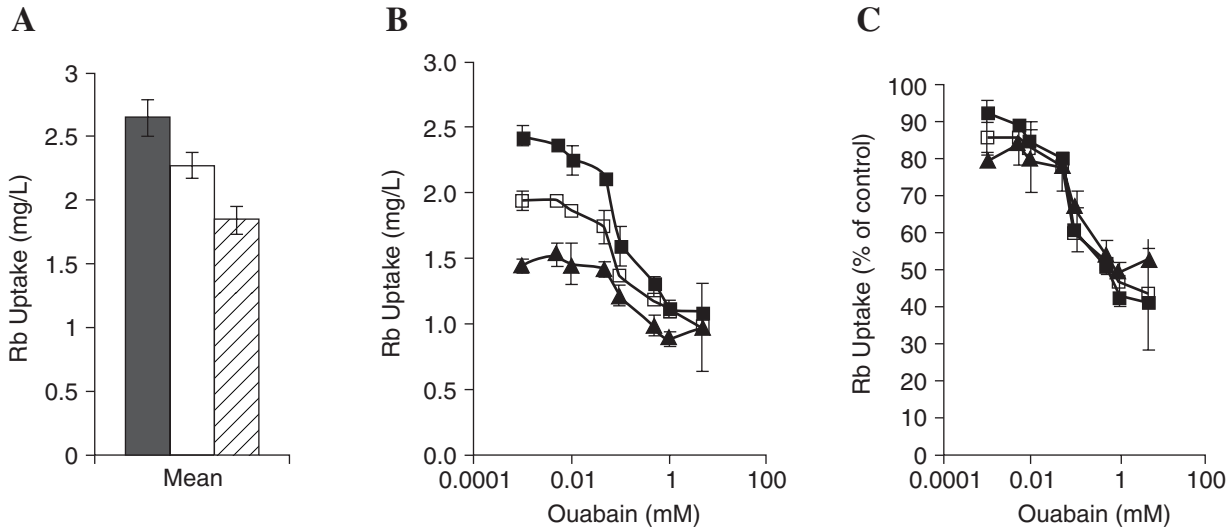

FIG. 4. Effect of K^+ on the Rb^+ uptake assay in the presence and absence of varying ouabain concentrations. (**A**) Hyperpolarization due to lack of K^+ in the uptake buffer raises the uptake of Rb^+, and depolarization caused by 60 m*M* KCl lowers the uptake in the absence of ouabain. (**B**) Blocking profile of Rb^+ uptake in the presence of 0 (■), 5 (□), and 60 m*M* KCl (▲). (**C**) Normalized data on the block of Rb^+ uptake by ouabain in the presence of 0 (■), 5 (□), and 60 m*M* KCl (▲).

Na^+,K^+-ATPase transient currents from different cells has already been investigated,[21,41] leading to the conclusion that the Na^+,K^+-ATPase significantly contributes to the maintenance of membrane potential. Ouabain application has been reported to depolarize the membrane potential by 5–10 mV in different tissue preparations.[21,42,43] Thus, the depolarization induced by 60 m*M* KCl has caused the blockage of Rb^+ uptake. Extracellular K^+ is known to contribute to the dephosphorylation of the E_2P subunit of Na^+,K^+-ATPase and is in addition known to counter the action of glycosides that bind preferentially to the phosphorylated subunit. These actions of K^+ decrease the affinity of ouabain on the E_2P subunit by a type of kinetic competition leading to an increase of the K_D for ouabain with a rise in K^+ concentration.[21,44,45]

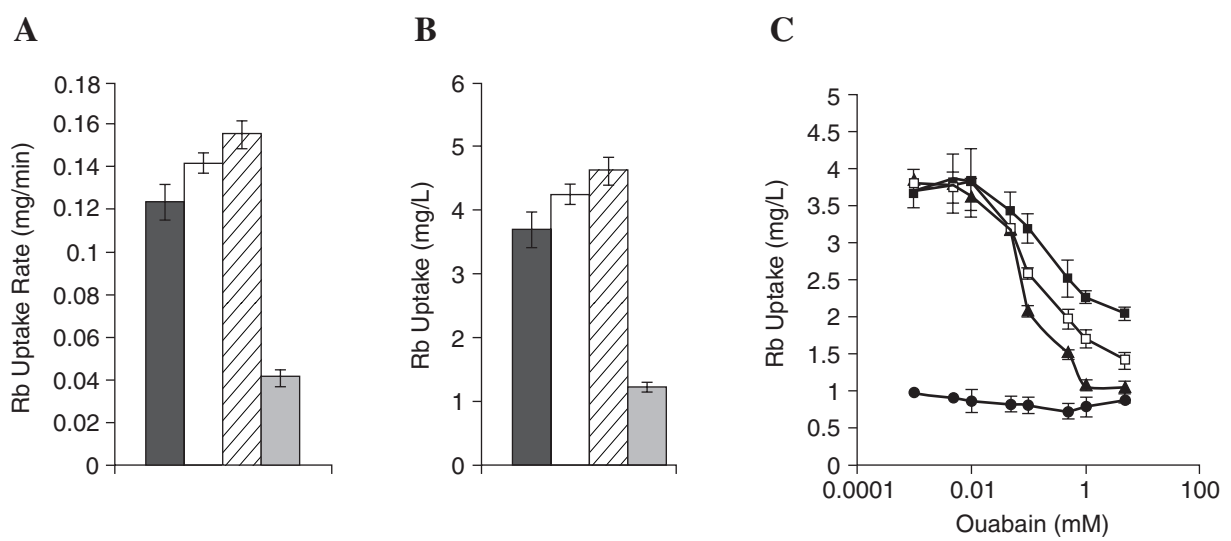

FIG. 5. Effect of extracellular Rb^+ and depolarization on Rb^+ uptake assay. (**A**) In the absence of ouabain, total Rb^+ uptake at 2 (black bars), 5.4 (open bar), and 10 mM (hatched bar) indicates that an increase in extracellular Rb^+ concentration results in a significant rise both in the total Rb^+ uptake and (**B**) in relative Rb^+ uptake rates in comparison to the control that contains 10 mM Rb^+ in the presence of 60 mM KCl (dotted bar). (**C**) The effect of ouabain at 2 (■), 5.4 (□), and 10 mM (▲) Rb^+ suggests that the raise in extracellular Rb^+ decreases the potency of ouabain. The effect of ouabain was not clear at 10 mM Rb^+ in the presence of 60 mM KCl (●).

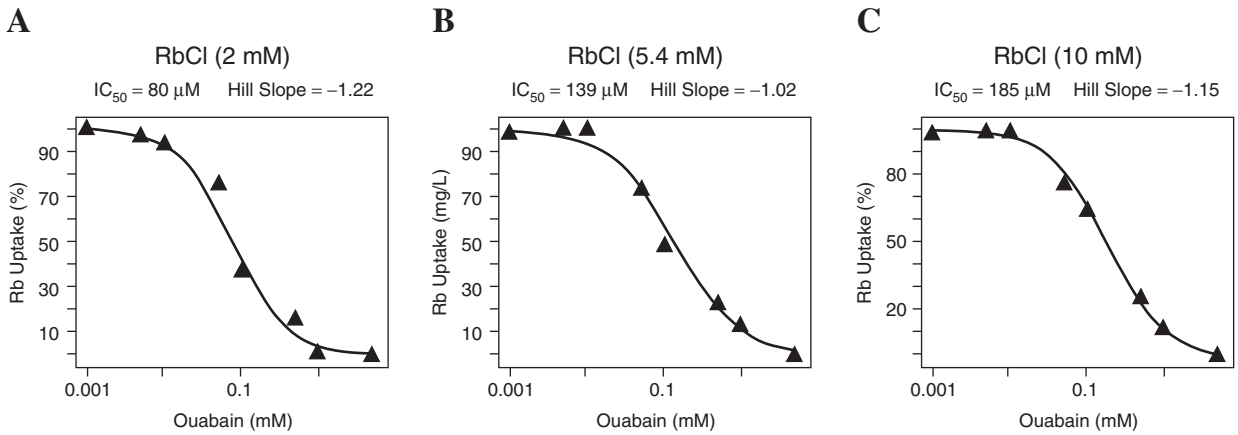

FIG. 6. The effect on the electrochemical gradient of 2, 5.4, and 10 m*M* extracellular Rb^+ was also reflected in the observed IC_{50} values of 80 (**A**), 139 (**B**), and 185 μM (**C**).

Effect of extracellular Rb^+ concentration

The effect of RbCl electrochemical gradient in the assay was also tested for effects on Rb^+ uptake and ouabain inhibition by varying extracellular concentrations of Rb^+. The increase of extracellular Rb^+ concentration resulted in the rise in the total and relative Rb^+ uptake rates. This observation suggests that the chemical gradient of RbCl has a significant effect on the Rb^+ influx. However, such a result can be offset by raising the membrane potential through the addition of 60 m*M* KCl (Fig. 5). The effect on electrochemical gradient of 2, 5.4, and 10 m*M* extracellular Rb^+ concentrations was also reflected in the observed IC_{50} values of 80, 139, and 185 μM, respectively (Fig. 6). The IC_{50} values for ouabain in the presence of 60 m*M* KCl could not be calculated because of the uptake block caused by the rise in the membrane potential. External monovalent cations such as Rb^+, Cs^+, and Li^+, as well as K^+, are reported to activate the Na^+,K^+-ATPase in various non-human isoforms with the order: $K^+ \geq Rb^+ >> Cs^+ >> Li^+$.[44,46–48] However, it is not clear whether the increase in Rb^+ uptake was a result of the electrochemical gradient or due to the activation of the Na^+,K^+-ATPase in the presence of monovalent ions as reported in other studies as well.

Effect of extracellular Na^+ ions

Since Na^+,K^+-ATPase involves the interplay of Rb^+, K^+, and Na^+ ions in its activity, the effect of extracellular Na^+ ions on the transporter's activity in the assay was also studied. The Na^+ in the extracellular solution was observed to have a significant influence on the activity of Na^+,K^+-ATPase; it was noted that Rb^+ uptake was affected by lowering the extracellular NaCl concentration from 145 m*M* to 50, 10, and 0 m*M*. These changes resulted in a 11, 30, and 46% decrease of Rb^+ uptake, correspondingly (Fig. 7). The effect of the Rb^+ flow at 0 m*M* NaCl in the presence of 60 m*M* KCl was very different from the previous observations as only 18% Rb^+ uptake was noted. In the absence of external Na^+ and presence of extracellular K^+, the Na^+,K^+-ATPase has been reported to operate as voltage-independent.[49]

Rank order of blockers

Following characterization of Na^+,K^+-ATPase with ouabain in the assay, the inhibitory potency of other compounds like NPPB, glibenclamide, and tolbutamide was also tested. These compounds are known to inhibit some chloride transporters and were also observed to significantly inhibit the activity of the Na^+,K^+-ATPase (Fig. 8). The IC_{50} values for ouabain, NPPB, glibenclamide,

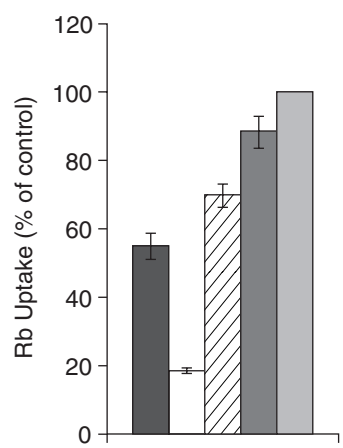

FIG. 7. Effect of extracellular Na^+ and depolarization on Rb^+ uptake assay. The Rb^+ uptake was affected by lowering the extracellular Na^+ concentration from 145 m*M* (■) to 50 (▩), 10 (▨), and 0 m*M* (■). These changes resulted in a decrease in 11, 30, and 46% of Rb^+ uptake, respectively. However, 60 m*M* KCl in the absence of Na^+ (□) resulted in an 81% decrease in Rb^+ uptake.

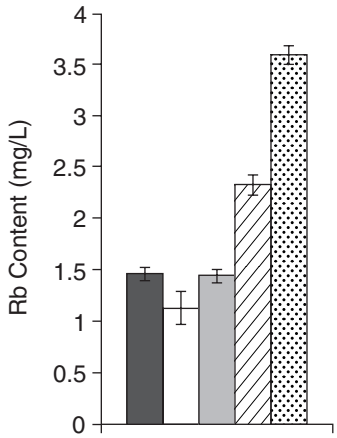

FIG. 8. Determining potency of blockers on Na^+,K^+-ATPase: 5 mM dose of each compound, ouabain (■), NPPB (□), glibenclamide, (■), and tolbutamide (▨) resulted in a decreased of 40, 31, 40, and 65% of the total Rb^+ uptake (⊞), respectively. The IC_{50} values for ouabain, NPPB, glibenclamide and tolbutamide were calculated to be 89, 170, 147 and 379 μM.

and tolbutamide were calculated to be 89, 170, 147, and 379 μM, respectively. The 2β-carboxymethoxy-3β-(4-fluorophenyltropane) receptor blockers NPPB and tolbutamide have been reported to inhibit the activity of Na^+,K^+-ATPase.[50] Thus, the compounds can be screened for their activity on Na^+,K^+-ATPase and ranked on the basis of potency.

Comparison of isoforms

The Rb^+ uptake assay was also carried on two other cell lines, HEK-293 and L(t)K, to determine the effect of ouabain on human and non-human isoforms of Na^+,K^+-ATPase.[44,46–48] The human ATPase isoform in HEK-293 cell line was found to be more sensitive than the non-human isoforms in L(t)K (mouse) and CHO-K1 (hamster) cell lines. The IC_{50} values for HEK-293 were determined to be 1.3 μM, while L(t)K and CHO-K1 values were 202 and 94 μM (Fig. 9). These data are supported with the reported K_D of 0.35, 21, and 0.5–10 μM for ouabain in HeLa cells, human vascular endothelial cells, and chick cardiac cells, re-

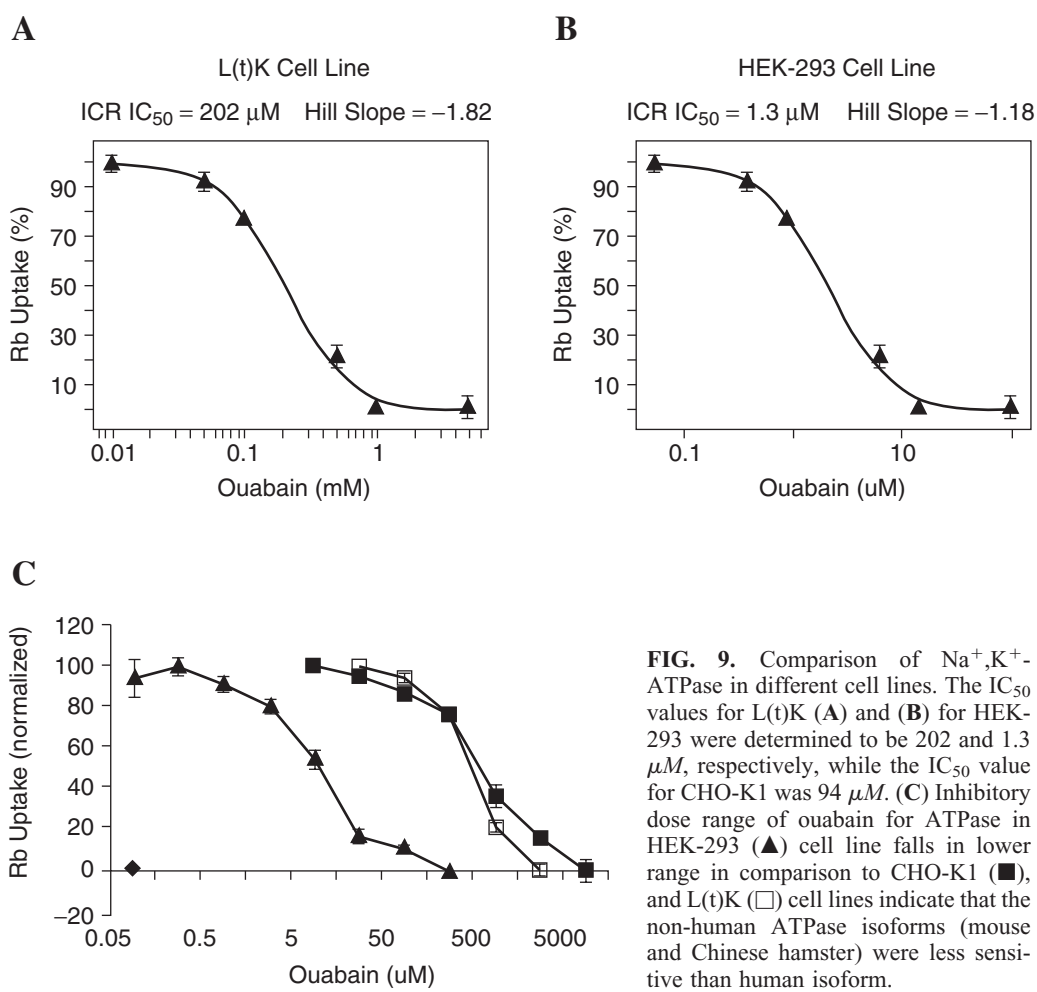

FIG. 9. Comparison of Na^+,K^+-ATPase in different cell lines. The IC_{50} values for L(t)K (**A**) and (**B**) for HEK-293 were determined to be 202 and 1.3 μM, respectively, while the IC_{50} value for CHO-K1 was 94 μM. (**C**) Inhibitory dose range of ouabain for ATPase in HEK-293 (▲) cell line falls in lower range in comparison to CHO-K1 (■), and L(t)K (□) cell lines indicate that the non-human ATPase isoforms (mouse and Chinese hamster) were less sensitive than human isoform.

spectively,[21,45,51] in patch-clamp studies. Similarly, a K_D of 0.05 and 65 μM for guinea pig ventricular cells has been reported in the previous studies.[52] The differences in the K_D values of dissimilar tissues and species are likely due to the presence of different α-subunits of Na^+,K^+-ATPase.[21] The ouabain sensitivity obtained in the present studies is in good accord with the reported IC_{50} values. The assay resulted in a Z' factor value higher than 0.7 throughout these studies. Thus, it shows that the flame atomic absorption-based uptake assay has values that are severalfold higher than the ones obtained from the patch-clamp studies. This protocol is considered robust and sensitive as it has generated reproducible IC_{50} numbers.

Conclusions

The preliminary data demonstrated that the rubidium uptake flux assay has both sensitivity and accuracy. These two parameters, together with information content, throughput, robustness, flexibility, and physiological relevance, are considered essential in a functional and useful assay.[53] The rubidium uptake flux assay is sensitive as it is capable of detecting even minute amounts of the Rb^+ ions in the solution. Further, this rubidium assay is robust and can be employed in screening compounds for modulation of transporters like Na^+,K^+-ATPase, including an accurate drug order ranking of the blockers. The IC_{50} values for ouabain, a known blocker, are severalfold higher than the published ones obtained via patch-clamp, but the rank order of tested compounds (NPPB, glibenclamide, and tolbutamide) is comparable to the data obtained via electrophysiology.[53] This confirms the accuracy of the assay for the Na^+,K^+-ATPase activity. In correlation to the high speeds, the cost associated with this assay system is significantly lower than average price of the similar assays.[38,53] Finally, the obtained data are physiologically relevant as they are based on the recordings from the whole cell, rather than the isolated membrane portion. Overall, this newly developed assay is well rounded and has the potential to serve the research community as an additional tool for the measurement of the functional activity and modulation of the Na^+,K^+-ATPase exchanger.

Acknowledgments

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