Video Article

Measuring Cation Transport by Na,K- and H,K-ATPase in *Xenopus* Oocytes by Atomic Absorption Spectrophotometry: An Alternative to Radioisotope Assays

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Abstract

Whereas cation transport by the electrogenic membrane transporter Na^+,K^+-ATPase can be measured by electrophysiology, the electroneutrally operating gastric H^+,K^+-ATPase is more difficult to investigate. Many transport assays utilize radioisotopes to achieve a sufficient signal-to-noise ratio, however, the necessary security measures impose severe restrictions regarding human exposure or assay design. Furthermore, ion transport across cell membranes is critically influenced by the membrane potential, which is not straightforwardly controlled in cell culture or in proteoliposome preparations. Here, we make use of the outstanding sensitivity of atomic absorption spectrophotometry (AAS) towards trace amounts of chemical elements to measure Rb^+ or Li^+ transport by Na^+,K^+- or gastric H^+,K^+-ATPase in single cells. Using *Xenopus* oocytes as expression system, we determine the amount of Rb^+ (Li^+) transported into the cells by measuring samples of single-oocyte homogenates in an AAS device equipped with a transversely heated graphite atomizer (THGA) furnace, which is loaded from an autosampler. Since the background of unspecific Rb^+ uptake into control oocytes or during application of ATPase-specific inhibitors is very small, it is possible to implement complex kinetic assay schemes involving a large number of experimental conditions simultaneously, or to compare the transport capacity and kinetics of site-specifically mutated transporters with high precision. Furthermore, since cation uptake is determined on single cells, the flux experiments can be carried out in combination with two-electrode voltage-clamping (TEVC) to achieve accurate control of the membrane potential and current. This allowed *e.g.* to quantitatively determine the $3Na^+/2K^+$ transport stoichiometry of the Na^+,K^+-ATPase and enabled for the first time to investigate the voltage dependence of cation transport by the electroneutrally operating gastric H^+,K^+-ATPase. In principle, the assay is not limited to K^+-transporting membrane proteins, but it may work equally well to address the activity of heavy or transition metal transporters, or uptake of chemical elements by endocytotic processes.

Video Link

The video component of this article can be found at http://www.jove.com/video/50201/

Introduction

We wanted to develop a sensitive, safe and inexpensive alternative to radioactive tracer experiments to investigate the specific transport activity of ion translocating membrane proteins in order to circumvent restrictions regarding the access to isotope laboratories, safety requirements or the use of costly radioisotopes, which - as in the case of lithium - may even be unavailable due to extremely short decay times. We were particularly interested in determining the activity of the electroneutrally operating gastric H^+,K^+-ATPase, because the enzyme does not generate current and its activity can therefore not be addressed by electrophysiological methods. Since Na^+,K^+- and H^+,K^+-ATPase transport Rb^+ as efficient as K^+ (and Li^+ as well), the high sensitivity of the AAS technique for rubidium or lithium should facilitate sensitive detection of transport activity. Atomic absorption spectrophotometers are common analytical devices, which are widely distributed in chemical laboratories and should be accessible to a large number of interested scientists. Furthermore, we wanted to take advantage of the *Xenopus* oocyte expression system, which utilizes large single cells (about 1.0-1.5 mm diameter) that allow to achieve a remarkably low cell-to-cell variability regarding the protein expression level within a single batch. A simple calculation demonstrates the feasibility of the AAS assay: The detection limit (characteristic mass) for rubidium with the THGA-AAS technique is 10 pg or $1.2 \cdot 10^{-13}$ mol (Rb: 85.47 g/mol), for lithium 5.5 pg or $7.9 \cdot 10^{-13}$ mol (Li: 6.94 g/mol). Upon heterologous expression of Na^+,K^+-ATPase in *Xenopus* oocytes, pump currents of 100 nA can be achieved (which equals about 6.2·1011

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elementary charges per second, or $1.03 \cdot 10^{-12}$ mol s^{-1}), thus resulting in a transport of $6 \cdot 10^{-6}$ C of charge within 1 min. Since the transport of one net charge corresponds to the uptake of two Rb^+ ions (due to the $3Na^+/2K^+$ stoichiometry), 100 nA current for 1 min corresponds to an uptake of $1.2 \cdot 10^{-10}$ mol Rb^+. Thus, even upon a 1,000-fold dilution (homogenization of an oocyte with about 1 µl volume in 1 ml water), a typical THGA-AAS sample (20 µl) contains $2.4 \cdot 10^{-12}$ mol Rb^+ (or 204 pg), which is far above the detection threshold. Therefore, even transporters with more than 100-fold lower transport activity or plasma membrane expression can be assayed with the technique by appropriately adjusting the flux time of the experiment.

Since the pumping rate is sensitively dependent on temperature (typical activation energies for Na^+,K^+-ATPase are in the range of 90 kJ/mol to 130 kJ/mol[1-3], which results in an about 30% increase in the turnover rate upon a change from 20 °C to 22 °C), it is mandatory to carry out the flux measurements under precise temperature control (air conditioning) with well equilibrated buffer solutions. Furthermore, oocytes should be carefully selected regarding homogenous size for the expression of an ion transporter. With these precautions, it is possible to routinely achieve experimental standard errors of less than 10 percent with about 10 cells per experimental condition. Using this technique, we were able to determine *e.g.* the apparent Rb^+ affinities of cation transport[4-6], the influence of extra- and intracellular pH[7] and the effect of mutations of residues involved in cation coordination during transport[4,8]. An advantage of the technique is that ion fluxes can also be determined in combination with two-electrode voltage clamping of the oocytes, which on one hand assures accurate control of the membrane potential during transport and on the other hand allows to correlate ion flux with membrane current. Thus, it was possible to verify the $3Na^+/2K^+$ stoichiometry of the Na^+,K^+-ATPase (see exemplary results below) and to determine the voltage dependence of cation transport of the gastric H^+/K^+-ATPase[7].

Protocol

1. cDNA Constructs and Protein Expression in *Xenopus* Oocytes

The cDNA of the membrane protein of interest should be cloned into a vector suitable for expression in *Xenopus laevis* oocytes such as pTLN[9] or pcDNA3.1X[10]. Such optimized vectors contain the 5'- and 3'-untranslated regions (UTR) of the *Xenopus laevis* β-globin gene flanking the multiple cloning site (MCS), an RNA polymerase promoter sequence (pTLN: SP6, pcDNA3.1X: T7) located before the 5' UTR, a poly-A stretch in 3' direction of the MCS to ensure cRNA stability in cells, and further downstream another sequence of single-cutting restriction endonuclease sites for linearization of the plasmid in order to serve as a template for *in vitro* cRNA transcription.

To distinguish the activity of the overexpressed human Na^+/K^+-ATPase (α_2-subunit+β_1-subunit) from the endogenous Na^+/K^+-ATPase of the oocytes, the mutations Q116R and N127D were introduced to obtain an ouabain-resistant protein with an IC50 in the millimolar range[11]. In the case of H^+/K^+-ATPase, flux measurements were carried out using the H^+/K^+-ATPase α-subunit mutant S806C, since Rb^+ fluxes were correlated with kinetic data from voltage-clamp fluorometry (VCF) experiments. The VCF technique (http://www.jove.com/video/2627/ examining-the-conformational-dynamics-of-membrane-proteins-in-situ-with-site-directed-fluo rescence-labeling)[12] is based upon site-specific attachment of a fluorophore to a strategically introduced cysteine residue, which reports conformational changes of the enzyme.

2. Linearization and Purification of the DNA Template

1. For linearization of the DNA template, digest 3 µg of plasmid DNA in 50 µl reaction volume with 5 µl 10x-concentrated buffer appropriate for the restriction enzyme and 10 U of restriction enzyme for 1 hr at 37 °C.
2. Purify the linearized DNA using the High-Pure PCR Product Purification Kit (Roche Applied Science). First, add 350 µl binding buffer (containing guanidiniumisothiocyanate) to the digest vial. After this purification step, the linearized DNA template is 'RNA grade' because guanidiniumisothiocyanate is an efficient protein denaturant and destroys in particular RNAse, which is a frequent contaminant in molecular biological laboratories.
3. Insert spin columns into collection tubes, load the column with DNA solution from step 2, centrifuge at 13,000 x g for 30 sec and discard flow-through.
4. Add 500 µl of washing buffer to the column. Centrifuge 1 min at 13,000 x g and discard flow through.
5. Add 200 µl of washing buffer to the column. Centrifuge 1 min at 13,000 x g to fully dry the column.
6. Place the spin column in a fresh 1.5 ml Eppendorf tube and add 50 µl of nuclease-free water (Ambion Product # AM9937) to the column. Elute by centrifuging for 1 min at maximum speed.
7. After elution, concentrate the DNA-containing solution to about 15 µl. This step ensures that the solution contains 0.5 µg linearized DNA in 3 µl, which is the recommended concentration for the following *in vitro* cRNA transcription reaction.

3. *In Vitro* cRNA Synthesis

1. Choose the appropriate mMessage mMachine Kit (Ambion Product #1340, #1344) according to the promoter sequence on the plasmid (SP6, T7). In 10 µl total reaction volume, add 3 µl linearized DNA from the previous step and 1 µl transcription buffer to pre-aliquoted 5 µl-samples of NTP-cap mix. Pre-aliquoting of the NTP-cap mix is necessary to avoid frequent freeze-thaw-cycles, which may lead to degradation of the nucleoside-triphosphates. Finally, add 1 µl of the appropriate RNA polymerase, mix briefly and spin-down the reaction mix.
2. Incubate the reaction mix for 1 to 2 hr at 37 °C. The incubation time should not exceed 2 hr, because this may lead to lower cRNA yield or quality.
3. Add 15 µl of ice-cold 3 M LiCl solution from the kit, 15 µl cold nuclease-free water, mix briefly (do not vortex), briefly spin down the sample at 11,000 x g for 10 sec, and store at -20 °C for at least 30 min to allow precipitation of the cRNA.
4. Pre-cool centrifuge to 4 °C and centrifuge at least 15 min at 13,000 x g after precipitation

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5. Remove the supernatant carefully and completely from the pale brown pellet at the bottom of the tube and add 150 µl of 70% RNA-grade ethanol cooled to -20 °C. Centrifuge at 4 °C for 5 min at 13,000 x g.
6. Remove the supernatant completely, briefly dry (1-2 min) the remaining cRNA pellet in a centrifugal evaporator (*e.g.* Eppendorf Concentrator model 5301) at room temperature.
7. Dissolve the pellet in 12 µl of cold nuclease-free water. CAUTION: All subsequent steps should be carried out with the cRNA sample stored on ice.
8. Determine the cRNA concentration using a Spectrophotometer (*e.g.* Eppendorf BioPhotometer).
9. Check the integrity of the cRNA by agarose gel electrophoresis. CAUTION: This cross-check is necessary, since even partially degraded RNA yields non-zero UV absorption. For cRNA electrophoresis, use a thoroughly cleaned electrophoresis chamber newly filled with TAE buffer and a freshly prepared 1% agarose gel. If RNAse contamination is suspected in the electrophoresis chamber or buffer, fill the chamber with 0.1 M HCl for 1 hr for cleaning and prepare TAE buffer with nuclease-free water.
10. Store cRNA at -20 °C until use. cRNA can be stored for at least one year at -20 °C.

4. Obtaining Ovary Material from *Xenopus laevis* Females by Partial Ovariectomy

This protocol has been approved by the responsible state authority (Landesamt für Gesundheit und Soziales Berlin, The Senate of Berlin, Germany).

1. Do not feed the frog within at least 12 hr prior to surgery to prevent vomiting.
2. Prepare a solution of 2 g of the local anesthetic tricaine (MS-222) dissolved in 1 L water adjusted to pH 7.0 with 5 mM HEPES buffer. CAUTION: Wear nitrile gloves at all times while handling MS-222 and prepare the solution in a chemical safety cabinet.
3. Immerse the frog in the tricaine solution (max. 10 min) until the animal no longer shows righting reflexes. To check unresponsiveness, try to turn the frog on its back in your hands and watch for defense movements.
4. Take the frog out of the tricaine solution by hand and rinse the animal with cold tap water to remove the anesthetic.
5. Place the frog with its back on top of a bed of ice covered with a wet paper towel to avoid damage to the frog's skin by direct contact to ice. Keep the skin of the animal wet at all times during the operation to avoid drying. Placement on ice prolongs the time of anesthesia.
6. Make a small abdominal incision (8-10 mm length) with sharp scissors on either the left or the right side of the frog's midline. CAUTION: Be careful not to injure the lateral line. Subsequent surgeries should be made on alternating sides to allow for good recovery of the underlying ovary tissue. Usually, three layers of tissue (the outer skin, a thin layer of white connective tissue - the fascia - and a muscle layer) have to be penetrated.
7. After opening the abdomen, seize parts of ovarian lobes with blunt forceps and pull gently to the exterior. As a guideline, remove about 3-5 cm of ovary material from the ovary by incision. In case of injury of small blood vessels and bleeding, apply pressure with a sterile plastic rod or cool the injury with ice until the bleeding stops.
8. Surgically close the incision using an interrupted suture pattern with 4.0 Ethicon Vicryl suture (Johnson & Johnson #V633H). Use surgical instruments for suturing and tie two or three knots separately along the incision. CAUTION: It is important for each knot to suture the three tissue layers in one step.
9. After operation, place the frog into a slightly inclined water basin (30 L), which contains just enough water to cover its bottom so that the animal's nostrils are above water level to permit breathing. Cover the animal during the wake-up period (about 1-2 hr) with a wet paper towel to avoid drying of the sensitive skin.
10. When the animal is active again, fill the water basin and keep the frog separately from the other animals for 3-4 days to monitor recovery and wound healing.
11. After surgery, save the frog from subsequent operations for at least 3 months to allow for complete recovery of the animal and its ovarian tissue. As a guideline, a maximum of 6-8 surgeries can be performed on each frog.

5. Enzymatic Isolation of Individual Oocytes

1. Cut the ovarian material into smaller pieces with scissors and transfer the tissue into *Oocyte Ringer Solution without* Ca^{2+} (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) containing collagenase (20 mg/ml, type 1A from *Clostridium hystolyticum*, Sigma #C9891) and trypsin inhibitor (10 mg/ml, Type III-O from chicken egg white, Sigma #T2011).
2. Gently shake the digest solution for 2.5 hr at 18 °C. Afterwards, follow the progress of the digest in 15 min intervals until most oocytes are separated from the follicular tissue.
3. Wash the oocytes several times in a 50 ml Falcon tube with *Oocyte Ringer Solution without* Ca^{2+} until the solution is sufficiently cleared from tissue debris.
4. Store the oocytes in *Oocyte Ringer Solution containing* Ca^{2+} (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.4) including gentamycin (50 µg/ml) at 18 °C.

6. cRNA Injection

1. For expression of Na^+,K^+- or H^+,K^+-ATPase, inject oocytes with 50 nl cRNA solution containing 25 ng α-subunit and 5 ng β-subunit cRNA using an electrically driven Nanoject II injection pump (Drummond Scientific Inc.), with 3.5" hematocrit tubes (Drummond #3 000-203-G/X). CAUTION: The amount of mRNA may vary according to the target protein and might need to be adjusted for maximal expression by Western blotting.
2. For each flux condition (*e.g.* at a specific Rb^+ concentration), inject 18-22 oocytes. From these, 50% will be assayed under "+flux" conditions (*e.g.* a certain Rb^+ concentration), and 50% under "-flux" conditions (*e.g.* with the chosen Rb^+ concentration and a specific inhibitor of the enzyme in saturating concentration, such as 10 mM ouabain for Na^+,K^+-ATPase or 10 µM SCH28080 at pH 5.5 for H^+,K^+-ATPase).

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3. After injection, transfer oocytes separately into 96-well plates half-filled with Oocyte Ringer Solution containing Ca^{2+} and 50 µg/ml gentamycin at 18 °C.
4. Cover the 96-well plate with Parafilm to prevent excessive evaporation.
5. Incubate the oocytes for 2-7 days (depending on the protein of interest) at 18 °C. If oocyte storage for more than 3 days is required, it is recommended to exchange the incubation buffer within the microwells every 3 days.
6. For each set of flux data (*e.g.* a [Rb^+] titration experiment), about 10 non-injected (or injected with 50 nl nuclease-free water) control oocytes should be assayed under "+flux" and "-flux" conditions. Thus, incubate a sufficient number of control cells in the same way as cRNA-injected oocytes prior to flux analysis.

Pretreatment of heterologous ATPase-expressing oocytes

Na^+ loading procedure

For functional measurements of Na^+/K^+-ATPase, it is important to elevate the intracellular Na^+ concentration[13], as follows:

1. Transfer oocytes into *Na^+ Loading Buffer* (110 mM NaCl, 2.5 mM Na-citrate, 2.5 mM MOPS, 2.5 mM TRIS, pH 7.4) for 45 min on ice.
2. For subsequent recovery, incubate oocytes in *Post Loading Buffer "PLB"* (100 mM NaCl, 1 mM $CaCl_2$, 5 mM $BaCl_2$, 5 mM $NiCl_2$ and 2.5 mM MOPS, 2.5 mM TRIS, pH 7.4) for at least 30 min on ice.
3. Store oocytes in *PLB* on ice until use.

7. Pre-blocking of Endogenous Na^+,K^+-ATPase

CAUTION: Since *Xenopus* oocytes express an endogenous Na^+,K^+-ATPase, it is important to block this endogenous pump prior to Rb^+ flux experiments, as follows:

1. Now to block the endogenous Na^+,K^+-ATPase by incubating the oocytes in Post Loading Buffer containing 100 µM ouabain for 15 min at room temperature.

8. Electrophysiology

1. Prepare microelectrodes by pulling borosilicate capillaries (GB150F-8P, Science Products GmbH) using a two-step micropipette puller (Model PC-10, Narishige).
2. Fill glass electrodes from the back with 3 M KCl. For TEVC experiments with H^+,K^+-ATPase, we recommend to use 3M NaCl instead, which led to more stable current recordings. Mount pipette over the Ag/AgCl wire of the microelectrode holders of the two-electrode voltage-clamp setup.
3. Insert the glass microelectrodes and two agar bridges (these should be made with NaCl/agar instead of commonly used KCl/agar), which connect the Ag/AgCl reference electrodes to the bath solution into the recording chamber (Warner Instr., model RC-10) pre-filled with measuring buffer (*e.g.* PLB with 10 µM ouabain for Na^+,K^+-ATPase).
4. Switch the voltage-clamp amplifier (Turbo TEC-10CX, NPI Electronics, Tamm, Germany) into the "OFF" mode.
5. Check the resistance of each glass electrode separately (both should be in the range of 0.5-2 MΩ).
6. Check offset potentials of voltage and current electrode on the appropriate TEVC amplifier display. In case of discrepancy from zero adjust the offset with the corresponding offset regulators of the amplifier.
7. Place an oocyte in the middle of the chamber and penetrate the membrane gently with the current and the voltage electrode.
8. Check potential reading of the amplifier. The reading for the potential of the voltage electrode now gives the value for the resting potential of the cell.
9. Switch amplifier to VC (voltage-clamp) mode.
10. Using the pCLAMP software (Molecular Devices Corp., Sunnyvale, CA), clamp the cell to a pre-defined membrane potential. This can be either the value of the previously measured resting potential (for recording under zero membrane current conditions) or any desired test potential value. Pay attention to the amplitude and stability of the leak current (in the absence of K^+/Rb^+/Li^+), which - as a general guideline - should not exceed -200 nA at -30 mV.
11. Perfuse the oocyte for a certain time (*e.g.* 3 min for Na^+,K^+-ATPase) with Rb^+-containing solution (to achieve maximal current use saturating concentrations from flux measurements under non-clamped conditions) and record pump current with pCLAMP software in the continuous recording mode. Make sure that Rb^+ uptake is completely stopped afterwards by perfusing the cell with Rb^+-free solution for 30 sec.
    a. Stop the two-electrode voltage-clamp experiment, remove the oocyte from the recording chamber and proceed to the washing step 4 of paragraph "Rb^+ uptake experiments" to analyze Rb^+ uptake with Atomic Absorption Spectrophotometry.
12. Perform offset current subtraction and evaluate the time integral of the pump current using the Clampfit program of the pCLAMP package.

9. Rb^+ Uptake Measurements

1. Cut and melt yellow pipette tip on a 200 µl pipette to about 2 mm diameter. Usage of such tips ensures minimal transfer of solution when the oocytes are moved between incubation or washing dishes. Briefly melt the tip with the flame of a pocket lighter. This polishing step prevents oocyte damage.

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2. Prepare for each oocyte to be measured a 1.5 ml Eppendorf tube filled with 1 ml Millipore water. Arrange three Petri dishes with Rb^+-free washing buffer (*e.g. PLB*) and one with Millipore water.
3. Transfer five preincubated oocytes simultaneously into a 3.5 cm Petri dish filled with Rb^+ flux buffer (concentrations from 0 to 15 mM Rb^+) and incubate for 3 min (Na^+,K^+-ATPase) or 15 min (H^+,K^+-ATPase) under temperature control (*e.g.* 21 °C in an air-conditioned room or on a heating plate).
4. Transfer oocytes simultaneously into the first dish with Rb^+-free washing buffer (in case of Na^+,K^+-ATPase PLB can be used). It is mandatory to keep the deviations between the incubation times smaller than 5 sec. Rinse gently.
5. Move oocytes into the second and third dish with Rb^+-free washing buffer, rinse gently.
6. Transfer oocytes into the dish with Millipore water, rinse gently.
7. Transfer each oocyte individually into one of the prepared Eppendorf tubes filled with 1 ml Millipore water.
8. Homogenize oocyte by pipetting up and down with a 200 µl pipette tip until solution is homogenously turbid.

10. Preparation of AAnalyst 800 Utilizing the THGA Furnace System

1. Turn on valve of the required inert gas (argon) and insert an appropriate hollow cathode lamp (single-element HCLs for Li^+ or Rb^+; Photron, Melbourne, Australia) in arbitrary socket position.
2. Start WinLab32 control software (Perkin Elmer) and switch on the appropriate hollow cathode lamp. Perkin Elmer Lumina hollow cathode lamps are recognized automatically and appear on the selection list. Non-compatible hollow cathode lamps need to be entered manually.
3. Allow for a warm-up time of approximately 10-15 min for equilibration of the lamps.
4. Ensure that the THGA tube (transversely-heated graphite atomizer tube, Perkin Elmer, #B3000641) is in good order by visual inspection. THGA furnaces should be replaced every 300 shots (average life-time stated by the manufacturer), but may well endure larger sample numbers.
5. Select the "THGA furnace" technique in WinLab32.
6. Prepare at least five calibration samples with Rb^+/Li^+ concentrations between 10 and 50 µg/L (*e.g.* by appropriate dilution from a 1 M RbCl or LiCl solution) and one blank probe with Millipore water only. Note: Salts should be of highest available purity ("puriss." or from commercially available calibration standards for AAS purpose).
7. Choose the appropriate initialisation file (termed "method" file, which provides a reference to calibration parameters and loads instrument settings, *e.g.* the AAS detection wavelength, the wavelengths for Rb^+ and Li^+ are 780 nm and 670.8 nm, respectively) then enter the positions and concentrations of calibration samples. For this purpose, select the appropriate autosampler (sample tray with 88 or 148 sampling positions for the AAnalyst 800 device).
8. Measure calibration curve.
9. Now transfer each oocyte homogenate into individual 1.2 ml polypropylene sample cups (Perkin Elmer, #B0510397) and place these test tubes into the autosampler tray.
10. Create a sample information file to identify the probes in the autosampler positions, then adjust sample volume to 20 µl, and start the instrument.
11. After completion of the run (or during visual inspection every hour for long series of samples), check for probes in which the measured amount of Rb^+ exceeds the maximum of the calibration curve. If so, dilute the respective samples appropriately and measure them again.

11. Remarks for Measuring Large Sample Numbers (Above 50)

- For an overnight run of the instrument, it is recommended to fill the reservoir of the autosampler with Millipore water to prevent evaporation of homogenates in test tubes.
- It is recommended to place between each 20 samples a test tube with 2% HNO_3 to prevent clogging of the autosampler's injection capillary.

Recommended THGA-AAS temperature protocol for rubidium

Step	Temperature (°C)	Ramp Time (sec)	Hold Time (sec)	Argon Gas Flow (ml/min)
Dry 1	110	1	20	250
Dry 2	130	5	30	250
Pyrolysis	300-600	10	20	250
Atomization	1700-1800	0	5	0
Clean-out	2400	1	2	250

Recommended THGA-AAS temperature protocol for lithium

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Step	Temperature (°C)	Ramp Time (sec)	Hold Time (sec)	Argon Gas Flow (ml/min)
Dry 1	110	1	30	250
Dry 2	130	15	30	250
Pyrolysis	900	10	20	250
Atomization	2200	0	5	0
Clean-out	2450	1	3	250

Data analysis

1. For each measuring condition, calculate mean Rb^+ uptake. The AAS spectrophotometer yields values in µg/L, which need to be converted into pmol/oocyte/min.
2. For kinetic analyses (apparent KM values), subtract background Rb^+ uptake determined from uninjected control oocytes from the same batch at the given RbCl concentration.
3. Plot with Origin software, apply fit of a Hill $A = A_{max} \cdot \frac{[S]^{n_H}}{K_{0.5}^{n_H} + [S]^{n_H}}$ or Michaelis-Menten function $A = A_{max} \cdot \frac{[S]}{K_{0.5} + [S]}$ to the data. In these equations, [*S*] is the substrate concentration, nH the Hill coefficient, and $K_{0.5}$ the substrate concentration needed for half-maximal activation.

Representative Results

Quantification of Rb^+ uptake by K^+- (or Rb^+)-countertransporting P-type ATPases by AAS in the *Xenopus* oocyte expression system permits reliable determination of enzyme kinetic parameters.

Determination of the transport stoichiometry of the Na^+/K^+-ATPase

For the electrogenic Na^+/K^+-ATPase, Rb^+ fluxes can be determined in two-electrode voltage-clamp experiments aimed at the correlation between net charge transport (time integral of pump current) and Rb^+ transport. **Figure 1A** shows the recording of pump current (about 40 nA stationary current amplitude) induced by perfusion of a Na^+,K^+-ATPase-expressing oocyte with a solution containing 1 mM Rb^+. Integration of the current signal (grey shaded area under the current trace) yielded 7839 nC of total transported charge (equivalent to 0.0822 nmol of charge). When the same oocyte was subsequently subjected to the AAS determination method, a total amount of 0.176 nmol of Rb^+ was found. The ratio between total charge and Rb^+ flux was 0.47, which is close to 0.5, the value expected from the 3:2 $Na^+/K^+(Rb^+)$ transport stoichiometry of the Na^+,K^+-ATPase (net outward transport of one elementary charge corresponds to the uptake of two Rb^+/K^+ ions). Repetition of such a single-cell experiment several times (**Figure 1B**) yields a good linear correlation between total charge and Rb^+ transport, with a slope factor of 0.49. Thus, even on a single cell level, the AAS technique yields excellently reliable results.

Rb^+ flux experiments for analysis of H^+/K^+-ATPase

For the electroneutrally operating H^+/K^+-ATPase, the [Rb^+] dependence of Rb^+ uptake and its sensitivity towards the specific inhibitor SCH28080 can be determined. The data listed in **Table 1** have been acquired in a set of three experiments on different batches of oocytes, in which Rb^+ fluxes (15 min flux time) mediated by H^+,K^+-ATPase were measured in Na^+-free buffers at pH_{ex} 5.5 for different external Rb^+ concentrations. Unspecific uptake into uninjected oocytes is less than 10% of the maximally detected Rb^+ uptake, and this is insensitive to the addition of SCH28080, whereas the fluxes in H^+,K^+-ATPase-expressing oocytes are reduced to background level upon addition of 10 µM SCH28080 (at pH_{ex} 5.5). Subtraction of the non-specific Rb^+ uptake (from uninjected oocytes) yields data as shown in **Figure 2**. Black squares in **Figure 2** refer to the data from **Table 1**, whereas the open squares were acquired in a similar set of experiments, in which the Rb^+ fluxes were determined in Na^+-containing buffers. These data yield (1) values for the apparent affinities of H^+,K^+-ATPase turnover transport for Rb^+, and (2) how the apparent affinity is modified by extracellular Na^+, showing that extracellular Na^+ ions compete for the extracellular facing cation binding pocket during transport, which leads to a decrease in the apparent affinity for Rb^+ [4].

The combination of Rb^+ uptake experiments under membrane potential control by TEVC and AAS determination can also be applied to determine the voltage-dependence of $H^+/K^+(Rb^+)$ turnover transport under different pH conditions. Data originating from such a combined experiment are listed in **Table 2** showing that Rb^+ transport by H^+,K^+-ATPase is only weakly affected by changing the membrane voltage from about -10 to -100 mV. Furthermore, the data in **Table 2** show that Rb^+ transport by H^+,K^+-ATPase is stimulated by about a factor of 2 at pH_{ex} 5.5 compared to pH_{ex} 7.4. On the first sight, this result is counter-intuitive, since an increase in the extracellular H^+ concentration should rather lead to a decreased turnover rate because the enzyme has to pump against a steeper H^+ gradient. However, it could be shown by additional experiments that a controlled decrease of the cytoplasmic pH by about 0.5 units had the same stimulating effect on transport, irrespective on extracellular pH[7]. This stimulation is due to a slight acidification of the oocytes' interior upon incubation in a solution with pH_{ex} 5.5[7]. Further information to be drawn from **Table 2** is that the addition of 10 µM SCH28080 inhibitor causes different levels of inhibition at pH_{ex} 7.4 and 5.5.

This is due to the fact that only the protonated form of SCH28080 is pharmacologically active. At pH_{ex} 5.5, 50% of the compound is in the protonated, active form, whereas at pH_{ex} 7.4, it is only 1%, which leads to a much weaker inhibition[7].

Rb^+ flux experiments to investigate functional effects of H^+/K^+-ATPase mutations

Another example for the achievement potential of the AAS detection technique is shown in **Figure 3**. **Figure 3A** compares the Rb^+ fluxes for several H^+,K^+-ATPase constructs in which one (ΔY), two (ΔYY), or five (ΔQELYY) amino acids at the C-terminus of the H^+,K^+-ATPase α-subunit were deleted. For Na^+,K^+-ATPase, it has been shown that the C-terminus is a distinct structural element, which is critical for structural stabilization of the cation binding sites [14-16]. The deletion of the two C-terminal Tyr residues (ΔYY) or of the last five amino acids (ΔKETYY) dramatically affected voltage-dependent parameters of cation transport and led to the occurrence of H^+- and Na^+-stimulated stationary inward currents in the absence of extracellular K^+ [17-19]. In order to elucidate whether C-terminal truncations also affect cation transport by H^+,K^+-ATPase, we assayed Rb^+ transport activity of H^+,K^+-ATPase wild-type and C-terminal truncation constructs as shown in **Figure 3A**. These data show that Rb^+ uptake activity gradually decreases with the extent of the C-terminal deletion. Moreover, the extent of Rb^+ uptake inhibition by 10 μM SCH28080, which is high for the WT enzyme, is very much reduced for the ΔY construct and essentially absent for constructs ΔYY and ΔQELYY. At this point, it can not be directly concluded that the decrease in specific Rb^+ uptake activity is due to a characteristic effect of the mutations on the transport turnover rate, since also other explanations have to be taken into account, such as general effects on protein folding, stability, or plasma membrane targeting. To correlate the measured Rb^+ fluxes with the amount of H^+,K^+-ATPase protein in the plasma membrane, we prepared plasma membrane (PM) fractions and total cellular membrane fractions (TM) from oocytes expressing the constructs from **Figure 3A**, and subjected the samples to SDS-PAGE (http://www.jove.com/video/758/electrophoretic-separation-of-proteins)[20] and Western blotting with antibodies directed against the H^+,K^+-ATPase α-subunit. This plasma membrane purification procedure has first been described by Kamsteeg and Deen21 and, later, with some modifications in[4,22]. **Figure 3B**, lower panel, shows that the relative amounts of total H^+,K^+-ATPase α-subunit protein in the TM fraction are very similar for all H^+,K^+-ATPase constructs investigated. However, in the PM fraction (**Figure 3B** upper panel), the relative amount of protein also gradually decreases with increasing extent of the C-terminal deletion. Therefore, one can conclude from this combination of experiments that truncations at the C-terminus of the H^+,K^+-ATPase's α-subunit affect plasma membrane expression indicating that folding and structural stability of the α-subunit or association with the β-subunit might be affected by the truncations. This also serves as an explanation for the reduction of specific Rb^+ uptake. However the SCH28080 inhibition data yield additional information for the truncation constructs. The SCH28080 inhibitor targets a specific conformational substate of the catalytic cycle, the so-called $E_2(P)$ conformation (P denotes a phosphorylated intermediate) with cation binding sites oriented towards the extracellular fluid and with high affinity for Rb^+/K^+. Substantially reduced sensitivity of the C-terminally truncated proton pumps towards 10 μM SCH28080 in the Rb^+ uptake experiments (black bars in **Figure 3A**) suggests that the steady-state distribution of reaction cycle intermediates is shifted to $E_1(P)$-like conformational substates, in which the cation binding pocket is exposed to the intracellular medium[4]. These data also show that it is important to correlate functional with biochemical data, if profound mechanistic conclusions are desired.

Temperature dependence of Rb^+ fluxes

Rb^+ fluxes can also be utilized to measure thermodynamic parameters like activation energies. As shown in **Figure 4A**, Rb^+ uptake by H^+,K^+-ATPase-expressing oocytes is strongly dependent on temperature. Notably, even at 34 °C incubation temperature during Rb^+ uptake, non-specific Rb^+ uptake is still low, as can be seen from the low value of the flux at 34 °C in the presence of the SCH28080 inhibitor. Plotting the decadic logarithm of the Rb^+ uptake against the reciprocal temperature (Arrhenius plot) yields a good linear correlation of the data, resulting in an activation energy of 96 kJ/mol from the slope of the curve, a value, which is typical for enzyme-catalyzed reactions and also agrees well with values determined for pump currents of Na^+,K^+-ATPase measured in the *Xenopus* oocyte expression system[1].

Li^+ uptake measurements

Besides Rb^+, also Li^+ uptake by H^+,K^+-ATPase can sensitively be determined by the AAS technique. **Figure 5** shows that unspecific Li^+ uptake in uninjected oocytes is quite low, and that uptake increases for H^+,K^+-ATPase-expressing oocytes with increasing Li^+ concentrations up to 60 mM. This is different for Rb^+, for which saturation of Rb^+ uptake is observed already at concentrations above 5 mM. Notably, Li^+ uptake activity is larger than Rb^+ uptake activity. Furthermore, 10 μM SCH28080 hardly reduces Li^+ uptake (compare bars for 30 mM Li^+ with and without inhibitor), whereas in the case of Rb^+, inhibition is stronger. Notably, this experiment has been carried out at pH_{ex} 7.4, at which only a small fraction of the SCH28080 compound is in the active (protonated) form. Thus, Li^+ could also act in terms of inducing preference of the enzyme towards $E_1(P)$ state(s)[7] during stationary H^+/Li^+ turnover cycling.

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$[Rb^+]$ / mM	normalized Rb^+ flux for HKα-S806C + HKβ	
	no inhibitor	+ 10 μM SCH28080
5 (uninjected oocytes)	0.07 ± 0.01	0.074 ± 0.002
0.05	0.17 ± 0.01	0.05 ± 0.02
0.1	0.31 ± 0.01	0.05 ±0.03
0.25	0.50 ± 0.03	0.05 ± 0.03
0.5	0.64 ± 0.03	0.07 ± 0.04
1	0.80 ± 0.03	0.08 ± 0.05
2.5	0.83 ± 0.06	0.15 ± 0.05
5	1.00 ± 0.05	0.13 ± 0.04
Normalization value: mean Rb^+-flux (at 5 mM Rb^+) from 3 separate experiments, 29.2 pmol/oocyte/min (10-15 oocytes per data point in each experiment)		

Table 1. SCH28080 sensitivity of Rb^+ uptake by oocytes expressing HKα-S806C/HKβ. Rb^+ uptake was determined at pH 5.5 in extracellular Na^+-free solution containing different Rb^+ concentrations in absence or presence of 10 μM SCH28080. Data were normalized to Rb^+ uptake at 5 mM $[Rb^+]$ and are given as means ± S.E.M. from 10-15 oocytes out of a single batch.

	pH_{ex} 7.4 (in pmol/ooc./min)	pH_{ex} 5.5 (in pmol/ooc./min)
Vm = -100 mV	11.9 ± 1.1	24.5 ± 1.3
unclamped (-10...-20 mV)	13.9 ± 1.5	26.5 ± 1.2
control: pH_{ex} 5.5, -100 mV + 10 μM SCH28080		2.4 ± 0.8

Table 2. Voltage dependence of Rb^+ uptake by oocytes expressing HKα-S806C/HKβ. Values represent Rb^+ uptake (in pmol/oocyte/min) at 5 mM Rb^+ and pH_{ex} 7.4 or 5.5 for oocytes expressing HKαS806C/βWT, which either were held at -100 mV by two-electrode voltage-clamping, or subjected to Rb^+ uptake without voltage control (the "resting" membrane potential was measured to be between 10 and -20 mV, as determined independently). Data are means ± S.D. from oocytes of one cell batch (N=12...23).

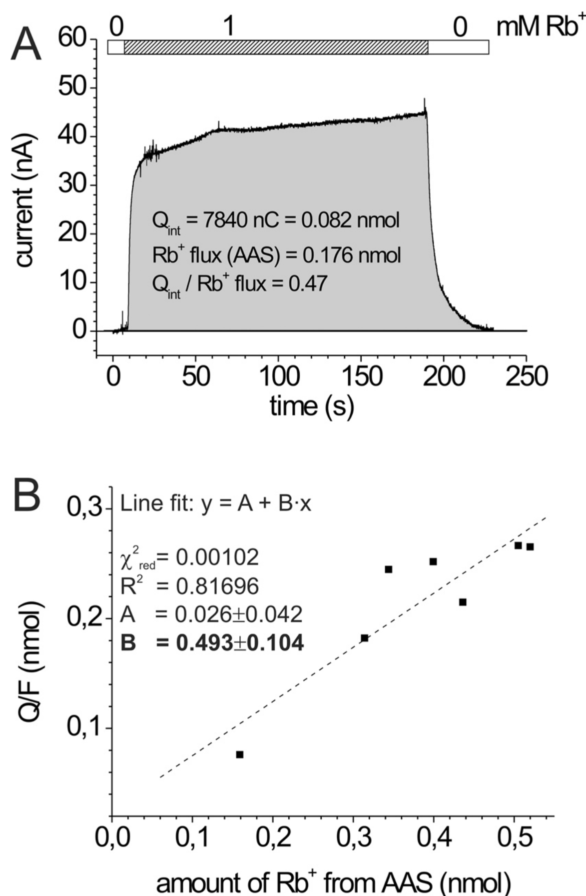

Figure 1. Correlation of Rb^+ uptake by Na^+,K^+-ATPase with pump current in two-electrode voltage-clamp (TEVC) experiments. **(A)** Stationary pump current recording at -10 mV membrane potential measured on an oocyte expressing ouabain-resistant human Na^+,K^+-ATPase α_2/β_1-subunit complexes. The pump current was initiated by solution exchange from a Rb^+-free to a 1 mM Rb^+-containing solution (white and hatched bars above current trace, respectively). The time integral of the current (here: 7840 nC) corresponds to a net outward transport of 0.082 nmol of elementary charges. When the measured oocyte was removed from the recording chamber and subjected to the AAS procedure, a total amount of 0.176 nmol Rb^+ was found in the cell, which is about 2.1 times the amount of transported charge. **(B)** Correlation between total transported charge (time integral of pump current) measured in two-electrode voltage-clamp Rb^+ flux experiments on single oocytes with the amount of Rb^+ per cell measured subsequently by AAS. The graph indicates that even with single cells, a good linear correlation (see values for the reduced χ^2 and correlation factor R^2) between charge and Rb^+ uptake can be obtained. The slope factor derived from a linear fit to the data (0.49 ± 0.10) is consistent with the uptake of 2 Rb^+ ions per transported charge, in agreement with the $3Na^+/2K^+(Rb^+)$ transport stoichiometry of the Na^+,K^+-ATPase.

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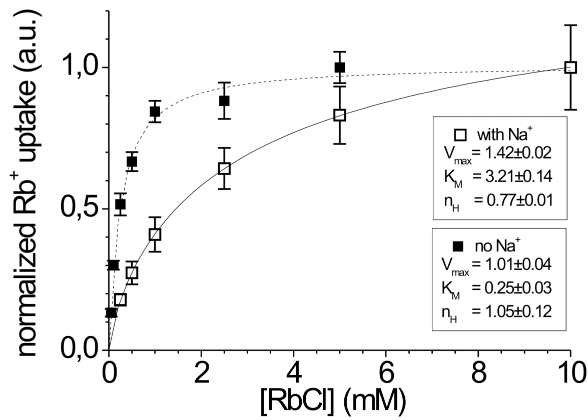

Figure 2. Concentration-dependent Rb^+ uptake by H^+,K^+-ATPase. Normalized Rb^+ uptake in the absence (filled squares) or presence (open squares) of extracellular Na^+ at pH 5.5 is depicted. Oocytes were injected with cRNA for the HKα-S806C mutant and the HKβ-subunit. Data were normalized to Rb^+ uptake at the maximal RbCl concentrations used, with the following values (in pmol/oocyte/min: 29.3, 27.4, 30.8 in absence of Na^+; 21.1, 23.4 in presence of Na^+). Data are means ± S.E.M., resulting from 8-12 oocytes out of three experiments. Apparent half-maximal activation constants $K_{0.5}$ were obtained from a fit of a Michaelis-Menten function to the data (fit curves shown as dashed and dotted line, respectively).

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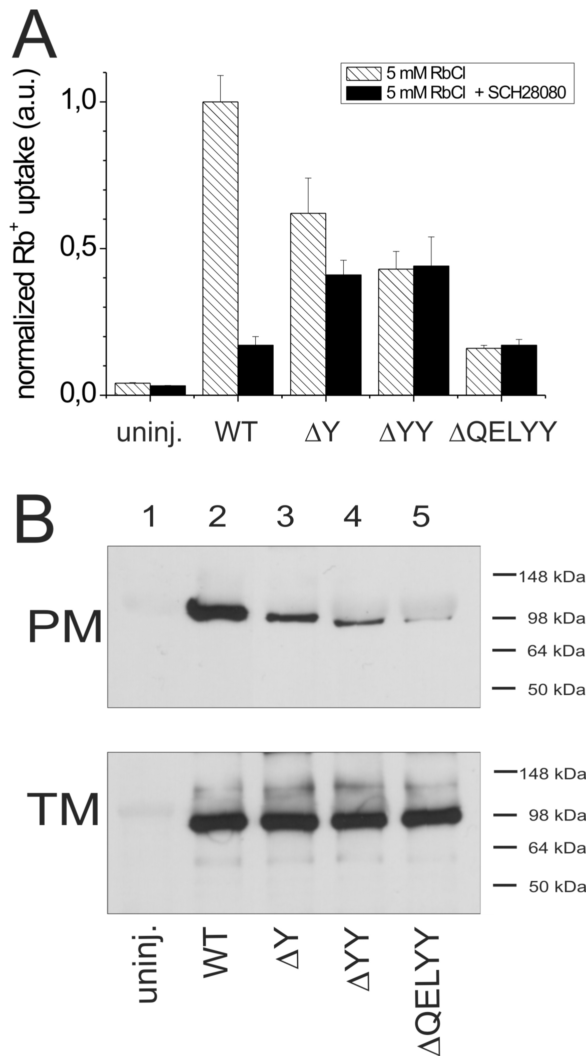

Figure 3. Rb^+ uptake by C-terminally truncated H^+,K^+-ATPase constructs. **(A)** H^+,K^+-ATPase-mediated Rb^+ uptake at 5 mM RbCl in the absence (hatched bars) or presence (black bars) of 10 µM SCH28080. Results from uninjected control oocytes, oocytes injected with cRNA of the HKβ-subunit and either HKα-S806C or HKα-constructs with the indicated C-terminal truncations are shown. Data are means ± S.E.M. from 3 individual experiments with 15-20 oocytes, normalized to Rb^+ uptake of the HKα-S806C/HKβ (corresponding to 20.4, 23.7 and 29.5 pmol/oocyte/min, respectively). **(B)** Western blot analysis of plasma membrane (PM, upper panel) and total membrane fractions (TM, lower panel) isolated from H^+,K^+-ATPase-expressing oocytes. Detection used anti-HKα-subunit antibody HK12.18[23]. One representative Western blot out of at least 3 from different oocyte batches is shown.

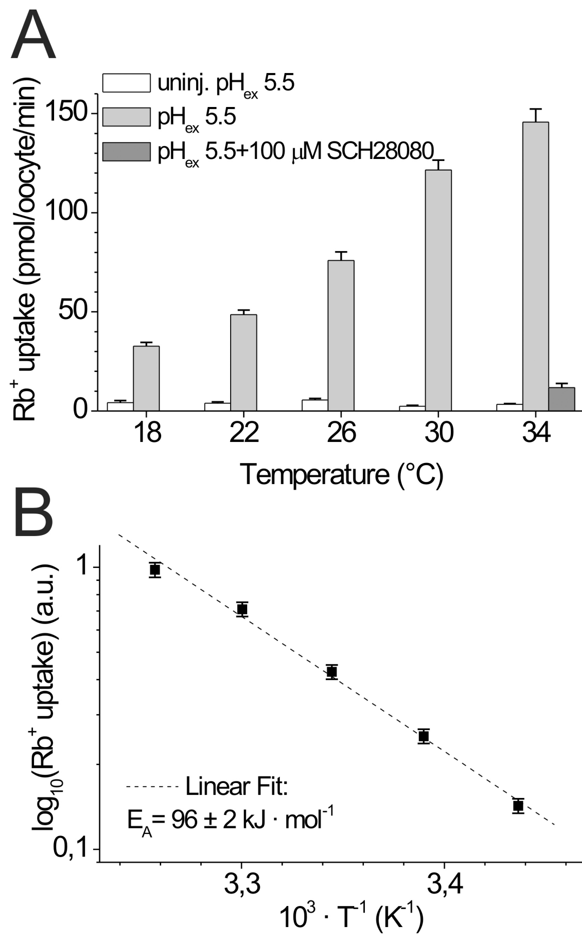

Figure 4. Temperature dependence of Rb^+ uptake by gastric H,K-ATPase. (A) H^+,K^+-ATPase-mediated Rb^+ uptake (in pmol/oocyte/min) at 5 mM Rb^+ and pH_{ex} 5.5 (light gray bars) at temperatures between 18 and 34 °C, as indicated. White bars represent Rb^+ uptake of non-injected control oocytes at each temperature under the same conditions. The gray bar at 34 °C shows Rb^+ uptake at pH_{ex} 5.5 in the presence of 10 µM SCH28080 inhibitor. Data in each column are means of 20-25 oocytes from oocytes of one cell batch. **(B)** Arrhenius diagram (plot of the decadic logarithm of Rb^+ uptake versus the reciprocal temperature) for temperature-dependent Rb^+ uptake from the data in **(A)**. Each data point is given as the mean ± S.E.M. of three independent experiments. For normalization, the Rb^+ uptake at 34 °C was taken for each experiment. The activation energy obtained from a fit of a straight line to the data (dashed line) is about 96 kJ/mol.

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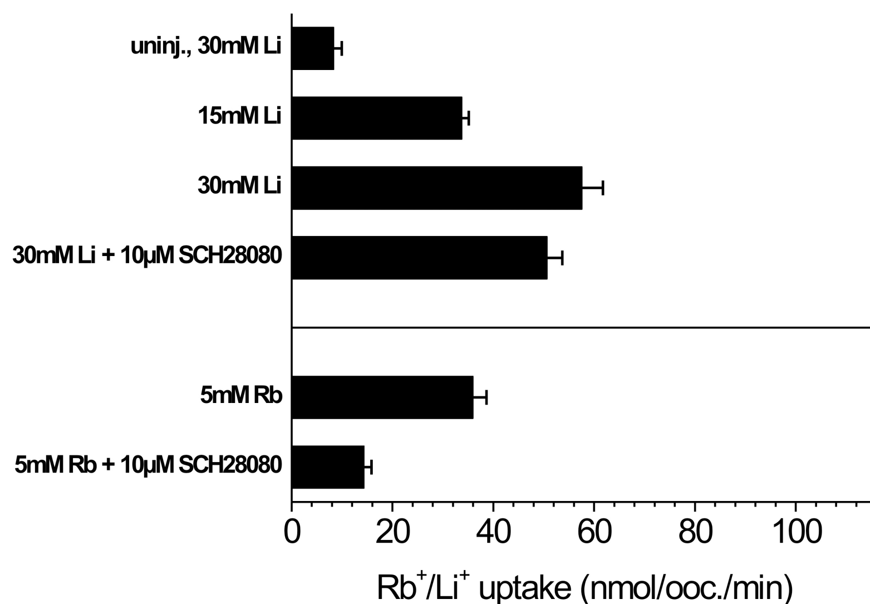

Figure 5. Comparison of Li^+ and Rb^+ uptake by H^+,K^+-ATPase. H^+,K^+-ATPase-mediated uptake of Li^+ at the indicated concentrations of LiCl, and of Rb^+ at 5 mM RbCl. The extracellular pH was 7.4. Results from uninjected control oocytes, and of H^+,K^+-ATPase-expressing oocytes in the presence of a certain $[Li^+]$ or $[Rb^+]$and 10 µM inhibitor SCH28080 are also included. Data are means ± S.E.M. from 8-15 oocytes of a single batch.

Discussion

The described method to measure the amount of Rb^+ (or Li^+) taken up into individual *Xenopus* oocytes expressing Na^+,K^+- or H^+,K^+-ATPase has proven to be a versatile, flexible and accurate technique to determine the kinetic or thermodynamic parameters of transport for cation-countertransporting P-type ATPases[1,4,5,7,8]. It is a safe and reliable alternative to radioactive tracer flux assays, and allows addressing a large scope of experimental questions within considerably short time. In accordance, only very limited data have been acquired with radiotracers[24-27]. The AAS method is particularly useful for H^+,K^+-ATPase, which - due to its electroneutral transport activity - cannot be analyzed by standard electrophysiology. However, the exemplary data shown in this protocol demonstrate that the combination of flux measurements with voltage-clamping can even resolve the voltage dependence of electroneutral transporters. From our experience, it is sufficient to employ flux times of 3 min (for Na^+,K^+-ATPase) or 15 min (for H^+,K^+-ATPase). These times could easily be reduced by adjusting the volume of fluid used to homogenize individual oocytes after uptake.

We consider the following points as crucial for the technique:

1. Oocytes should be carefully selected for integrity and homogenous size prior to cRNA injection. Integrity of oocytes should be checked thoroughly again after flux experiments, directly prior to homogenization for analysis by AAS.
2. During the Rb^+ uptake experiment, it is mandatory to keep the temperature constant. Even a diurnal ΔT of 1 degree produces significant differences in cation flux (with 90 kJ/mol activation energy more than 13% at 20 °C can be expected).
3. For Rb^+/Li^+ flux experiments (or Cs^+, which may also be counter-transported by Na^+,K^+-ATPase) in *Xenopus* oocytes, it is absolutely mandatory to pre-incubate the cells in 10 µM ouabain containing buffer and to have 10 µM ouabain present in all flux buffers during the experiment to inhibit the endogenous Na^+,K^+-ATPase.
4. Time intervals for incubation of oocytes in flux buffers should be kept constant as exactly as possible. It is recommended to acquire some dexterity regarding the fast transfer of oocytes from dish to dish before starting an experiment.
5. For uptake measurement under voltage-clamp control, sharp glass electrodes with preferably small tip opening should be used, mechanical damage to the cell membrane *e.g.* from vibrations of the electrode holders avoided, and flux times should be as short as possible to prevent unspecific entry of cations from the external buffer.
6. Plasma membrane expression of a transporter protein of interest is a critical parameter, which - if it varies much from cell to cell - can lead to a large scattering of flux data. In such a case, we recommend to measure pools of *e.g.* 5 oocytes and cross-check the measured fluxes with the amount of transporter protein at the cell surface from similar cell samples of the same batch (plasma membrane extraction, subsequent Western blotting and densitometric analysis of protein bands. CAUTION: Consider saturation effects of protein gels or films). For other types of experiments, such as those in **Figure 3**, in which the transport properties of various mutants is compared, we recommend to normalize the measured fluxes with reference to the plasma membrane expression level, to exclude ambiguities arising from divergent numbers of transporters per cell surface area due to mutations.

The described technique is not limited to Rb^+ and Li^+ transport, but could easily be extended to other trace elements, especially transition or heavy metals of biological relevance, for which AAS hollow cathode lamps are available, such as Cs, Mn, Mo, Fe, Co, Ni, Cu, or Zn.

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Furthermore, the method is not limited to K^+-countertransporting P-type ATPases. Essentially any metal ion transporter that can be expressed in the plasma membrane of *Xenopus* oocytes, and even endocytotic uptake mechanisms can in principle be investigated. In the special case of H^+,K^+-ATPase, which is not accessible by standard electrophysiology, the AAS flux method has provided deep insight into the underlying transport kinetics and structure-function relationships.

Of note, the technique also supports pharmacological screening. The gastric H^+/K^+-ATPase is an important therapeutic target for drugs known as acid suppressants or proton pump inhibitors. While there is still a strong need for improved compounds with better bioavailability, pharmacokinetics, or less side effects, only very few assays are available that meet the strict legal approval regulations. Although the THGA-AAS method with *Xenopus* oocytes will not allow for high-throughput screening, it is excellently suited for profiling *e.g.* hundreds of derivatives of promising lead structures with high information content and in reasonably short time.

Due to the high relevance of Na^+ in natural transport processes across membranes, a similarly sensitive detection of Na^+ would be highly desirable. Although we paid considerable effort on determining Na^+ by THGA-AAS, the element has proven to be very problematic, since Na^+ is a frequent and ubiquitous environmental contaminant. Even the preparation of absolutely Na^+-free reference buffers in laminar flow benches did not lead to reliable results, since our AAS device is not installed in a clean room. Thus, in the case of Na^+, other experimental approaches such as inductive coupled plasma emission spectroscopy (ICP-ES) might give better results.

Disclosures

The authors declare that they have no competing financial interests.

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