Control of Gastric H,K-ATPase Activity by Cations, Voltage and Intracellular pH Analyzed by Voltage Clamp Fluorometry in Xenopus Oocytes

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Abstract

Whereas electrogenic partial reactions of the Na,K-ATPase have been studied in detail, much less is known about the influence of the membrane potential on the electroneutrally operating gastric H,K-ATPase. In this work, we investigated site-specifically fluorescence-labeled H,K-ATPase expressed in Xenopus oocytes by voltage clamp fluorometry to monitor the voltage-dependent distribution between E1P and E2P states and measured Rb+ uptake under various ionic and pH conditions. The steady-state E1P/E2P distribution, as indicated by the voltage-dependent fluorescence amplitudes and the Rb+ uptake activity were highly sensitive to small changes in intracellular pH, whereas even large extracellular pH changes affected neither the E1P/E2P distribution nor transport activity. Notably, intracellular acidification by approximately 0.5 pH units shifted \( \Delta V_{0.5} \), the voltage, at which the E1P/E2P ratio is 50:50, by \(-100 \) mV. This was paralleled by an approximately two-fold acceleration of the forward rate constant of the E2P→E1P transition and a similar increase in the rate of steady-state cation transport. The temperature dependence of Rb+ uptake yielded an activation energy of \( \sim 90 \) kJ/mol, suggesting that ion transport is rate-limited by a major conformational transition. The pronounced sensitivity towards intracellular pH suggests that proton uptake from the cytoplasmic side controls the level of phosphoenzyme entering the E2P→E1P conformational transition, thus limiting ion transport of the gastric H,K-ATPase. These findings highlight the significance of cellular mechanisms contributing to increased proton availability in the cytoplasm of gastric parietal cells. Furthermore, we show that extracellular Na+ profoundly alters the voltage-dependent E1P/E2P distribution indicating that Na+ ions can act as surrogates for protons regarding the E2P→E1P transition. The complexity of the intra- and extracellular cation effects can be rationalized by a kinetic model suggesting that cations reach the binding sites through a rather high-field intra- and a rather low-field extracellular access channel, with fractional electrical distances of \( \sim 0.5 \) and \( \sim 0.2 \), respectively.

Introduction

Gastric H,K-ATPase, the main transporter responsible for acid secretion in the stomach, belongs to the family of P-type ATPases. A hallmark of this ATPase family is the formation of phosphorylated enzyme intermediates during the transport cycle, which is achieved by reversible phosphorylation of a highly conserved aspartate residue (Asp-385 in rat gastric H,K-ATPase). The phosphorylation and dephosphorylation reactions are coupled to conformational transitions between the two principal conformations E1 and E2 (and the respective phosphoenzyme forms E1P and E2P, respectively), for which the ion binding pocket is exposed to different sides of the membrane. Furthermore, the conformational changes are linked to characteristic changes in the affinities for the transported cations. For the reaction mechanism of Na,K-ATPase, a cyclic scheme of reversible partial reactions has been proposed, which is known as Post-Albers scheme [1,2]. Although Na,K-ATPase exchanges 3 Na+ against 2 K+ ions in an overall electrogenic transport reaction, which is in contrast to the 2:2 (or 1:1), hence electroneutral, H+ /K+ exchange mediated by the gastric H,K-ATPase, the pump cycle of gastric H,K-ATPase presumably proceeds according to a very similar reaction scheme (Fig. 1A).

Voltage-dependent partial reactions of the Na,K-ATPase have been studied in detail. It is well established that the major electrogenic event in the Na,K-ATPase cycle occurs during extracellular Na+ release (or re-uptake). This has first been inferred from the voltage-dependent inhibition of K+-stimulated stationary pump currents by extracellular Na+ [3,4,5]. Furthermore, ouabain-sensitive presteady-state currents, which occur in the absence of extracellular K+ in response to voltage pulses under conditions favoring phosphoenzyme formation (intracellular Na+ and ATP present), critically depend on the presence of extracellular Na+ [6,7,8]. These findings were interpreted in terms of a high-field ion access channel or “ion well” [9,10,11] through which the Na+ ions travel upon extracellular release from the binding sites. Since the E1P→E2P transition is rate-limiting for Na+ deocclusion/release as well as Na+ uptake/occlusion, the voltage dependence of the major charge component of the
Due to its overall electroneutral transport, much less is known about the voltage-dependent steps of gastric H,K-ATPase. Experiments in which H,K-ATPase-containing parietal cell membrane fragments were adsorbed to black lipid membranes [19,20,21] provided evidence for electrogenicity in the H⁺ limb of the transport cycle, since rapid release of ATP from caged ATP in the absence of K⁺ induced transient currents. To account for the overall electroneutrality, it was proposed that electrogenic H⁺ translocation is counter-balanced by another partial reaction of opposite electrogenicity during K⁺ translocation. Indeed, K⁺ inhibition experiments on inside-out gastric vesicles [22] revealed that an electrogenic step exists in the K⁺ branch (steps 5–9 in Fig. 1A). These studies showed that the inhibitory effect of high intracellular [K⁺] on ATPase activity was prevented at intracellularly negative K⁺ diffusion potentials, but was restored upon dissipation of the diffusion potential. Moreover, equilibrium titration experiments using the electrochromic dye RH421 on gastric membrane vesicles confirmed the electrogenicity of both K⁺ and H⁺ binding steps [23].

Several studies have shown that Na⁺ modulates function of the gastric H,K-ATPase. However, interpretation of the results was hampered because the used vesicle or membrane preparations did not allow a differentiation between intra- and extracellular effects [24,25,26,27]. We have previously found in Rb⁺ uptake experiments using Xenopus oocytes that extracellular Na⁺ reduces the apparent affinity for Rb⁺ about 7-fold, thus indicating a competition between Na⁺ and Rb⁺ [28]. This behavior is quite similar to the Na,K-ATPase, which exhibits significantly decreased apparent K⁺ affinity in the presence of extracellular Na⁺ as well [14]. In order to understand the function of H,K-ATPase within its physiological context, it is mandatory to study the complexity of extra- and intracellular cation effects and their voltage dependence in intact cells.

A suitable technique for this purpose is voltage clamp fluorometry (VCF), which senses voltage-dependent partial reactions even in transporters that operate net electroneutrally. Initially, VCF has been pioneered for the detection of conformational rearrangements of voltage sensing segments in voltage-gated cation channels [29,30]. To enable SH-reactive coupling of fluorescent dyes for site-specific labeling, cysteine mutations are introduced into extracellular loops of the protein, usually at the interface between the membrane and the extracellular space. Here, conformational transitions may change in the dye’s microenvironment and induce variations in fluorescence intensity, which, depending on the photophysical properties, can be due to local changes in the electrostatics, hydrophobicity, pH, or differential access of fluorescence quenchers. Tetramethylrhodamine-maleimide (TMRM) has proven distinctly useful, since it is particularly sensitive to solvent polarity and collisional quenching by water [29]. Thus, its fluorescence increases upon movement into a sheltered, hydrophobic environment, and quenching occurs upon exposure to the aqueous phase. When TMRM was used to label the Shaker K⁺ channel (mutation M536C at the N-terminal part of the S4 segment, which carries most of the gating charge), a good kinetic correlation between fluorescence changes and the gating charge integral was found. Other dyes like fluoresceine-maleimide or Oregon Green maleimide attached to the same residue produced kinetically more complex responses [29] indicating that these fluorophores encounter a series of different microenvironments, which are sometimes difficult to correlate with functional properties. For the Na,K-ATPase, it has been shown that TMRM labeling close to the extracellular end of the central M5 helix (mutation N790C, sheep α₁-subunit), leads to fluorescence signals with properties similar to those of presteady-state currents [31] indicating that the same molecular event, the E₁P→E₄P transition, is reported. For H,K-ATPase, TMRM

**Figure 1. Reaction cycle of gastric H,K-ATPase.** (A) Reaction mechanism of gastric H,K-ATPase adapted from the Post-Albers scheme [1,2], which had originally been postulated for the related Na,K-ATPase. Upon intracellular binding of protons to the E₁ conformation (step 1), a phosphorylintermediate with occluded H⁺ ions (E₁P(H⁺)) is formed (step 2), and after a conformational change to E₂P (step 3), protons dissociate to the extracellular space (step 4). Subsequently, K⁺ ions bind from the extracellular side (step 5) and become occluded, a process which stimulates dephosphorylation (step 6), and after a conformational change from E₂ to E₃ (step 7) the K⁺ ions are intracellularly released (step 8). The gray box indicates the reaction sequence which can be studied by voltage pulses at [K⁺]ext = 0 in VCF experiments. (B) Pseudo-three-state model for the reaction sequence including steps 1 to 4 in (A). A detailed description and analysis of this kinetic scheme is provided in Supporting Information (Appendix S2).

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labeling at homologous position (mutation S806C, Figure S2), produces similar fluorescence signals upon voltage pulses in the absence of extracellular K\(^+\), which presumably reflect the E_{1P}\text{--}E_{2P} relaxation as well [28,32,33,34].

In the present study, we used the VCF technique and carried out Rb\(^+\) uptake measurements under various pH and ionic conditions upon expression of gastric H,K-ATPase in *Xenopus* oocytes. This dual approach allowed us to gain information about the voltage dependence of the overall pump process as well as that of a subset of partial reactions involving the E_{1P}\text{--}E_{2P} conformational change and the ion translocation steps linked to it. Our data show that the E_{1P}\text{--}E_{2P} distribution and stationary cycle.

### Materials and Methods

**Ethics statement**

Surgical removal of ovary tissue from adult *Xenopus laevis* females followed registered protocols approved by the relevant state authority (Landesamt für Gesundheit und Soziales Berlin, Reg. No. O 0308/06) and the local ethics committee (Tierschutzberat), in strict accordance with the German Animal Protection Act (Tierschutzgesetz). Animals were anesthetized by immersion in water containing 0.2% w/v tricaine (MS-222, Sigma, Deisenhofen, Germany) for 5 min, and subsequently placed on ice during surgical treatment. All efforts were made to minimize animal suffering.

**Protein expression in *Xenopus* oocytes**

*Xenopus* oocytes were obtained by collagenase treatment after partial ovariectomy from *Xenopus laevis* females. cRNAs were prepared using the SP6 mMessage mMACHINE Kit (Agilent Technologies, Santa Clara, CA). A 50 nl aliquot containing 20–25 ng rat gastric H,K-ATPase \(\alpha\)-subunit cRNA and 5 ng wild-type H,K-ATPase \(\beta\)-subunit cRNA was injected into each cell. The variant HK\(_{\alpha}5806C\), which carries the mutation S806C within the MS/M6 loop to enable site-specific labeling with TMRR (see Figure S2), was used as parent construct for mutagenesis and is termed “wild-type” herein. The S806C mutation does not affect ion translocation activity [32,33]. Mutagenesis was performed by recombinant PCR and verified by DNA sequencing (Euromix MGW Operon, Ebersberg, Germany). After cRNA injection, oocytes were kept in ORI buffer (110 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 5 mM HEPES, pH 7.4, plus 50 mg/l gentamycin) at 18°C for two days.

**Experimental solutions**

Experimental solutions were Na buffer, TMA buffer, NMDG buffer, (20 mM TEACL, 5 mM BaCl\(_2\), 5 mM NiCl\(_2\) and 90 mM NaCl, TMA-Cl or NMDG-Cl, respectively). For measurements at extracellular pH (pH\(_{\text{ex}}\)) of 7.4, the solution was buffered with 10 mM HEPES, whereas 10 mM MES was used for experiments at pH\(_{\text{ex}}\) 5.5. The pH of the buffers used is indicated by a lower index. For measurements in presence of extracellular K\(^+\), 5 mM NaCl of the Na\(_2\) buffer (or Na\(_5\) buffer) were replaced by 5 mM K\(^+\). For intracellular acidification measurements in Na\(_2\) buffer, 40 mM NaCl were replaced by 40 mM Na-butyrate. At pH\(_{\text{ex}}\) 7.4, a concentration of 40 mM butyrate corresponds to 100 \(\mu\)M undissociated (thus membrane-permeable) butyric acid, which was reported to increase cytosolic [H\(^+\)] of oocytes from 50 nM to 160 nM (corresponding to an intracellular pH change from \(\sim 7.3\) to \(\sim 6.0\) [35]). All solutions contained 100 \(\mu\)M ouabain to inhibit the endogenous *Xenopus* Na,K-ATPase.

**Voltage clamp fluorometry**

Site-specific labeling of H,K-ATPase \(\alpha\)-subunit mutant S806C (HK\(_{\alpha}5806C\)) upon expression oocytes was achieved by incubating oocytes in Na\(_2\) buffer with 5 mM TMRR (tetramethylrhodamine-6-maleimide, Molecular Probes) for 5 min at room temperature in the dark, followed by extensive washes in dye-free Na\(_2\) buffer. Labeled oocytes were transferred into an oocyte perfusion chamber (model RC-10, Warner Instr., Hamden, CT), which was mounted on the stage of an epifluorescence microscope (Axioskop 2FS; Carl Zeiss, Göttingen, Germany) equipped with a 40× water immersion objective (numerical aperture = 0.8). Fluorescence was excited with a 100 W tungsten lamp using a 535DF50 excitation filter, a 565 EFLP emission filter and a 570DRLP dichroic mirror (Omega Optical, Ballstorough, USA). Fluorescence monitoring used a PIN-022A photodiode (United Detector Technologies, Torrence, CA) mounted to the microscope camera port, whose photocurrents were amplified by a DLPCA-200 low-noise current amplifier (FEMTO Messtechnik GmbH, Berlin, Germany). Control of transmembrane voltage was achieved by means of a Turbotec 05 two-electrode voltage clamp amplifier (npi, Tamm, Germany). Fluorescence and current signals were recorded simultaneously using a Digidata 1322A interface and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA).

**Rb\(^+\) uptake assay**

Two days after injection, non-injected control oocytes and H,K-ATPase-expressing oocytes were preincubated for 15 min in TMA\(_2\) buffer, containing 100 \(\mu\)M ouabain for complete inhibition of the endogenous Na,K-ATPase. Oocytes were then incubated for 15 min in Rb\(^+\)-flux-buffer (5 mM RbCl, 85 mM TMACl (NMGC1 or NaCl), 20 mM TEACL, 5 mM BaCl\(_2\), 5 mM NiCl\(_2\), 10 mM MES, pH 5.5 or pH 7.4, 100 \(\mu\)M ouabain). For intracellular acidification measurements in Na\(_2\) buffer, 40 mM NaCl in the Rb\(^+\)-flux-buffer were replaced by 40 mM Na-butyrate. In NMDG- or TEA-based solutions, 40 mM NaCl in the Rb\(^+\)-flux-buffer were replaced by 40 mM Na-butyrate. For intracellular acidification measurements in Na\(_2\) buffer, 40 mM NaCl in the Rb\(^+\)-flux-buffer were replaced by 40 mM Na-butyrate. For intracellular acidification measurements in Na\(_2\) buffer, 40 mM NaCl in the Rb\(^+\)-flux-buffer were replaced by 40 mM Na-butyrate. For intracellular acidification measurements in Na\(_2\) buffer, 40 mM NaCl in the Rb\(^+\)-flux-buffer were replaced by 40 mM Na-butyrate. For intracellular acidification measurements in Na\(_2\) buffer, 40 mM NaCl in the Rb\(^+\)-flux-buffer were replaced by 40 mM Na-butyrate. For intracellular acidification measurements in Na\(_2\) buffer, 40 mM NaCl in the Rb\(^+\)-flux-buffer were replaced by 40 mM Na-butyrate.
**Results**

**Effect of extra- and intracellular pH on presteady-state fluorescence changes**

The aim of this study was to investigate electrogenic partial reactions within the H⁺ translocating branch of gastric H,K-ATPase and to scrutinize, whether and how the concept of high-field access channels established for Na,K-ATPase [12] can be transferred to the H⁺ pump. For that purpose, we performed voltage clamp fluorometry on gastric H,K-ATPase mutant S806C under various ionic conditions. Figure 2A shows typical fluorescence signals at pH<sub>ex</sub> 7.4 resulting from voltage pulses to a series of test potentials between −180 mV and +60 mV, which were recorded in the absence of K⁺ and with 90 mM Na⁺ in the extracellular solution. The fluorescence of the dye attached to the extracellular end of helix M5 (see structural model in Figure S2), increases upon jumps to negative potentials and decreases at depolarizing membrane voltage. In analogy to Na,K-ATPase, positive voltages should favor the transition to E₂P, whereas negative voltage steps drive the enzyme into E₁P. According to the crystal structures of several reaction intermediates of the related SERCA Ca⁺²-ATPase [36,37,38,39], the central helix M5 moves in relation to the surrounding helices during the cycle. Since TMRM is sensitive to hydrophobicity and collisional quenching, the observed fluorescence signals presumably result from a motion of the extracellular end of M5 from a buried, sheltered environment in E₁P into a more aqueous, quenching environment in E₂P.

Although it would be desirable to study the H⁺ pump under physiological working conditions (i.e. pH<sub>ex</sub> down to −1), it was not possible to apply such large [H⁺] gradients in our experiments. The recording of a single set of VCF traces as shown in Fig. 2A requires absolutely stable fluorescence for at least 90 s, but at pH values lower than 5.3 the signal quality was too poor for kinetic analyses. Furthermore, as we show below, extracellular acidification leads to significant proton leakage into the cells, which not only impairs long-term stability of the cells, but also leads to an ill-defined [H⁺] gradient. For the sake of reliable working conditions, we had to restrict our analyses to the pH<sub>ex</sub> range between 7.4 and 5.5, which - in terms of H⁺ concentration - is still an about 100-fold difference.

A change in the pH<sub>ex</sub> from 7.4 to 5.5 altered the fluorescence signals profoundly. At pH<sub>ex</sub> 7.4 (Fig. 2A), the largest fluorescence changes occurred at positive voltages, at which the transition to E₂P should be favored, whereas the opposite was observed at pH<sub>ex</sub> 5.5 (Fig. 2B), with fluorescence changes being largest upon negative voltage steps, which should drive the enzyme into E₁P. Plotting the steady-state fluorescence amplitudes against the membrane potential resulted in sigmoidal (1/ΔF/F)-V distributions, which could be approximated by a Boltzmann-type function (Fig. 2C). Of note, the pH<sub>ex</sub> change from 7.4 to 5.5 resulted in a

![Figure 2. Effects of extracellular pH on the E₁P/E₂P distribution of gastric H,K-ATPase.](image-url)

(A,B) Fluorescence responses of site-specifically labeled gastric H,K-ATPase under K⁺-free conditions (90 mM Na⁺ in the extracellular solution) upon voltage jumps from −40 mV to voltages between −180 mV and +60 mV in (−20 mV steps, see inset in A) at an extracellular pH of 7.4 (A) or 5.5 (B). (C) Voltage dependence of normalized fluorescence amplitudes (1-ΔF/F) from experiments as in (A,B) for pH<sub>ex</sub> 5.5 (●), and for pH<sub>ex</sub> 7.4 (○). Data are means ± S.E. of 11–14 oocytes. Superimposed as dashed lines are curves resulting from fits of a Boltzmann-type function to the data sets (pH<sub>ex</sub> 7.4: V<sub>0.5</sub> = −19.7±5.4 mV, z<sub>q</sub> = 0.26±0.02; pH<sub>ex</sub> 5.5: V<sub>0.5</sub> = −126.4±16.6 mV, z<sub>q</sub> = 0.27±0.04). The fluorescence amplitudes 1-ΔF/F were normalized to the difference between the saturation values at positive or negative potentials, respectively, as obtained from the fits. (D) Reciprocal time constants (1/t<sub>2</sub>) from fits of a single exponential function to voltage jump-induced fluorescence changes under K⁺-free conditions at pH<sub>ex</sub> 5.5 (●) and pH<sub>ex</sub> 7.4 (○). Data are means ± S.E. from 15–17 oocytes. (E,F) Graphs showing the forward (k<sub>f</sub>) and reverse (k<sub>b</sub>) rate constants of the E₁P→E₂P transition at pH<sub>ex</sub> 7.4 (E) and pH<sub>ex</sub> 5.5 (F), as calculated from the observed k<sub>f</sub> and k<sub>b</sub> values, as summarized in Table 1. As summarized in Table 1, the following parameters (rate constants at 0 mV: k<sub>f</sub>(0), k<sub>b</sub>(0), and z<sub>q</sub> values), as summarized in Table 1, were: pH<sub>ex</sub> 7.4 (E): k<sub>f</sub>(0) = 2.61±0.05 s<sup>−1</sup>, z<sub>q</sub> = −0.06±0.01; k<sub>b</sub>(0) = 2.31±0.10 s<sup>−1</sup>, z<sub>q</sub> = 0.21±0.01; pH<sub>ex</sub> 5.5 (F): k<sub>f</sub>(0) = 4.56±0.05 s<sup>−1</sup>, z<sub>q</sub> = −0.056±0.003; k<sub>b</sub>(0) = 1.22±0.10 s<sup>−1</sup>, z<sub>q</sub> = 0.230±0.004.

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strong negative $V_{0.5}$ shift of the $(1-AF/F)-V$ distribution by $-103$ mV (Fig. 2C and Table 1). This observation is puzzling since it would indicate that an increase of the extracellular proton concentration increases formation of E2P, in contrast to established paradigms about the electrogenicity of Na,K-ATPase. For the Na$^+$ pump, an increase of [Na$^+$]$_{ext}$ shifts the voltage-dependent distribution of the slow charge from ouabain-sensitive transient currents (Rakowski, 1993, Holmgren et al. 2000) towards positive potentials. Hence, high [Na$^+$]$_{ext}$ drives the E1P/E2P equilibrium of the Na$^+$ pump towards E1P, in line with the concept of an extracellular access channel for Na$^+$ ions.

**pH effects on presteady-state kinetics of gastric H,K-ATPase**

To delineate the processes underlying this behavior of the H,K-ATPase, we analyzed the kinetics of the voltage step-induced conformational changes. Figure 2D shows the voltage dependence of the reciprocal time constants ($τ^{-1} = k_{o0}$) obtained from fitting single exponential functions to the fluorescence signals at the two different pH$a$ values. Assuming that the fluorescence signals directly reflect the redistribution between E1P and E2P in response to voltage steps, a simplified two-state kinetic model can be used to derive information about the kinetics of the forward and the backward reaction (see Appendix S1 and [40]). Within this framework, the observed reciprocal time constants ($τ^{-1} = k_{o0}$) represent the sum of the voltage-dependent rate constants for the forward ($k_f$) and the backward reaction ($k_b$) of the E1P→E2P transition ($τ^{-1} = k_{o0} = k_f + k_b$), which is coupled to extracellular cation uptake or release steps. From the actual poise of the E1P/E2P distribution (reflected by the $(1-AF/F)-V$ curve) and $k_{tot}$ the individual forward and backward rate constants $k_f$ and $k_b$ at each membrane potential $V$ can be calculated (Eq. A6 and Eq. A7 in Appendix S1). Subsequently, the voltage-dependent values $k_f(V)$ and $k_b(V)$ can be fitted by a single exponential function (according to Eq. A1 and Eq. A2 in Appendix S1). From these fits, the parameters characterizing the voltage dependence of $k_f$ and $k_b$ can be determined, such as the values for the equivalent charge, $z_{q,f}$ and $z_{q,b}$, and the rate constants at 0 mV membrane voltage, $k_f(0)$ and $k_b(0)$. Table 1 summarizes these parameters for all data sets analyzed in this work. Notably, at strongly negative potentials, the reciprocal time constants ($τ^{-1} = k_{o0}$) were similar for both pH$a$ values (Fig. 2D). Since the total rate constant $k_{o0}$ at negative potentials should mainly be determined by $k_b$ of the backward reaction, this observation indicates that extracellular acidification does not accelerate the reverse reaction (E2P→E1P). The calculated $k_b$ values (Fig. 2E and 2F) are even lower at acidic pH$a$, with $k_b(0)$ values showing a reduction by about 50% upon a change from pH$a$ 7.4 to 5.5 (Table 1). At positive voltages, however, the reciprocal time constants ($τ^{-1} = k_{o0}$) at pH$a$ 5.5 were nearly two-fold larger than at pH$a$ 7.4 (Fig. 2D), which is reflected by a similar increase in $k_f$ (Fig. 2E,F and Table 1 for $k_f(0)$ and $k_f(0)$ values). These observations indicate an acceleration of the forward reaction (E1P→E2P) by extracellular acidification, again contradicting the expectations from an extracellular access channel concept.

The slope factors $z_{q,f}$, which characterize the voltage dependence of the $k_b$ values in Fig. 2E and 2F, are very similar. Notably, the backward rate constant carries most of the voltage dependence ($z_{q,b}$ values 0.21 to 0.23, similar to the slope factor $z_q$ from fits of the corresponding $(1-AF/F)-V$ curves with a Boltzmann-type function), whereas the forward reaction is only weakly voltage-dependent ($z_{q,f}$ values 0.05 to 0.06).

**Effects of intracellular pH changes on the E1P/E2P distribution**

Since the H,K-ATPase binds protons intracellularly at around pH 7 but releases them extracellularly against a luminal pH of up to $\sim$1, it is conceivable that the proton pump might be rather unaffected by pH$a$ changes from 7.4 to 5.3, whereas the enzyme may be much more sensitive to minute pH changes at the intracellular side. Therefore, we asked whether the observed $V_{0.5}$ shifts of the $(1-AF/F)-V$ curves could be due to an influence of the extracellular pH on the pH inside the oocytes. In fact, several studies on Xenopus oocytes reported small pH$a$ changes upon extracellular acidification [35,41,42], with pH$a$ 5.3 causing a drop in the intracellular pH by about 0.5 units [42]. To test this hypothesis experimentally, we carried out an “acid-bath procedure” by adding the weak organic acid butyric acid to the extracellular solution. Butyric acid can permeate the plasma membrane in its neutral form and dissociate intracellularly, thereby allowing a controlled intracellular acidification to be achieved (see Material and Methods). To acidify the oocyte interior by $\sim$0.5 pH units, 40 mM NaGl was replaced by an equal amount of Na-butyrate at pH$a$ 7.4, as pioneered elsewhere [35]. In Figure 3, the effects on the voltage-dependent fluorescence signals after a solution exchange from butyrate-free (Fig. 3A) to a butyrate-containing solution (Fig. 3B), and back to butyrate-free solution (Fig. 3C,D) are shown. Notably, the fluorescence changes at pH$a$ 7.4 in presence of 40 mM butyrate (Fig. 3B) were very similar to the ones observed at pH$a$ 5.3 (Fig. 3E). The $(1-AF/F)-V$ curves (Fig. 3F) show that at the same pH$a$ of 7.4, the $V_{0.5}$ value of the conformational distribution in presence of butyrate is shifted to negative potentials in essentially the same way as observed at pH$a$ 5.3 in butyrate-free solution. This supports the hypothesis that the observed $V_{0.5}$ shift of the $(1-AF/F)-V$ distribution at pH$a$ 5.3 entirely results from intracellular acidification. The concept that the H,K-ATPase is tightly regulated by intracellular pH is further supported by the fact that the reciprocal rate constants of the E1P→E2P relaxation in the presence of 40 mM butyrate at pH$a$ 7.4

**Table 1. Parameters characterizing the voltage dependence of the E1P→E2P conformational transition.**

| ion, pH | $V_{o.5}$/mV | $z_q$ | $k_f(0)$s$^{-1}$ | $z_{q,f}$ | $k_b(0)$s$^{-1}$ | $z_{q,b}$ |
|---------|------------|-------|----------------|--------|----------------|--------|
| Na$^+$, pH$a$ 7.4 | $-19.7 \pm 5.4$ | 0.26 ± 0.02 | 2.61 ± 0.05 | 0.06 ± 0.01 | 2.31 ± 0.10 | 0.21 ± 0.01 |
| TMA$^+$, pH$a$ 7.4 | $-89.6 \pm 3.3$ | 0.48 ± 0.04 | 3.34 ± 0.12 | 0.11 ± 0.01 | 0.87 ± 0.07 | 0.30 ± 0.02 |
| Na$^+$, pH$a$ 7.4 | $-126.4 \pm 16.6$ | 0.27 ± 0.01 | 4.56 ± 0.06 | 0.06 ± 0.01 | 1.22 ± 0.02 | 0.23 ± 0.01 |
| TMA$^+$, pH$a$ 7.4 | $-125.2 \pm 11.4$ | 0.49 ± 0.07 | 3.98 ± 0.18 | 0.09 ± 0.02 | 0.53 ± 0.06 | 0.33 ± 0.02 |

Parameters from fits of a Boltzmann-type function to the data in Fig. 2C and 5A,B ($V_{o.5}$ and $z_q$) and parameters characterizing the voltage dependence of forward and backward rate constants $k_f$ and $k_b$ from data in Figure 2EF and 5D,E,G,H (see Appendix S1). 
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7.4 were very similar to those measured at pHex 5.5 (Fig. 3G). As already outlined for the rate constants at pHex 5.5 (Fig. 2F), the observed increase in $k_{\text{tot}}$ at positive potentials must largely be due to an increased rate constant $k_f$ for the forward reaction, for which a dependence on intracellular pH is rather straightforward. The effects of butyrate on intracellular pH and the conformational
distribution of the gastric H,K-ATPase were strictly reversible. Already a few minutes after a solution exchange to butyrate-free solution (Fig. 3C,D), the fluorescence signals were almost identical to the initially observed fluorescence signals at pH₆ₓ 7.4 (Fig. 3A), in agreement with a time constant of ~315 s determined by Stewart et al. for the recovery of intracellular pH after withdrawal of butyrate [35].

Effect of extra- and intracellular pH on steady-state cation pumping

To test the significance of the described pH effects on stationary H⁺/K⁺ exchange transport, we measured the Rb⁺ transport activity of the H,K-ATPase under turnover conditions at saturating Rb⁺ concentrations (5 mM). As shown in Figure 3H, Rb⁺ uptake of the gastric H,K-ATPase at pH₆ₓ 5.5 was more than two-fold larger than the transport activity at pH₆ₓ 7.4. Again, the effect of the extracellular acidification could be attributed to an intracellular pH decrease, since almost the same increase in transport activity was observed at pH₆ₓ 7.4 in presence of 40 mM butyrate. These findings strongly suggests that the availability of protons at the intracellular side is not only rate-limiting for the EᵢP→E₂P transition (Fig. 3G), but also for the turnover rate during stationary cation pumping. Notably, the relatively increased EᵢP preference of the enzyme at neutral intracellular pH [pH₆ₓ 7.4 without butyrate, Fig. 2C] compared to conditions of slight intracellular acidification [pH₆ₓ 5.3 or pH₆ₓ 7.4 with butyrate] was also reflected by distinct differences regarding the inhibition by 10 μM SCH28080 (black bars in Fig. 3H). To understand the effects of this reversible, EᵢP/E₂P-specific, K⁺-competitive inhibitor, two aspects have to be kept in mind. First it must be considered that only the protonated form of SCH28080 is pharmacologically active, with deprotonation occurring with a pKᵣ of 5.5 [43,44]. Consequently, at pH₆ₓ 7.4 only about 1% of the total inhibitor concentration (i.e. ~0.1 μM) is in the protonated, active form, which is in the same range as the IC₅₀ values determined for this K⁺-competitive antagonist (67 nM for [K⁺] = 0, and 460 nM for [K⁺] = 10 mM [45]; 0.18 μM and 0.66 μM for K⁺-stimulated ATPase activity [43]), whereas at pH₆ₓ 5.5 about 50% of the compound is active. The second important point is the fractional amount of enzyme molecules in E₁P or E₂P, because the inhibitor is specific for these intermediates. Therefore, the highly effective inhibition at pH₆ₓ 5.5 is on one hand due to the higher abundance of the active compound, and on the other hand due to the strong shift towards the E₁P-state (at potentials around −10 mV, which are relevant for the Rb⁺ flux measurements without voltage control, see Fig. 2C and 3F) caused by the concomitant decrease in intracellular pH. However, the higher extent of SCH28080 inhibition at the same pH₆ₓ of 7.4, depending on whether the cytoplasm is acidified by the addition of butyrate or not (Fig. 3H, black bars), indicates that intracellular acidification indeed entails a higher E₁P preference of the H,K-ATPase.

Changes in the conformational distribution in response to extracellular K⁺

Of note, all VCF experiments shown so far were done under K⁺/Rb⁺-free conditions and therefore do not reflect the conditions of the Rb⁺ fluxes in Fig. 3H. In order to discriminate the effects of different monovalent cations on the E₁P/E₂P distribution, we first measured the changes of the voltage-dependent fluorescence signals under H⁺/K⁺ turnover conditions (Fig. 4). After a change from K⁺-free solution to 5 mM K⁺, the magnitudes of fluorescence changes were substantially reduced at both investigated pH₆ₓ normal values (Fig. 4A,B), with the effect being more pronounced at pH₆ₓ 7.4 (compare black bars in Fig. 4D). This is very similar to the effect of K⁺ on the fluorescence changes of TMRM-labeled Na,K-
ATPase [31]. Cyclic turnover at high K+ concentrations results in a redistribution of enzyme molecules over all reaction cycle intermediates, thereby increasing the accumulation of states (e.g. dephosphorylated E1-type intermediates), whose occupancies are insensitive to transmembrane voltage. This, in effect, diminishes the number of pump molecules that contribute to the fluorescence changes related to the voltage-dependent redistribution between E1P and E2P states. Due to the proposed acceleration of the rate-limiting step early within the H+ branch of the cycle by slight intracellular acidification (as a result of the pHex change to 5.5), fewer molecules are kinetically trapped in voltage-insensitive intermediates, which explains the significantly larger fluorescence changes under turnover conditions at pHex 5.5. A comparison of the time course of the fluorescence responses to −180 mV and +60 mV from Figure 4A in the absence of K+ and at 5 mM K+ (see normalized signals in Fig. 4C) shows that K+ accelerates the conformational relaxation at negative as well as positive potentials. This global acceleration of rate constants indicates that K+ opens up a second relaxation pathway (E2P→E2→E1) that occurs in addition to the E1P→E2P relaxation.

Na+ effects on presteady-state fluorescence changes of gastric H,K-ATPase

In a previous publication, we found indications for a competition between Na+ and Rb+ at the extracellular binding sites [28], since the apparent affinity for extracellular Rb+ in Rb+-uptake experiments was reduced about 7-fold in the presence of extracellular Na+. To scrutinize, whether such competitive effects of extracellular Na+ ions also affect the voltage dependence and kinetics of the E1P→E2P transition, we compared the voltage dependence of the fluorescence amplitudes and of the respective reciprocal time constants in extracellular Na+-free and Na+-containing solutions (Fig. 5). Notably, the parameters zq for the (1-ΔF/F)-V distributions were larger in the absence than in the presence of Na+ at both pHex values (Fig. 5A,B), which will be rationalized in the Discussion and Supporting Information (Appendix S2 and Appendix S3). Furthermore, the presence of extracellular Na+ had a large effect on the V0.5 values of the E1P/E2P distribution at pHex 7.4 (Fig. 5A and Table 1), but not at pHex 5.5 (Fig. 5B and Table 1). From the voltage dependence of the reciprocal time constants (τ−1=k−1) (Fig. 5C,F) and the k−1 and
$k_b$ values calculated thereof (Fig. 5D,E and Fig. 5G,H), it is evident that extracellular $Na^+$ accelerates the reverse rate constants $k_f(0)$ by a factor of $\sim 2.5$ at both $pHex$ values. This indicates that extracellular $Na^+$ ions, which are by far more abundant than protons at $pHex$ 7.4 as well as 5.5, can act as $H^+$ analogs when the binding sites face the extracellular medium (see Discussion). In contrast, $k_f(0)$ is only slightly changed ($\sim 22\%$ decrease at $pHex$ 7.4, Fig. 5D,E; $\sim 15\%$ increase at $pHex$ 5.5, Fig. 5G,H) compared to $Na^-$-free conditions (Table 1). Therefore, extracellular $Na^+$ mainly accelerates the reverse rate constant $k_b$ whereas $k_f$ is essentially unchanged, and the total rate constant is consequently increased only at negative voltages, in notable contrast to the effect of $K^+$ on the relaxation kinetics (see Discussion).

Temperature dependence of steady-state pump activity

$Rb^+$ uptake measurements were carried out at different temperatures to determine the activation energy of $Rb^+$ transport at $pHex$ 5.5 and $pHex$ 7.4. As shown in Figure 6A, the $Rb^+$ transport activity at $pHex$ 5.5 was substantially larger than at $pHex$ 7.4 in the whole temperature range covered by our experiments (18–34°C). Arrhenius plots yielded linear relationships at both investigated $pH$ (Fig. 6B). At $pHex$ 7.4, we consistently observed in several independent experiments that the data points corresponding to a temperature of 34°C significantly diverged from the linear function defined by the other data points. Such a behavior is not uncommon, as exemplified by the temperature dependence of $K^+$-stimulated pump currents of Na,K-ATPase expressed in oocytes, for which a reduced slope of the Arrhenius plot at temperatures above 26°C was observed too [46]. Exclusion of the data point for 34°C at $pHex$ 7.4 yielded activation energies of similar magnitude at both $pHex$ conditions (95.8±1.7 kJ at $pHex$ 5.5 versus 91.7±3.7 kJ at $pHex$ 7.4). The close similarity of these values suggests that $Rb^+$ uptake of the gastric proton pump at both $pH$ values is rate-limited by the same partial reaction, and due to the high activation energy, this step is likely not to be diffusion-controlled, but might be related to a major conformational change.

Voltage dependence of steady-state cation transport

To assess the voltage dependence of the overall pump activity, we performed $Rb^+$ uptake experiments also under transmembrane voltage control ($Na^-$-free conditions). In Figure 6C, the $Rb^+$ uptake activity at saturating $Rb^+$ concentrations (5 mM) in not voltage-clamped oocytes ($V_m$ $\sim$ 10 to $\sim$ 20 mV, determined in independent experiments) is compared to the $Rb^+$ uptake activity of oocytes whose membrane potential was clamped to $\sim$ 100 mV by two-electrode voltage clamping. At $pHex$ 7.4 as well as $pHex$ 5.5, only a slight and hardly significant decrease of the $Rb^+$ transport activity was observed at $\sim$ 100 mV compared to unclamped oocytes. Importantly, however, the about two-fold increase of $Rb^+$ transport at $pHex$ 5.5 compared to $pHex$ 7.4, as observed previously in Fig. 3H, occurred irrespective of the membrane potential. This finding supports the hypothesis that an intracellular $pH$-sensitive and only weakly voltage-dependent event is not only rate-limiting for the $E1P\rightarrow E2P$ conformational transition (monitored by the VCF experiments) but also for the overall pumping rate.

Discussion

Effects of extracellular $pH$ on the $E1P/E2P$ distribution

It is generally accepted that the transport cycle of the H,K-ATPase proceeds according to a Post-Albers-type reaction scheme, as formulated for the Na,K-ATPase, despite some difference in detail, such as the strong $E2P$ preference of the gastric proton

**Figure 6. Temperature and voltage 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 a $pHex$ of 7.4 (light gray bars) or 5.5 (gray bars) at temperatures between 18 and 34°C, as indicated. White bars represent $Rb^+$ uptake of non-injected control oocytes at each $pHex$. Data in each column are means of 20–25 oocytes from oocytes of one cell batch. (B) Arrhenius plot for temperature-dependent $Rb^+$ uptakes from data as in (A) at $pHex$ 7.4 ( ), and $pHex$ 5.5 ( ). Data represent means±S.E. of three independent experiments (similar to the one shown in A), after normalization to $Rb^+$ uptake at 34°C for each experiment. Activation energies obtained from linear fits to the data (superimposed lines) are given for each $pHex$ ( ). (C) $Rb^+$ uptake (in pmol/oocyte/min) at 5 mM $Rb^+$ and $pHex$ 7.4 or 5.5 for oocytes expressing HxKs806C/WT, which had either been clamped to a membrane potential of $\sim$ 100 mV, or subjected to $Rb^+$ uptake without voltage clamping ($V_m$ $\sim$ 10 to $\sim$ 20 mV). Black bars represent $Rb^+$ uptake of HxK-ATPase-expressing oocytes clamped at $\sim$ 100 mV in the presence of 100 $\mu M$ SCH28080. Data are means±S.D. from several oocytes of a single batch (numbers stated on each column). doi:10.1371/journal.pone.0033645.g006
pump under physiological conditions [26,28,47,48]. Although the H,K-ATPase carries out net electroneutral transport, experiments using H,K-ATPase-containing parietal cell membrane fragments attached to black lipid membranes have shown transient current signals upon ATP concentrations jumps in the absence of K⁺ [19,20,21] suggesting that an electrogenic event takes place during H⁺ translocation. Thus, as a first approach, one could assume that electronegativity in the H,K- and the Na,K-ATPase follows the same mechanism. For the Na⁺ pump, the slowest phase of presteady-state Na⁺ movement, which is kinetically coupled to the E₃→E₂ transition, arises from extracellular Na⁺ release (or reverse binding to) a site located at ~70% of the electrical distance from the extracellular side. According to the high-field access channel hypothesis, changes in membrane potential are kinetically equivalent to changes in the effective ion concentration at the binding sites deep within the ion well [49,50]. Thus, an about 100-fold increase in the extracellular H⁺ concentration (change from pH₆.4 to 5.5) should shift the Vₒ₅ value of the E₁P/E₂P distribution towards E₁P. The resulting shift (∆Vₒ₅) could then be predicted from a Nernst-like equation [9,12,50]:

$$\Delta V_{o_5} = \frac{R T}{z_q F} \ln \left( \frac{[H^+]_o}{[H^+]_r} \right) - \frac{59 mV}{z_q} \Delta pH \ (\text{with } \Delta pH = pH_{H^+}/pH_0)$$  

(1)

Thus, using an equivalent charge or fractional well depth (z_q) of 0.26–0.27 as derived from the Boltzmann curve parameters in Fig. 2C, a pH change from 7.4 to 5.5 should result in a Vₒ₅ of ~90 to ~110 mV, which is in good agreement to the observed shift of ~105 mV (Fig. 2C and 3F). For the (1-AF/F)-V distributions measured in the absence of extracellular Na⁺ (Fig. 5A,B), the observed A1Vₒ₅ (~35 mV) agrees less well with theoretical values (~48 to ~61 mV, with z_q between 0.48 and 0.49). However, considering the strongly negative Vₒ₅ values of the (1-AF/F)-V curves in question, it must be noted that oocyte TEVC experiments at voltages below ~180 mV become increasingly problematic.

Extracellular Na⁺ ions compete with protons for access to E₂P, and kinetic analysis elicits the fractional depth of intra- and extracellular access channels

Since extracellular Na⁺ ions not only reduce the apparent affinity for extracellular Rb⁺ as K⁺ congeners in Rb⁺ uptake studies [20], but also profoundly change the conformational distribution (compared to the relatively small effect achieved by extracellular acidification, Fig. 5A,B), an extracellular cation access channel still has to be considered for the H,K-ATPase. At pH₆.4 to 7.4, a Na⁺ concentration of 90 mM leads to a stronger accumulation of E₁P at physiological potentials (around ~70 mV), as actually expected for high [H⁺], by a combined effect on Vₒ₅ and a decrease in the slope factor z_q (Fig. 5A,B). The larger fraction of E₃P correlates with an increase of the reciprocal rate constants at hyperpolarizing potentials (Fig. 5C), which indicates an increase of the rate constant for the backward reaction (Fig. 5D,E), whereas the forward rate constant is hardly changed. These observations agree with the notion that Na⁺ ions, which are 10⁻³ to 10⁻⁶-fold more abundant than protons in our experiments, can act as H⁺ analogs within an extracellular-facing ion well. At pH₆.5, the Na⁺ effect on Vₒ₅ of the conformational distribution was no longer present (Fig. 5B), although the reciprocal time constants at negative potentials were also increased (Fig. 5F) suggesting that the effect of Na⁺ ions on the E₁P→E₂P kinetics is present even upon a 100-fold increase of the extracellular [H⁺]. But, at this lower pH₆.5 of 5.5, the E₃P-shifting effect of the increased kₒ values is counteracted by the simultaneous increase of the forward rate constants (Fig. 5G,H) that occurs due to the intracellular acidification. The fact that Na⁺ ions exert H⁺-like effects on H,K-ATPase is another example for the promiscuity of the external-facing cation binding sites in P-type pumps, as outlined recently for Na,K-ATPase, in which some alkali metal ions or monovalent organic cations were shown to induce Na⁺-like or K⁺-like functional effects [51].

Since it is reported in the literature that for both Na,K- and H,K-ATPase Na⁺ ions can mimic the effect of K⁺ ions in the dephosphorylation limb of the cycle [27], one could argue that the observed kinetic effects of Na⁺ on the conformational distribution might be due to an alternative reaction branch. However, if Na⁺ ions would act like K⁺ ions to stimulate the E₄P→E₃P→E₁P pathway, Na⁺ addition should result in a global increase of the total relaxation rate constant (as indeed seen for K⁺, Fig. 4G), which is not observed. In fact, Na⁺ mainly affects kₒ, but not k₄ and thus increases the total rate only at negative potentials (Fig. 5). Furthermore, a significant entry of enzyme molecules into the K⁺ limb of the cycle should lead to an accumulation of E₄ states,
which cannot contribute to the voltage-dependent E$_1$P$\leftrightarrow$E$_2$P relaxation. Thus, similar to the results of K$^+$ addition in Fig. 4A,B, the absolute fluorescence amplitudes should decrease, which is also not observed with Na$^+$. Therefore, we conclude here that under the conditions of our experiments there is no indication for a significant effect of Na$^+$ on the dephosphorylation branch of the H,K-ATPase cycle.

Notably, at both investigated pH$_{ex}$, Na$^+$ had a strong effect on the slope factor $\theta$ of the $(\Delta A/F)/V$ distribution ($0.26-0.27$ vs. $0.48-0.49$ without extracellular Na$^+$). As outlined in Supporting Information (see Appendix S2, Appendix S3, and Figure S1), such a situation can arise from a superposition of effects resulting from cation binding through an intra- and an extracellular access channel. For the pseudo-three-state model depicted in Fig. 1B, the following assumptions are made: First, the $\theta$ factor of $\sim0.5$ measured in the absence of external Na$^+$ exclusively represents the fractional depth of an intracellular H$^+$ access channel. Second, external Na$^+$ ions exert their effect on the conformational distribution by binding through a shallower extracellular ion well with a $\varphi$ of $\sim0.2$. This is reasonable, since the H,K-ATPase lacks the third ‘unique’ cation binding site characteristic for the Na$^+$ pump, which is responsible for the major electrogenic release of the third Na$^+$ ion with a fractional charge of $\sim0.8$, whereas the release/uptake of cations to the two ‘common’ sites occurs with a smaller apparent valence of $\sim0.2$. The model simulations in Appendix S3 qualitatively reproduce the experimental observations (Figure S1): First, the inclusion of an additional electrogenic extracellular Na$^+$ uptake step enforces a positive shift in $V_{0.5}$, Second, such uneven $\theta$ factors distort the voltage dependence of the resultant conformational distribution in a way that fitting by a simple Boltzmann-type function yields an ‘effective’ $\theta$ value of even less than 0.5 (Appendix S3 and Figure S1), exactly as observed in Fig. 5A.

Thus, the voltage dependence of the calculated rate constants $k_{f}$ and $k_{b}$ of the forward and backward reaction (Fig. 2E,F and Fig. 5D,E,G,H) can be reconciled with the concept of a high-field intracellular and a shallower extracellular access channel. Although more detailed kinetic information would be required to correlate the calculated rate constants $k_{f}$ and $k_{b}$ with individual rate constants within the pseudo three-state model of Appendix S2, a tentative assignment appears feasible. The data in Fig. 5D,E,G,H show that one of the rates, $k_{b}$, is rather strongly dependent on membrane potential (with $\theta$ values of $\sim0.23$), whereas the voltage dependence of the other, $k_{b}$, is weak. In case of the Na$^+$ pump, the voltage insensitivity of the forward rate constant from ouabain-sensitive transient currents is attributed to a voltage-independent reaction step (the E$_1$P$\leftrightarrow$E$_2$P conformational transition in conjunction with Na$^+$ deocclusion) that is rate-limiting for the subsequent Na$^+$ release step(s). The increase of rate constants upon hyperpolarization results from the fact that negative potentials favor the entry of Na$^+$ ions to the binding pocket through an extracellular high-field access channel. With an hypothetical intracellular access channel in the case of H,K-ATPase, the fact that the forward rate constant $k_{f}$ is independent from voltage (and pH$_{ex}$) suggests that a voltage-independent step preceding intracellular H$^+$ binding (step 1 in Fig. 1A) is rate-limiting for H$^+$ uptake and the subsequent E$_1$P$\leftrightarrow$E$_2$P conformational transition. Conversely, the relatively steep voltage dependence of $k_{b}$ results from the fact that negative voltages speed up the intracellular release of H$^+$ through the access channel. The [Na$^+]_{ex}$-dependence of the partial reaction represented by $k_{b}$ might be the consequence of Na$^+$ ions traversing a shallow extracellular access channel to reach the binding pocket, which, in effect, will also speed up the E$_2$P$\rightarrow$E$_1$P transition.

Proposed mechanism for the effect of intracellular pH on the H$^+/K^+$ pumping rate

The dependence of the proposed voltage-independent step preceding intracellular H$^+$ binding on the intracellular pH could mean that prior to the electrogenic binding of H$^+$ to the transport site(s) a proton must bind to a ‘regulatory’ site (with a pK$_{a}$ around neutral), which is in rapid equilibrium with the intracellular pH. Neutralization of a protonatable residue might change the local electrostatics, eventually leading to the formation of the access channel itself or to control the accessibility of the ion well for intracellular protons. Alternatively, the ‘regulatory’ proton could even be one of the presumably two protons that are transported in each reaction cycle. Of note, our Rb$^+$ uptake experiments suggest that the availability of protons at the intracellular side is not only rate-limiting for the E$_1$P$\rightarrow$E$_2$P conformational transition (Fig. 3G), but also for the turnover rate during stationary cation pumping (Fig. 3H). This conclusion can be drawn from the fact that both the stationary turnover number (Rb$^+$ uptake) and the forward rate constant of the E$_1$P$\rightarrow$E$_2$P relaxation (monitored by the VCF experiments) show an about two-fold increase upon intracellular acidification by 0.5 pH units. Importantly, at pH$_{ex}$ 7.4 as well as pH$_{ex}$ 5.5, only a very small decrease of the Rb$^+$ transport activity was observed at $-100$ mV compared to unclamped oocytes, whereas the about two-fold increase of Rb$^+$ transport at pH$_{ex}$ 5.5 compared to pH$_{ex}$ 7.4 occurred irrespective of the membrane potential. The weak voltage sensitivity of cation transport is, first, reflected by the hardly voltage-sensitive rate constants $k_{f}$ (Figure 5D,E,G,H). Second, if the rate constant for K$^+$(Rb$^+$)-dependent dephosphorylation is much faster than the voltage-dependent relaxation between E$_1$P and E$_2$P, the majority of H,K-ATPase molecules on average will dwell in states (e.g. dephosphorylated intermediates like E$_3$), whose occupancies are insensitive to transmembrane voltage, as indicated from the small voltage-dependent fluorescence changes under turnover conditions (Fig. 4).

The close similarity of the activation energies at pH$_{ex}$ 7.4 and 5.5 also suggests that Rb$^+$ uptake of the gastric proton pump is rate-limited by the same pH$_{ex}$-dependent partial reaction, and the high $E_A$ values suggest that this step is likely not diffusion-controlled, but might be related to a major conformational change. The activation energies at pH$_{ex}$ 7.4 and pH$_{ex}$ 5.5 reported here are remarkably close to the 93 kJ/mol determined by Stengelin and co-workers in BLM experiments at an intermediate pH of 6.2. [21]. This value was obtained from the temperature-dependence of a time constant ($\tau_0$) that was assigned to the phosphorylation reaction and covered an even larger temperature range between 3°C and 40°C. The agreement between these values corroborates the idea that the overall pump activity monitored by the Rb$^+$ uptake measurements is indeed rate-limited by partial reactions of the H$^+$ outward moving branch, i.e. the phosphorylation reaction (that strongly depends on the intracellular H$^+$ concentration) and the subsequent E$_1$P$\rightarrow$E$_2$P conformational transition reflected by the presteady-state fluorescence measurements.

Proposed role of Glu-820 for intra- and extracellular proton sensitivity

In a recent study, we have identified an acidic residue belonging to the putative cation binding pocket of the gastric H,K-ATPase (Glu-820 in M6) that might be crucial for the sensitivity towards intracellular acidification described here. Upon replacement of Glu-820 by non-protonatable residues (e.g. Gln or Ala), Rb$^+$ uptake did not increase in presence of butyrate at pH$_{ex}$ 7.4 (see...
H,K-ATPase. In contrast, intracellular acidification by saturation value of protons and K ions with Rb two charge-neutralizing mutants was even reduced indicating that the channel for protons has a fractional depth of extracellular access channel indicates that the intracellular access dependent (un)binding/(de)occlusion steps through an intra- and an on a pseudo-three state model that simultaneously includes voltage-dependent parameters and rate constants on the conformational distribution of H,K-ATPase.

Concluding remarks

In the absence of extracellular K\(^+\), extracellular acidification from pH\(_{37}\) 7.4 to 5.5 has no effect on the E\(_{1P}\)-to-E\(_{2P}\) relaxation of gastric H,K-ATPase. In contrast, intracellular acidification by \(~\sim 0.5~\) pH units speeds up the forward relaxation rate and increases the H\(^+\)/K\(^+\) pumping rate two-fold. Extracellular Na\(^+\) ions compete with protons and K\(^+\) ions for entry into the extracellular-facing access channel to the binding sites in E\(_{2P}\), but have no significant effect on the dephosphorylation branch of the cycle. Kinetic analysis based on a pseudo-three state model that simultaneously includes voltage-dependent (un)binding/(de)occlusion steps through an intra- and an extracellular access channel indicates that the intracellular access channel for protons has a fractional depth of \(~\sim 0.5~\) whereas the extracellular access channel, which is accessible for protons, Na\(^+\) and K\(^+\) ions, has a fractional depth of \(~\sim 0.2~\). The overall H\(^+\)/K\(^+\) pumping rate is essentially voltage-insensitive indicating that a voltage-independent step is rate-limiting for the pump cycle. This intracellular pH-sensitive, rate-limiting step might be the intracellular binding of a proton to a regulatory binding site, which could be the transport site, to which the side chain of E820 is contributing.

Supporting Information

Figure S1 Model simulations. (A) Simulation curves for the function from Eq. B23 (see Appendix S2) with parameters \(B = 1\), \(z_{w} = 0.5\) and \(z_{p} = 0\) for the fractional depth of the intra- or extracellular access channel, respectively. Variation of \(A\) alters the saturation value of \(F(V)\) and leads to a shift in \(V_{0.5}\) (B) Simulation curves for the function from Eq. B23 with parameters \(A = 0.3\), \(z_{w} = 0.5\) and \(z_{p} = 0\) for the fractional depth of the intra- or extracellular access channel, respectively. Variation of \(B\) shifts the \(V_{0.5}\) value of the distribution in a logarithmic fashion. (C) Simulated data (dots) according to Eq. B23 with parameters \(A = 2\), \(B = 1\), \(z_{w} = 0.5\) and \(z_{p} = 0\) for the fractional depth of the intra- or extracellular access channel, respectively. Also included are fits of a Boltzmann-type function to the simulated data sets (solid lines) with fit parameters as indicated.

Figure S2 Structural model or rat gastric H,K-ATPase. Structural model of the rat gastric H,K-ATPase according to PDB structure entry 3B3E (Morth et al. (2007), Nature 450: 1043–1048; doi:10.1038/nature06419), which represents pig renal Na,K-ATPase in the E2P\(_{1}\) conformation with two bound Rb\(^+\) ions. The structure model was created using SwissModel (http://swissmodel.expasy.org/) after manual adjustment of the sequence alignment according to the data deposited in The P-type ATPase Database (http://traplabs.dk/pathbase/). The left panel shows an overview of the domain structure of H,K-ATPase with nucleotide binding (N), phosphorylation (P), actuator (A) and transmembrane (TM) domain indicated by different colors. Also shown is the transmembrane part of the \(\beta\)-subunit (light blue), the \(\beta\)-subunit’s ectodomain, which was not resolved in the 3B3E structure, is omitted for clarity. Highlighted in red is the central \(\beta\)-sheet of the P domain close to D395, the residue, which is intermediate phosphorylated during the reaction cycle. Furthermore, two bound Rb\(^+\) ions are shown within the putative binding pocket in the center of the block of transmembrane helices, and the enzyme’s C-terminus (dark blue) including the two terminal tyrosines, which have been shown to be pivotal for cation transport in Na,K-ATPase. Depicted in orange is the central transmembrane helix M5, whose upper part extends into the P domain, whereas in the TM region residue K791 is located, which contributes to cation coordination. Close to the extracellular end of M5 within the M5/M6 loop the Cys mutation S806C is shown, to which the fluorescent dye tetramethylrhodamine-maleimide (TMRM) is site-specifically bound. The right panel shows the transmembrane region in higher magnification using the same color coding as on the left. Here, the location of the putatively salt bridge-forming residues K791 (M5) and E820 (M6) in the vicinity of the bound Rb\(^+\) ions is shown in relation to the labeling position S806C, which resides at the extracellular mouth of the cation exit pathway.

Appendix S1 Simplified two-state kinetic model used for analysis of voltage-dependent fluorescence signals.

Appendix S2 Pseudo three-state model including charge translocation through intra- and extracellular-facing access channels used for model simulations to rationalize experimental observations.

Appendix S3 Model simulations to elucidate the impact of voltage-dependent parameters and rate constants on the conformational distribution of H,K-ATPase.

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Author Contributions

Conceived and designed the experiments: KLD NNT TF. Performed the experiments: KLD NNT TF. Analyzed the data: KLD TF. Contributed reagents/materials/analysis tools: KLD NNT TF. Wrote the paper: KLD NNT TF.
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