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Rb^+ occlusion stabilized by vanadate in gastric H^+/K^+-ATPase at 25 °C

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ABSTRACT

Despite its similarity with the Na^+/K^+-ATPase, it has not been possible so far to isolate a K^+-occluded state in the H^+/K^+-ATPase at room temperature. We report here results on the time course of formation of a state containing occluded Rb^+ (as surrogate for K^+) in H^+/K^+-ATPase from gastric vesicles at 25 °C. Alamethicin (a pore-forming peptide) showed to be a suitable agent to open vesicles, allowing a more efficient removal of Rb^+ ions from the intravesicular medium than $C_{12}E_8$ (a non-ionic detergent). In the presence of vanadate and Mg^{2+}, the time course of [^{86}Rb]Rb^+ uptake displayed a fast phase due to Rb^+ occlusion. The specific inhibitor of the H^+/K^+-ATPase SCH28080 significantly reduces the amount of Rb^+ occluded in the vanadate–H^+/K^+-ATPase complex. Occluded Rb^+ varies with [Rb^+] according to a hyperbolic function with $K_{0.5} = 0.29 \pm 0.06$ mM. The complex between the Rb^+-occluded state and vanadate proved to be very stable even after removal of free Mg^{2+} with EDTA. Our results yield a stoichiometry lower than one occluded Rb^+ per phosphorylation site, which might be explained assuming that, unlike for the Na^+/K^+-ATPase, Mg^{2+}-vanadate is unable to recruit all the Rb^+-bound to the Rb^+-occluded form of the Rb^+–vanadate–H^+/K^+-ATPase complex.

1. Introduction

The gastric H^+/K^+-ATPase is responsible for the active, ATP-dependent exchange of intracellular protons for extracellular potassium ions, thus generating the proton gradient associated with gastric acid secretion [1]. The reaction cycle of H^+/K^+-ATPase is proposed to be similar to that of the Albers–Post model, already used to describe the reactions catalyzed by Na^+/K^+-ATPase [2], but where protons (in lieu of sodium ions) are exchanged for potassium ions. The transport seems to be electroneutral, i.e. an equal number of H^+ and K^+ are exchanged [3]. It is generally believed that transport of ions by P-type ATPases occurs with the formation of intermediates in which the transported ions are held in an occluded state [4,5]. It has been shown that occlusion of K^+ in the Na^+/K^+-ATPase intermediate, $E_2(K_2)$, can occur either after K^+–dependent dephosphorylation (through the so-called "physiological route") or in media lacking ATP and Na^+, where K^+ is the only ligand present ("direct route"). Although Na^+/K^+-ATPase and H^+/K^+-ATPase are very similar in their amino-acidic sequence and kinetic mechanisms, isolation of a K^+-occluded state in the H^+/K^+-ATPase has been problematic [6]. A possible explanation for the repeated failure to observe K^+ occlusion in this enzyme is that H^+/K^+-ATPase exhibits a much faster rate of transition from the $E_2(K)$ to the E_1 states than the Na^+/K^+-ATPase [7,8].

While a Rb^+-occluded state has never been found at room temperature, Rabon et al. [9] reported the isolation of an intermediate holding Rb^+ occluded in the vanadate-inhibited H^+/K^+-ATPase at 4 °C. They calculated a stoichiometry of 2–3 K^+ per phosphorylation site, which agree with results of Rabon et al. [10], measuring transport stoichiometry. However, a value of 1 H^+:1 K^+ per hydrolyzed ATP has been proposed by Reenstra et al. [11] and Smith et al. [12], also from transport experiments. Additionally, Koenderink et al. [13], using homology modeling of the H^+/K^+-ATPase based on the X-ray structure of the sarcoplasmic reticulum calcium ATPase, found a single high affinity binding site for K^+.

As an attempt to shed light on these controversial results we measured the occlusion of the K^+-congener Rb^+ in the H^+/K^+-ATPase using a method that was successfully applied to isolate an occluded-Rb^+ state in the Na^+/K^+-ATPase [14]. Here we investigate Rb^+ occlusion through the "direct route" in H^+/K^+-ATPase from gastric vesicles permeabilized with $C_{12}E_8$ or the channel-forming peptide alamethicin [15].

As it has been shown for Na^+/K^+-ATPase that inhibition of activity by vanadate is caused by stabilization of the $E_2(K_2)$ occluded state [4,16,17], we therefore used this inhibitor to stabilize the conformational state of H^+/K^+-ATPase holding occluded Rb^+ ions [18].

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We found that permeabilization of vesicles with alamethicin and the enhancement of Rb^+ binding by vanadate allows the detection of a Rb^+ occluded state at 25 °C. However, the presence of vanadate seems not to be enough to completely recruit H^+/K^+-ATPase to the Rb^+-occluded state.

2. Materials and methods

2.1. Enzyme

Preparation of pig H^+/K^+-ATPase-enriched membrane vesicles was performed as described by Sachs [3] with some modifications. Briefly, the hog gastric fundus was scraped, homogenized in a glass homogenizer with Teflon pestle and a microsomal pellet was obtained by differential centrifugation. Purified gastric vesicles were then obtained in the light fraction of sedimentation through a 7% Ficoll in 0.25 M sucrose gradient. To assess the purity of H^+/K^+-ATPase we performed an SDS gel electrophoresis of the preparation after being incubated with 25 μM [γ-^{32}P]ATP. After gel staining with Coomassie Blue we observed a prominent band migrating at a molecular mass of approximately 110 kDa (close to the expected mass for the alpha subunit of the pig gastric H^+/K^+-ATPase).This band was the only one showing incorporation of ^{32}P. ATPase activity measured in gastric vesicles is shown in Table 1. It can be seen in the absence of K^+ that ATPase activity decreased to 15% of the control value and was not detectable when Mg^{2+} was omitted. Vanadate inhibited 86% of control activity added either as Na^+ or Tris salt. A similar degree of inhibition was obtained when media contained 20 μM SCH28080 (imidazole 1,2α pyridine) a specific inhibitor of H^+/K^+-ATPase [19]. We also observed that ATPase activity was no longer detectable after heat inactivation of the enzyme by incubating the vesicles during 35 min at 56 °C. The possibility of contamination of our preparation with Na^+/K^+-ATPase was ruled out by testing the effect of ouabain on ATPase activity and using specific antibodies in immunoblotting assays [20].

2.2. Reaction conditions

Unless otherwise stated, incubations were performed at 25 °C in media containing 25 mM imidazole-HCl (pH 7.4 at 25 °C) and 0.25 mM EDTA. The concentrations of other components varied according to the experiments and are indicated under "Results". In every experiment where vanadate was employed, reaction media also contained 2 mM $MgCl_2$, unless otherwise noted.

2.3. Reagents

^{86}[Rb]RbCl was obtained from PerkinElmer Life Science. Vanadate and ATP were from Sigma Chemical Co. (USA), and were freed of Na^+ by passing solutions of these reagents through a column containing a cation exchange resin (Bio Rad AG MP-50). Final concentration of Na^+ in the eluate, measured by flame photometry, was less than 0.05% of the vanadate or ATP concentration on a mol to mol basis. Alamethicin was from Sigma Chemical Co., USA. All other reagents were of analytical grade.

Table 1
ATPase activity. Experiments were performed at 25 °C in reaction media containing 25 μg protein/ml, 2.2 mM ATP, 4 mM $MgCl_2$ and 10 mM KCl (control). When other reagents were added, these were incubated with the enzyme during 20 min prior to the assay. Results are presented as mean ± SD.

	ATPase activity at 25 °C (μmol Pi × (mg protein)$^{-1}$ × h^{-1})
Control	29 ± 3
Absence of K^+	4.3 ± 0.4
Absence of Mg^{2+}	0.1 ± 0.3
+ 0.2 mM vanadate	4 ± 1
+ 20 μM SCH 28080	5.5 ± 0.4
Treated at 56 °C for 35 min.	1.1 ± 0.5
+ 0.0025% $C_{12}E_8$	48.8 ± 0.5
+ 0.5 g alamethicin/g protein	52.0 ± 0.2

2.4. Assay of ATPase activity

ATPase activity was assayed as the release of ^{32}Pi from [γ-^{32}P]ATP at 25 °C according to the method described by Schwarzbaum et al. [21], slightly modified to quantitatively extract the Pi present in the media. Incubation time was short enough to prevent the hydrolysis of more than 10% of the ATP present and to ensure initial rate conditions. Reaction media contained 7 to 10 μg of protein. Blanks were estimated by acid precipitation of the enzyme prior to addition of [γ-^{32}P]ATP.

2.5. Phosphorylation

The time course of formation of phosphorylated intermediates was measured in experiments with [γ-^{32}P]ATP carried out using a rapid mixing apparatus (SFM4 from Bio-Logic, France). The reaction was stopped by chemical quenching with trichloroacetic acid (final concentration 10 g/100 ml) as described by Schwarzbaum et al. [21].

2.6. Uptake of Rb^+ by H^+/K^+-ATPase-enriched vesicles

The determination of [^{86}Rb]Rb^+ retained by the vesicle preparation (Rb_{ret}) was performed according to Rossi et al. [14]. The incubation of the enzyme with [^{86}Rb]Rb^+ took place in the rapid mixing apparatus. After the appropriate time, reaction media were squirted into the stream of an ice-cold washing solution (containing 30 mM KCl and 10 mM imidazole-HCl, pH 7.4 at 0 °C) flowing through a quenching and washing chamber. Reactions were stopped by means of an almost instantaneous drop in temperature and in ligands concentration. The particulate material was retained by a Millipore filter (0.8 μm pore size) which was removed, dried under an incandescent lamp, and counted for radioactivity in a β-scintillation counter. Not significantly different results were obtained using 0.22 μm-pore-size filters. Blanks were estimated from the amount of [^{86}Rb]Rb^+ retained by the filters when the enzyme was omitted.

2.7. Permeabilizing agents

2.7.1. Octaethylenglycol dodecylether, C12E8

H^+/K^+-ATPase-enriched vesicles were incubated for 30 min with the non-ionic detergent $C_{12}E_8$, at concentration between 0.001% and 0.1% (w/v). A maximal activation of 70% was observed at 0.0025% $C_{12}E_8$ (Table 1). ATPase activity increased with the incubation time and remained constant after 20 to at least 50 min.

2.7.2. Alamethicin

This peptide was dissolved in 50% v/v ethanol to a concentration of 10 mg/ml. This solution was added to a suspension containing the protein. The final concentration of ethanol never exceeded 1% in volume. Maximal activation of the ATPase by alamethicin was reached at a concentration between 0.5 and 1 g/g protein. At 0.5 g alamethicin/g protein the increase in ATPase activity was approximately 75% (Table 1) and remained constant from 30 to at least 80 min of incubation time.

2.8. Data analysis

Equations were fitted to the experimental data by a non-linear regression procedure based on the Gauss-Newton algorithm with commercial software (Microsoft Excel and Sigma-Plot for Windows).

3. Results

In the following we will show that the combined use of permeabilizing agents and vanadate allows to determine the kinetics of Rb^+ binding to the H^+/K^+-ATPase which could be interpreted as the formation of a Rb^+-occluded state at 25 °C.

3.1. Permeabilization of vesicles with $C_{12}E_8$ and alamethicin

Addition of permeabilizing agents would facilitate the washing out of Rb^+ from the vesicles in the quenching and washing chamber thus decreasing the amount of Rb^+ in the intravesicular media, which could interfere with the detection of Rb^+-occluded states of H^+/K^+-ATPase. To investigate this, we tried the effect of $C_{12}E_8$ and alamethicin on the time course of retained [^{86}Rb]Rb^+ (Rb_{ret}). Fig. 1 shows that in vesicles pre-incubated with detergent (open circles) or alamethicin (triangles), the amount of retained Rb^+ was drastically reduced with respect to control samples (filled circles). By comparing the two treatments, it can be seen that alamethicin causes a greater decrease in the uptake than $C_{12}E_8$, being Rb_{ret} after 300 s one sixth of that measured in control vesicles. Note that in both cases, this decrease in Rb_{ret} is accompanied by an increase in the ATPase activity (Table 1). For control vesicles or vesicles with $C_{12}E_8$, description of the results seems to require more than the sum of two increasing exponential functions of time while a monoexponential function plus an independent term provides a good description of the results obtained with alamethicin (continuous lines in Fig. 1).

We investigated the ability of vanadate to stabilize the Rb^+-occluded form of H^+/K^+-ATPase. Fig. 2 shows results of the effect of the inhibitor on the time course of Rb_{ret} using untreated vesicles (panel A) and vesicles exposed to $C_{12}E_8$ (panel B) or alamethicin (panels C and D). In all cases, vesicles were incubated in media with or without vanadate and then exposed to [^{86}Rb]Rb^+. In untreated vesicles, although it is somewhat difficult to observe, Rb_{ret} obtained in media with vanadate tends to be higher than in controls during the first 4–5 s (inset in panel A), while for longer reaction times it falls below data obtained in the absence of the inhibitor. When vesicles were preincubated with $C_{12}E_8$, vanadate increased both the initial rate and the amplitude of the fast phase of Rb_{ret} (inset in panel B). In the case of alamethicin-treated vesicles (panels C and D), it is clear that the presence of vanadate adds a new fast component to the time course with respect to that observed in the absence of the inhibitor and that both curves rise from the same value at $t=0$ (panel C). The fast component of the time course obtained with vanadate (rate coefficient $=1.14\pm0.42\ s^{-1}$) is probably representing the formation of a Rb^+-occluded state whereas the slow one would be mainly due to binding to structures other than the H^+/K^+-ATPase or to the influx of Rb^+ in remnant sealed vesicles. Panel D shows that the vanadate-induced component remains stable up to at least 3 min, though in additional experiments we determined that this stability period can be extended to at least 1 h of incubation. The fact that the slow phase is shared by the results obtained both with and without vanadate (see legend to Fig. 2 for panels C and D) indicates that the latter could eventually serve as a blank for Rb^+ occlusion. This effect of vanadate was not observed in heat inactivated enzyme.

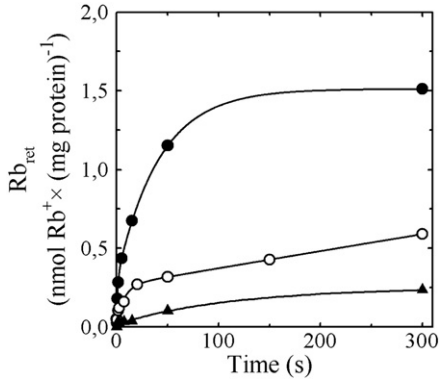

Fig. 1. Rb^+ uptake by H^+/K^+-ATPase-enriched membrane vesicles in the presence of $C_{12}E_8$ or alamethicin. Reaction media contained 50 μg protein/ml, 400 μM Rb^+ (●) and either 0.0025% $C_{12}E_8$ (○) or 0.5 g alamethicin/g protein (▲). Continuous lines are the plots of $f_{(t)}=A_o+A_1(1\text{-}e^{-k1t})+A_2(1\text{-}e^{-k2t})$ (● and ○) and $f_{(t)}=A_o+A_1(1\text{-}e^{-k1t})$ (▲). Assays were performed after 20 to 50 min of incubation between vesicles and detergent or alamethicin.

The results in Fig. 2 suggest that occlusion of Rb^+ will be difficult to measure when the proportion of tightly sealed vesicles is predominant, as in panels A and B. They also show the convenience of using alamethicin as a permeabilizing agent for the study of Rb^+ occlusion in the vanadate–H^+/K^+-ATPase complex.

3.2. Binding of Rb^+ to the vanadate–enzyme complex in alamethicin-treated vesicles

The effect of alamethicin on Rb_{ret} with (open circles) or without (filled circles) vanadate can be observed in Fig. 3. Enzyme was incubated with different alamethicin concentrations and exposed to 500 μM Rb^+ for 20 s. This time was selected due to the fact that under similar conditions the formation of the vanadate–enzyme complex holding Rb^+, i.e. the fast phase of the results with vanadate in Fig. 2C and D, was complete. The plots of Rb_{ret} as a function of alamethicin concentration show a small increase followed by a decrease for both groups of data. The decrease is much less pronounced for the results obtained in the presence of vanadate, a fact that can be explained by the existence of two processes: (i) higher alamethicin concentrations favor the opening of vesicles, thus allowing more [^{86}Rb]Rb^+ to be washed out from the preparation, (ii) vanadate increases the concentration of a state of H^+/K^+-ATPase holding occluded Rb^+. In the absence of vanadate, the values of Rb_{ret} as a function of the concentration of alamethicin approach zero, which suggests that under these conditions Rb^+-occluded states are very difficult to detect.

To evaluate if the effect of vanadate is due to its interaction with the proton pump, we measured the time courses of Rb_{ret} in the presence or absence of SCH28080 in vesicles that had been incubated with alamethicin and vanadate. Fig. 4 shows that SCH28080 substantially reduced Rb_{ret}, which is consistent with a specific interaction of Rb^+ and vanadate with the H^+/K^+-ATPase.

Since Mg^{2+} seems to be an activator for the binding of vanadate (Table 2 and reference 23), we studied the effect of EDTA on the Rb^+-occluded state of the vanadate–H^+/K^+-ATPase complex. In experiments carried out at 25 °C, addition of 5 mM EDTA plus unlabeled Rb^+ did not affect the level of Rb_{ret} during at least 10 min. The stability of the vanadate–H^+/K^+-ATPase form with occluded Rb^+ is in agreement with results obtained for occluded Rb^+ in the vanadate–Na^+/K^+-ATPase complex [23].

We investigated the dependence of the amount of Rb_{ret} on vanadate concentration. Alamethicin-treated preparations were incubated with vanadate in concentrations ranging from 5 to 200 μM and then briefly exposed to [^{86}Rb]Rb^+. Table 2 shows that the level of Rb_{ret} remains unchanged regardless of the vanadate concentration. This result is not unexpected since it has been shown that in the presence of Mg^{2+}, vanadate binds with very high affinities to both the Na^+/K^+-ATPase ($K_d=4$ nM [17,22]) and the H^+/K^+-ATPase ($K_d=5$ nM [18]). Results in Table 2 also show that, unlike the lack of effect of EDTA when added to the Rb^+–vanadate–enzyme complex, binding of [^{86}Rb]Rb^+ to the enzyme was abolished when the preparation was pre-incubated with vanadate and sufficient EDTA to complex the Mg^{2+} present in the solutions. When the enzyme was incubated with vanadate and ADP before adding [^{86}Rb]Rb^+, the level of Rb_{ret} was markedly reduced probably because the nucleotide poises the equilibrium between conformers toward E_1.

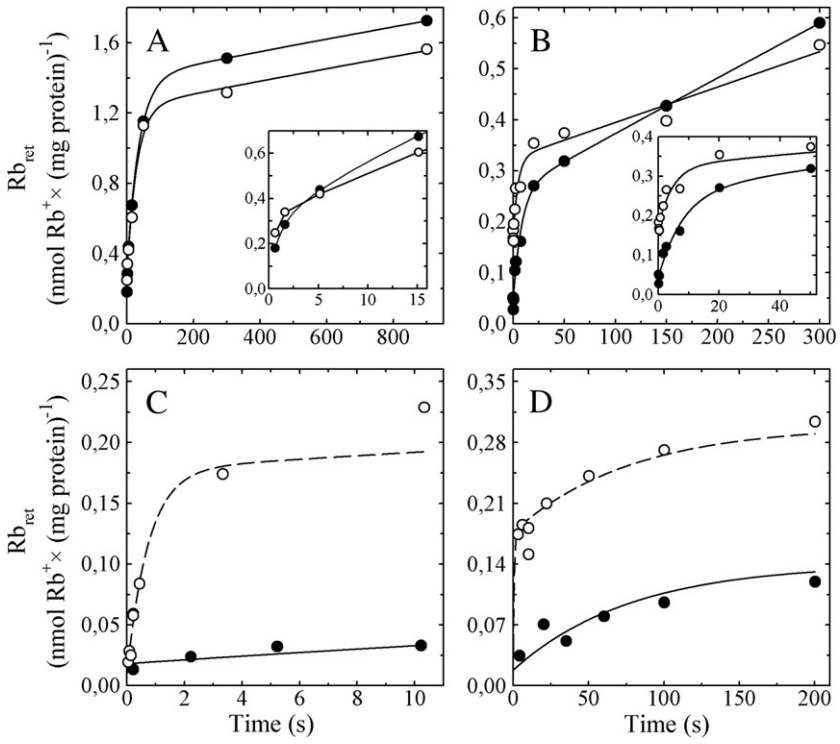

Fig. 2. Effect of vanadate on the time course of Rb^+ retention by H^+/K^+-ATPase-enriched vesicles. We measured the amount of Rb_{ret} by untreated vesicles (panel A) and by vesicles exposed to 0.0025% $C_{12}E_8$ (panel B) or 1 g alamethicin/g protein (panels C and D). In all cases vesicles were incubated in media without (●) or with 0.2 mM vanadate and 2 mM $MgCl_2$ (○) for at least 20 min, and then exposed to 400 (panels A and B) or 500 (panels C and D) μM Rb^+. Protein concentration was 50 μg/ml. The dashed lines in panels C and D are plots of $f_{(t)} = A_o + A_1(1\text{-}e^{-k1t}) + A_2(1\text{-}e^{-k2t})$ for the parameter values that gave best fit to the results (± SE): $A_o = 0.016 \pm 0.007$ nmol $Rb^+ \times (mg\ protein)^{-1}$, $A_1 = 0.160 \pm 0.013$ nmol × $(mg\ protein)^{-1}$, $k_1 = 1.14 \pm 0.42\ s^{-1}$, $A_2 = 0.122 \pm 0.017$ nmol $Rb^+ \times (mg\ protein)^{-1}$ and $k_2 = 0.013 \pm 0.005\ s^{-1}$. Best fit to the results obtained in the absence of vanadate (continuous lines) was achieved with the same equation but setting $A_1 = 0$.

3.3. Dependence of retained Rb^+ on Rb^+ concentration

Since K^+ occlusion in the H^+/K^+-ATPase should be a saturable process we measured Rb_{ret} in vesicles treated with alamethicin at different Rb^+ concentrations. The experiments were performed at 25 °C in the presence or absence of vanadate. Incubations lasted 8 min to ensure that the difference between Rb_{ret} with and without vanadate was constant even at low [Rb^+]. Fig. 5A shows the results for Rb^+ concentrations between 0.03 and 1.3 mM. To calculate the amount of occluded Rb^+ (Rb_{occ}) we subtracted the results obtained without vanadate (filled circles) from those obtained with the inhibitor (open circles). As it can be seen in Fig. 5B, Rb_{occ} was adequately described by a hyperbolic function of [Rb^+] (continuous line). These results are therefore consistent with the formation of a Rb^+-occluded state of the H^+/K^+-ATPase, with $K_{0.5} = 0.29 \pm 0.06$ mM and $Rb_{occMax} = 0.45 \pm 0.03$ nmol × $(mg\ of\ protein)^{-1}$.

Alternatively, we analyzed the data for Rb_{ret} measured in the presence of vanadate (open circles in Fig. 5A) according to the procedure described by Rabon et al. [9], using the following equation:

$$Rb_{occ} = Rb_{occMax} \times \left[Rb^+\right] / \left(K_{0.5} + \left[Rb^+\right]\right) + Nsp \times \left[Rb^+\right] \quad (1)$$

where the second term accounts for the non specific binding of Rb^+. This analysis yielded best fitting values for $K_{0.5} = 0.18 \pm 0.07$ mM, and

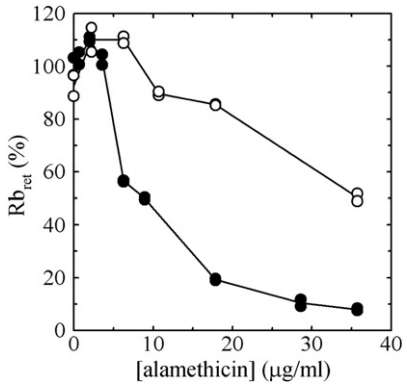

Fig. 3. Dependence of Rb_{ret} on alamethicin concentration. Protein preparation was incubated with alamethicin (●) or alamethicin plus vanadate (○) during 20 min before exposure to Rb^+ for 20 s. Final media contained 50 μg protein/ml, 500 μM Rb^+, 0.2 mM vanadate, 2 mM $MgCl_2$ and the indicated concentrations of alamethicin. Rb_{ret} was expressed as the percent of that measured in the absence of both vanadate and alamethicin.

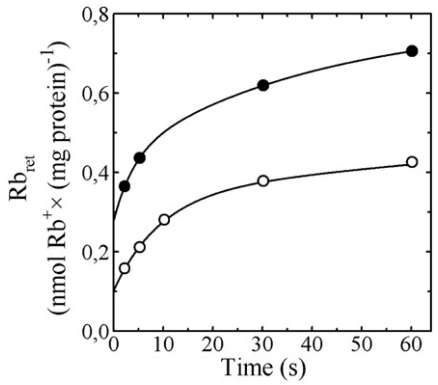

Fig. 4. Effect of SCH28080 on Rb_{ret} in alamethicin and vanadate treated vesicles. After incubating vesicles with alamethicin and vanadate for 20 min, we measured the time course of Rb_{ret} in media containing 600 μM Rb^+ and either 0 (●) or 50 μM (○) SCH28080. Final concentrations were 80 μg protein/ml, 0.1 g alamethicin/g protein, 0.2 mM vanadate and 2 mM $MgCl_2$.

Table 2
Effect of ligands on Rb_{ret}. Enzyme preparation was incubated during 20 min with 0.5 g alamethicin/g protein and the ligands indicated in the table before a 10-s exposure to $^{86}Rb^+$. Reaction media contained 100 μg protein/ml, 500 μM Rb^+ and, when vanadate was added, 2 mM $MgCl_2$. Results are presented as mean ± SD.

Ligands in the preincubation media	Rb_{ret} (% of maximal level)
None	15.0 ± 4.1
200 μM vanadate	100.0 ± 5.1
100 μM vanadate	100 ± 10
5 μM vanadate	99.8 ± 3.7
100 μM vanadate + 5 mM EDTA	13.55 ± 0.22
100 μM vanadate + 3 mM ADP	43.66 ± 0.80

$Rb_{occMax} = 0.48 \pm 0.12$ nmol $Rb^+ \times (mg\ of\ protein)^{-1}$, which are not significantly different from those obtained by fitting a single hyperbolic function to the results in Fig. 5B (see above).

To test possible effects of temperature on Rb^+ occlusion and compare our results with those obtained by Rabon et al. [9], an experiment similar to that shown in Fig. 5 was performed at 4 °C (Fig. 6). Results are well described by Eq. (1) with best fitting values for $K_{0.5} = 0.21 \pm 0.11$ mM, $Rb_{occMax} = 0.84 \pm 0.15$ nmol $Rb^+ \times (mg\ of\ protein)^{-1}$ and Nsp = 0.27 ± 0.03 nmol $Rb^+ \times (mg\ of\ protein)^{-1} \times mM^{-1}$ (continuous line in Fig. 6). We also plotted the specific, hyperbolic component (Nsp = 0, dashed line). It is interesting to note an increase in the maximal amount of occluded Rb^+ when the temperature was decreased from 25 °C to 4 °C.

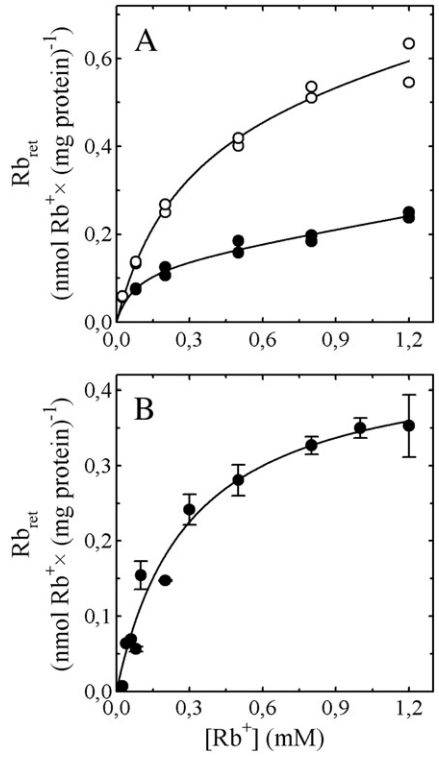

Fig. 5. Rb_{ret} and Rb_{occ} as a function of [Rb^+]. Enzyme preparation was incubated in media containing alamethicin (0.5 g/g protein) with (○) or without (●) vanadate for 20 min at 25 °C and then exposed to different [Rb^+]. Final media contained 50 μg protein/ml and when added, 0.2 mM vanadate and 2 mM $MgCl_2$. Panel A shows the plot of Rb_{ret} in the presence (○) or absence of vanadate (●). The continuous line that describes the results obtained with vanadate was drawn according to Eq. (1) where $K_{0.5} = 0.18 \pm 0.07$ mM, $Rb_{occMax} = 0.48 \pm 0.12$ nmol $Rb^+ \times (mg\ of\ protein)^{-1}$ and Nsp = 0.134 ± 0.08 nmol $Rb^+ \times (mg\ of\ protein)^{-1} \times mM^{-1}$. Panel B shows the amount of occluded Rb^+ (Rb_{occ}) calculated by subtracting Rb_{ret} obtained in the absence of vanadate from that obtained in the presence of the inhibitor, for each concentration assayed. The data shown are the average of five experiments and each error bar represents ± 1 SE. The continuous line is the plot of a hyperbolic function: $Rb_{occ} = Rb_{occMax}[Rb^+]/(K_{0.5} + [Rb^+])$ and the best fitting parameters were $K_{0.5} = 0.29 \pm 0.06$ mM and $Rb_{occMax} = 0.45 \pm 0.03$ nmol $Rb^+ \times (mg\ protein)^{-1}$.

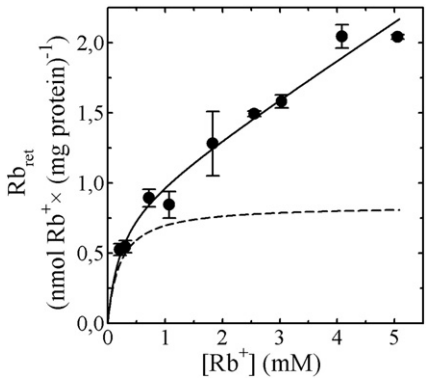

Fig. 6. Rb_{ret} as a function of [Rb^+] at 4 °C. Enzyme preparation (50 μg/ml) was incubated with 0.5 g alamethicin/g protein, 0.2 mM vanadate, 2 mM $MgCl_2$ and [^{86}Rb]RbCl during 5 min at 25 °C and 3 h at 4 °C. Lines were drawn according to Eq. (1), where $K_{0.5}$ (mM) = 0.21 ± 0.11, Rb_{occMax} (nmol $Rb^+ \times (mg\ of\ protein)^{-1}$ = 0.84 ± 0.15 and *Nsp* (nmol $Rb^+ \times (mg\ of\ protein)^{-1} \times mM^{-1}$) = 0.27 ± 0.03 (continuous line) or *Nsp* = 0 (dashed line).

3.4. Enzyme phosphorylation

In order to estimate the ATPase concentration in the preparation, we measured the amount of phosphorylated enzyme, EP, as a function of time in media with 40 μM ATP and 3 mM $MgCl_2$. Results in Fig. 7 show that formation of EP can be described by an increasing exponential function of time, $EP_{(t)} = EP_{max}(1 - e^{-kt})$ (continuous line), where EP_{max} is the steady-state amount of EP and k is the rate coefficient of phosphorylation. The parameter values that gave best fit to the results were $k = 36.6 \pm 2.2\ s^{-1}$, and $EP_{max} = 1.34 \pm 0.03$ nmol × (mg protein)$^{-1}$. Regarding the specific activity of 29 μmol Pi × (mg protein × h)$^{-1}$ at 25 °C, this yields a k_{cat} of about 6 s^{-1} which is comparable with the values of 10 s^{-1} [20] and 8.8 s^{-1} [24] obtained at 30 °C and 21 °C, respectively. The time course of EP formation when the enzyme was preincubated with alamethicin is very similar to that obtained without the ionophore (triangles in Fig. 7). It can be seen from these results and those in Table 3 that the steady-state level of EP obtained with alamethicin is slightly higher than that in the control preparation, suggesting that most of the ATP-binding sites are exposed to the outside of the vesicles as already described [9,20,25]. Table 3 also shows that incubation of H^+/K^+-ATPase with vanadate prevented phosphorylation by [γ-^{32}P]ATP, a result that is expected since vanadate is known to inhibit P-type ATPases by binding to the E_2 conformer [4,16].

4. Discussion

The results presented here are the first description of the time course of occlusion of Rb^+ in H^+/K^+-ATPase from gastric vesicles

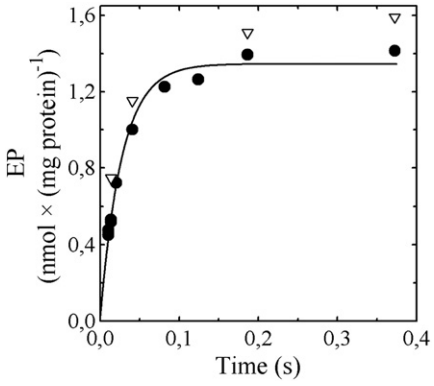

Fig. 7. Time course of phosphoenzyme formation. Phosphorylation was initiated by the addition of ATP and Mg^{2+} to vesicles. Final media contained 50 μg enzyme protein/ml, 40 μM ATP and 3 mM $MgCl_2$ without (●) or with 0.5 g alamethicin/g protein (▽).

Table 3
Phosphorylation of H^+/K^+-ATPase. EP was measured after 0.8 s of reaction between protein (50 μg/ml) and 40 μM [γ-^{32}P]ATP plus 3 mM $MgCl_2$ (control medium). Enzyme preparation was preincubated with alamethicin and EDTA or vanadate. Final media contained 0.5 g alamethicin/g protein and EDTA 5 mM or vanadate 0.2 mM as indicated. Results are presented as mean ± SD.

	EP at 25 °C (nmol Pi × (mg protein)$^{-1}$)
Control	1.34 ± 0.03
+ alamethicin	1.5 ± 0.1
+ alamethicin + EDTA	0.160 ± 0.003
+ alamethicin + vanadate	0.046 ± 0.003

measured at 25 °C. Detection of occluded Rb^+ required the presence of vanadate and the use of alamethicin to permeabilize vesicles.

4.1. Rb^+ uptake in alamethicin-treated vesicles

Measurement of Rb_{ret} as a function of time allowed us to evaluate the relative contribution of Rb^+ occlusion and Rb^+ influx to this magnitude, thus helping to follow the opening of vesicles after addition of permeabilizing agents and to find suitable conditions for occlusion time. Even a small Rb^+ influx can produce an accumulation of the cation into vesicles that might lead to overestimation of the amount of occluded Rb^+. However, this accumulation was drastically lowered using permeabilizing agents and extensive washing, which allowed measuring occluded cations that remain trapped into the transport system machinery. From transient experiments we obtained a rate coefficient of 1.14 s^{-1} for Rb^+-occlusion at 500 μM Rb^+, which is one order of magnitude lower than that we obtained for the Na^+/K^+-ATPase, though in the absence of Mg^{2+}-vanadate.

To permeabilize vesicles, Rabon et al. [9] used digitonin in a concentration that decreased Rb^+ uptake around 40%. We obtained about the same effect using enough $C_{12}E_8$ to achieve maximal ATPase activity, whereas alamethicin decreases Rb^+ uptake around 85% with an increase of 75% in the ATPase activity. This ionophore was previously successfully employed to permeabilize mitochondria [26] and vesicles enriched in sarco-endoplasmic reticulum calcium ATPase [27], as well as whole cells [28]. Its use on H^+/K^+-ATPase gastric vesicles was reported by Klodos [15], although little experimental detail was provided. The increase in ATPase activity by addition of $C_{12}E_8$ and alamethicin is probably due in the former case to the disruption of vesicles and in the latter to the formation of large pores [15], thus improving the access of ligands to their binding sites on the enzyme and preventing the accumulation of these ligands into vesicles. A curious feature of the curves of Rb_{ret} vs. alamethicin concentration is that they not only show a sigmoid decrease but also an increase in Rb^+ uptake at low concentrations of the antibiotic. A sigmoid response would be expected since it is known that six or more molecules of alamethicin are required to form a pore [29]. The increase in Rb_{ret} could be due to the opening of the outer membranes in bilamellar vesicles (see references [3] and [30]) thus favoring the accessibility of Rb^+ and its uptake by the inner compartment.

4.2. Specificity of the binding of Rb^+ to the vanadate–H^+/K^+-ATPase complex

Throughout this work we have demonstrated that, in a similar manner to that reported for the Na^+/K^+-ATPase [22,23], Mg^{2+}-vanadate binds specifically to the H^+/K^+-ATPase and stabilizes the Rb^+-occluded state since: (i) once it has been formed the complex is very stable, even after isotopic dilution of Rb^+ and removal of free Mg^{2+} by addition of EDTA, (ii) when free Mg^{2+} was absent in the preincubation medium we could not detect occluded Rb^+, (iii) preincubation with ADP reduces the amount of occluded Rb^+ probably by poising the conformational equilibrium of the enzyme to an E_1-like form, (iv) SCH28080 significantly reduces the amount of Rb^+ occluded in the vanadate–H^+/K^+-ATPase complex, and (v) vanadate inhibited phosphorylation of the H^+/K^+-ATPase. A further evidence in favor of the specificity, is that tightly bound Rb^+ is saturable with [Rb^+] according to a hyperbolic function with $K_{0.5}$ = 0.2–0.3 mM, these values being comparable to that obtained by Jackson et al. [31] measuring fluorescence of a FITC-labeled H^+/K^+-ATPase.

4.3. Stoichiometry

Regarding the stoichiometric ratio between occluded Rb^+ and phosphorylation of the H^+/K^+-ATPase, we obtain a value of 0.34 nmol Rb^+/nmol EP at 25 °C. The only precedent measuring Rb^+ occlusion in the H^+/K^+-ATPase (Rabon et al., [9]) reports a stoichiometric ratio of 2.6–3.1 occluded Rb^+ per phosphorylation site at 4 °C. Our experiments performed at 4 °C gave values of 0.63 nmol Rb^+/nmol EP, which is almost twice that obtained at 25 °C but still significantly lower than the one reported by Rabon et al. The difference between theirs and our results is not easy to explain although one possibility is that part of the bound $^{86}Rb^+$ they measured is not actually occluded but trapped inside closed vesicles.

It should be mentioned that from experiments measuring H^+ transport, Reenstra and Forte [11] and Smith and Sholes [12], calculated a stoichiometric ratio of one H^+ per ATP hydrolyzed whereas Rabon et al. [10] proposed a $2H^+$/ATP stoichiometry. Additionally, Koenderink et al. [13] using homology modeling with the sarcoplasmic reticulum calcium ATPase found only a single high affinity binding site for K^+. Therefore, there is no unequivocally accepted value for the number of cation-transport sites in the H^+/K^+-ATPase neither from transport experiments nor from structural analysis.

It has been proposed that the repeated failure to measure cation occlusion in the H^+/K^+-ATPase lies on the high velocity of the $E_2Rb_{occ} \rightarrow E_1 + Rb$ transition [6,23]. We have shown in the present work that the Rb^+-occluded state of the H^+/K^+-ATPase is strongly stabilized by Mg^{2+}-vanadate. However, unlike in the case of Na^+/K^+-ATPase, this stabilization seems insufficient to accumulate all H^+/K^+-ATPase in the Rb^+-occluded state. It has been shown for the Na^+/K^+-ATPase that occluded Rb^+ should be in equilibrium with bound (but not occluded) Rb^+ [32], and that the maximal amount of occluded Rb^+ will be equal to the number of transport sites only if the equilibrium between these two states is strongly poised toward the occluded one [33]. This should also apply for the Rb^+ bound to the vanadate–H^+/K^+-ATPase complex, where the Rb^+-occluded state would be in equilibrium with a state where the cation is simply bound, $E_2VaRb \leftrightarrow E_2VaRb_{occ}$. Vanadate could stabilize to similar degrees both occluded and non-occluded forms without leading to a complete conversion into any of them. This could explain why the ratio Rb_{occMax}/EP_{max} is lower than the number of transport sites, either one or two. Furthermore, a drop in temperature might shift the equilibrium between these two states toward the occluded one, thus aiding to explain the higher level of occlusion found at 4 °C. If this were the case, according to the results presented in this work, it seems not safe to calculate a value of Rb^+/ATP stoichiometry from the measurement of occluded Rb^+ into the vanadate–H^+/K^+-ATPase complex neither at 25 °C nor at 4 °C. We are at present investigating the use of inhibitors of the class of benzimidazole derivatives like omeprazole, which has been described to stabilize the H^+/K^+-ATPase in the E_2 conformation [34] and could help solving the stoichiometry question.

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