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Tertiary amines as antagonists of both the luminal and cytosolic K^+-site of gastric H,K-ATPase

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Abstract

Tertiary amines like imidazole and triallylamine lower the apparent affinity of K^+ in the ATP hydrolysis reaction of pig gastric H,K-ATPase in a pH and amine concentration dependent way. The mechanism and sidedness of this effect was studied by analyzing the partial reactions of the enzyme in both leaky and ion-tight vesicles. In leaky vesicles Tris and Hepes had nearly no effect on the apparent K_m for K^+ in the ATPase reaction, but imidazole ($K_i = 13$ mM) and triallylamine ($K_i = 1.6$ mM) markedly decreased the K^+ affinity. The steady-state ATP-phosphorylation level in the absence of K^+ was not or only slightly affected by these compounds. The reduction of the ATP-phosphorylation level by K^+, however, again depended on both the type and concentration of tertiary amine used. A comparable K^+-amine antagonism was observed in the dephosphorylation reaction. In tightly sealed vesicles, where no activation of K^+ at the luminal side could occur, K^+ reduced the affinity for ATP in the phosphorylation reaction. Triallylamine counteracted this effect. The K^+-activated *p*-nitrophenylphosphatase activity in these ion-tight vesicles also showed a K^+-triallylamine antagonism. Inhibition of H,K-ATPase activity in these vesicles by triallylamine was immediate (with nigericin present in order to allow intravesicular K^+ activation), suggesting the transmembrane feature of this inhibition. These results indicate that tertiary amines decrease the affinity for K^+ at both luminal and cytosolic binding sites by interaction at the cytosolic side of the membrane. This results in shifts in the equilibrium of both the $E_1 \cdot H \leftrightarrow E_1 \cdot K$ transition and in the dephosphorylation reaction, $E_2\text{-}P \rightarrow E_2 \cdot K$.

Key words: ATPase, H^+/K^+-; Tertiary amine; Potassium ion activation; Inhibitor

1. Introduction

Gastric H,K-ATPase is an intrinsic membrane protein complex, which is responsible for acid secretion. It contains a 114 kDa catalytic α-subunit [1] and a 60–90 kDa glycosylated β-subunit [2] and catalyses the active transport of 2 H^+ and 2 K^+ ions across the apical membrane per mol ATP hydrolysed [3]. In the H,K-ATPase reaction mechanism (Fig. 1) [4,5], based on the Post-Albers scheme for Na,K-ATPase two different effects of K^+ can be distinguished: (i) activation of the dephosphorylation process (reaction 5 + 6) via the luminal K^+-site and (ii) promotion of the $E_1 \cdot H^+$ to $E_1 \cdot K^+$ transition (reaction −1) via the cytosolic K^+-site; the latter site is also assumed to be responsible for the K^+-activated *p*-nitrophenylphosphatase activity.

The reported properties of the overall H,K-ATPase activity (reaction 1–7) vary with the conditions in which they are analyzed. In our hands the affinity of K^+ for H,K-ATPase, ranged from 2.7 to 4.8 mM [6,7], while other investigators reported values between 0.3 and 5.0 mM [8–16]. Such variations in K^+-affinity could be due to differences in experimental conditions like the ATP and Mg^{2+} concentration, temperature and pH. Another prominent variable is the type of buffer used in these studies. Since preliminary experiments indicated that amine buffers, similarly as with Na,K-ATPase [17,18], have a marked effect on the K^+-affinity of

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Abbreviations: H,K-ATPase, magnesium-dependent hydrogen ion transporting and potassium-stimulated adenosine triphosphatase (EC 3.6.1.36); Na,K-ATPase, sodium and potassium activated adenosine triphosphatase (EC 3.6.1.37); SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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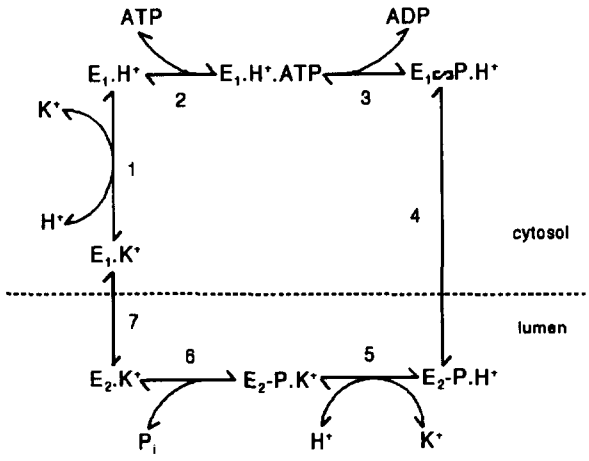

Fig. 1. Simplified reaction scheme of H,K-ATPase [4,5].

H,K-ATPase, we decided to investigate the mechanism of this effect in detail.

In both leaky and ion-tight vesicles the effect of different buffers on the overall K^+-activated ATPase activity (reaction 1–7), the K^+-activated *p*-nitrophenylphosphatase activity, the steady-state ATP-phosphorylation level (amount of phospho-enzyme) and the dephosphorylation reaction (reaction 5 + 6) was studied. Freshly prepared ion-tight H,K-ATPase vesicles, where the cytosolic side faces the extravesicular medium [19], made it possible to study the effects of tertiary amines exclusively at the cytosolic side of the membrane. In these vesicles the equilibrium between $E_1 \cdot H^+$ and $E_1 \cdot K^+$ (reaction −1) could also be studied via steady-state ATP phosphorylation, as in these preparations no activation by luminal K^+ can occur.

The data suggest that imidazole and triallylamine, both tertiary amines, react at the cytosolic side of the membrane and thereby decrease competitively the affinity for K^+ at both the cytosolic and the luminal side of the membrane.

2. Materials and methods

2.1. H,K-ATPase preparations

H,K-ATPase, from pig gastric mucosa, was prepared as reported previously [19]. Fresh (ion-tight) H,K-ATPase vesicles were collected at the 0.25 M sucrose and 7% Ficoll (w/v) in 0.25 M sucrose interface. These vesicles were stored at 4°C for maximally 7 days in 0.25 M sucrose, buffered with 20 mM Tris-HCl (pH 7.0), prior to use. Leaky H,K-ATPase vesicles were prepared by dilution of freshly prepared vesicles in 20 mM Tris-HCl (pH 7.0). After overnight storage at 4°C the vesicles were centrifuged at 100 000 × *g* for 60 min and the pellet was resupended in 0.25 M sucrose/20 mM Tris-HCl (pH 7.0). The latter fraction was again layered on a cushion of 7% Ficoll (w/v) in 0.25 M sucrose and centrifuged for 60 min at 100 000 × *g*. The pellet was collected, resuspended in 0.25 M sucrose/20 mM Tris-HCl (pH 7.0) and stored at −20°C. Occasionally, leaky vesicles were prepared by centrifugation of the ion-tight vesicles, followed by resupension in water and freeze drying. The preparation was stored at −20°C in 0.25 M sucrose/20 mM Tris-HCl (pH 7.0).

2.2. Protein determination

Protein was determined according to either the Lowry [20] method, following trichloroacetic acid precipitation [21], or the Bio-Rad protein assay [22]. In either case bovine serum albumin was used as a standard. All data are expressed in Lowry protein values.

2.3. Steady-state ATP-phosphorylation

H,K-ATPase (10–50 μg/ml) was incubated at 22°C for 3–10 s in 80 μl medium, containing: 1–20 μM [γ-^{32}P]ATP (spec. act. 0.03–0.3 Ci/mmol, Radiochemical Centre Amersham, UK), 0.1 mM $MgCl_2$ at a buffer concentration as indicated in the experiment. The reaction was stopped by adding 5 ml 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid and filtered over a Schleicher & Schüll filter (type AE95, 1.2 μm, Dassel, Germany). After washing two times with 5 ml stopping solution, the filters were analyzed for their ^{32}P-protein content. Blanks were prepared by denaturing the enzyme prior to incubation with the phosphorylation medium [19].

2.4. Dephosphorylation studies

After 10 s phosphorylation with 2 μM [γ-^{32}P]ATP (see above) 10 volumes of 20 μM non-radioactive ATP with the ligand to test were added and incubated for another 3–15 s at room temperature. The reaction was stopped by adding 5 ml stopping solution. The ^{32}P-phosphoenzyme was determined as described above. Dephosphorylation is either expressed as the decrease in acid-stable phosphoenzyme during 3 s incubation (% hydrolysis) or as rate, calculated by assuming first order kinetics.

2.5. K^+-activated ATPase assay

The K^+-activated ATPase activity was either determined as previously described [19], or with a modified radiochemical method. For this purpose 0.32–22 μg H,K-ATPase was added to 80 μl medium, which contained 2 mM Mg[γ-^{32}P]ATP (spec. act. 0.15 mCi/mmol and adjusted to the desired pH with Tris), 0.1 mM ouabain and imidazole-acetate, Tris-acetate or Hepes-Tris, at the indicated concentrations and pH values

and with varying concentrations of KCl and choline chloride. After incubation at 37°C the reaction was stopped by adding 500 μl ice-cold 10% (w/v) charcoal in 6% (w/v) trichloroacetic acid and after 10 min at 0°C the mixture was centrifuged for 10 s (10000 × *g*). To 0.2 ml of the clear supernatant, containing the liberated inorganic phosphate ([^{32}P]P_i), 4 ml OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added and the mixture was analyzed by liquid scintillation analysis. Blanks were prepared by incubating the enzyme in the absence of KCl.

2.6. *p*-Nitrophenylphosphatase assay

K^+-activated *p*-nitrophenylphosphatase activity was determined under the same conditions as the K^+-activated ATPase activity with *p*-nitrophenol phosphate (2.5 mM) as substrate, according to Schrijen et al. [6].

2.7. Calculations

Linear regression analysis was carried out in order to determine the K_m and $K_{0.5}$ values for ATP and K^+ from either Scatchard plots or Woolf-Augustinsson-Hofstee plots or Lineweaver-Burk plots [23]. $K_{0.5}$ is defined as the concentration of effector giving the half-maximal stimulation and I_{50} as the value giving 50% inhibition of the activity. From data on the phosphorylation level (E-P) and ATP hydrolysis rate (v) the dephosphorylation rate constant (k) was calculated using the equation: $v = k \cdot [E\text{-}P]$.

2.8. Chemicals

[γ-^{32}P]ATP (Amersham International, Amersham, UK) was diluted with non-radioactive ATP to a specific activity of 0.15–3000 mCi/mmol. Nigericin (Sigma, St. Louis, MO, USA) was dissolved in ethanol and diluted to its final concentration (maximally 10 μM in 0.1% ethanol). Triallylamine, neutralized with HCl, was from Merck, Darmstadt, Germany. SCH 28080 (2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-aceto nitrile) was kindly provided by Dr. B. Wallmark, Astra-Hässle, Sweden. All other chemicals were of analytical grade.

3. Results

3.1. The K^+-dependent ATP hydrolysis of H,K-ATPase

Incubation of gastric H,K-ATPase with varying concentrations of KCl at different pH values showed a K^+ and H^+ dependent hydrolysis of ATP. Similarly to Ljungström et al. [12] two different effects of K^+ could be observed: (i) activation at low and (ii) inhibition at high concentrations of this ligand (Fig. 2A). In the presence of 50 mM Tris-acetate the optimal H,K-ATPase activity was observed at pH 7.0 with a $K_{0.5}$ value for K^+ of 1.3 mM. At both pH 6.0 and pH 8.0 the maximal velocity was 40–50% of the value at pH 7.0, while the $K_{0.5}$ value for K^+ at these pH values was 2.6 and 0.2 mM, respectively (Fig. 2B). The inhibition of the H,K-ATPase activity at high concentrations of K^+ was also pH dependent: at pH 6.0 inhibition occurred only at high [K^+] (> 100 mM), while at pH 8.0 the activity was already reduced at 4 mM KCl. The H^+-K^+ antagonism in the activation of the H,K-ATPase activity was also observed with either 50 mM imidazole-acetate or 50 mM Hepes-Tris (Fig. 3A). The type of buffer had hardly any effect on the maximal H,K-ATPase activity. The $K_{0.5}$ value for K^+ on the other hand depended strongly on the type of buffer used. In

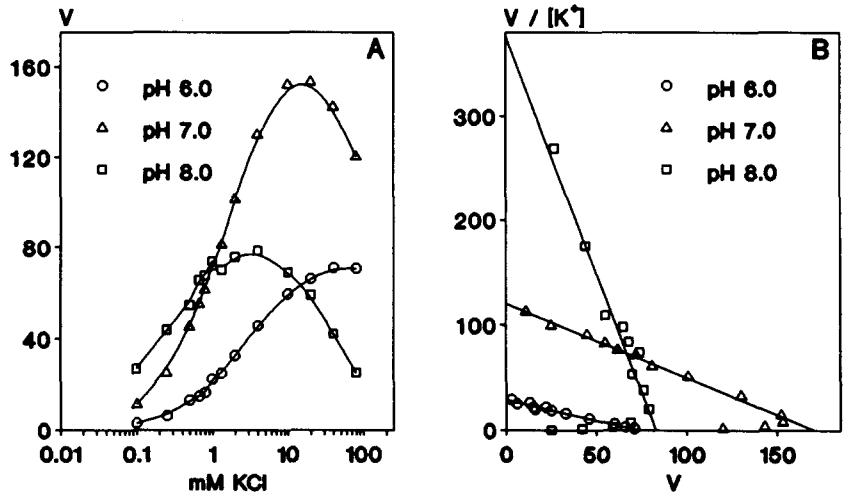

Fig. 2. K^+ dependence of the H,K-ATPase activity, at variable pH. (A) Leaky H,K-ATPase vesicles (20 μg per ml) were incubated, at 37°C, under hypo-osmotic conditions with 5 mM MgATP, 50 mM Tris-acetate and the KCl concentrations, as indicated, at pH 6.0 (○), pH 7.0 (△) and pH 8.0 (□). V is expressed as μmol P_i/mg protein per h. (B) Scatchard plot of the data from (A). The lines are calculated by linear regression analysis using the activating parts of the curves in (A).

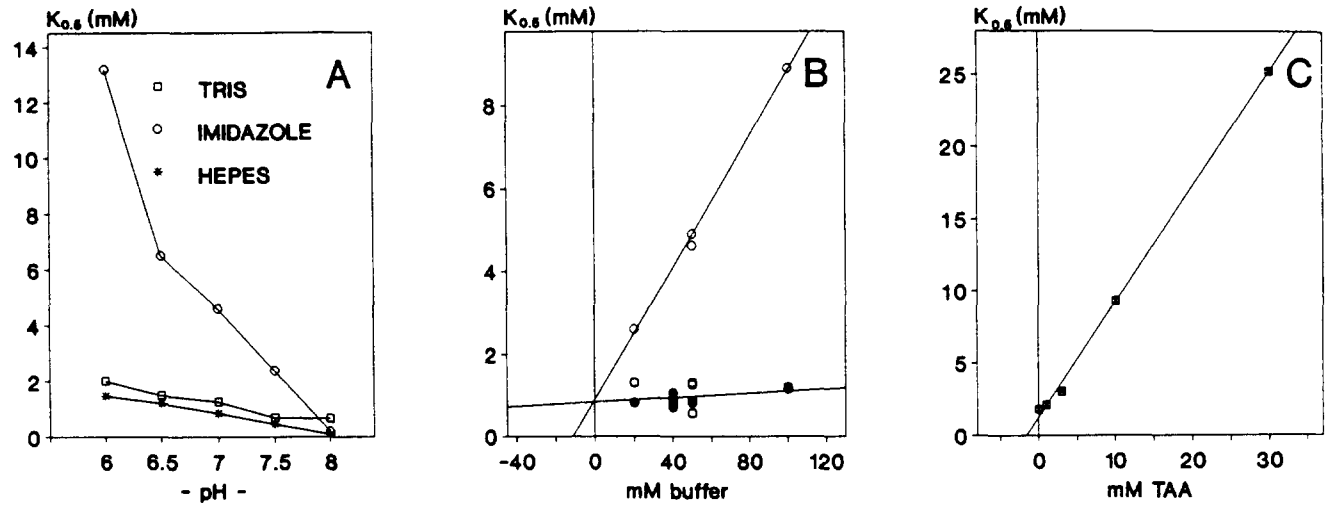

Fig. 3. The effect of pH (A), the concentration of different buffers (B) and triallylamine (C) on the $K_{0.5}$ for the activating effect of K^+ in the overall H,K-ATPase reaction. (A) Leaky H,K-ATPase vesicles (4 μg per ml) were incubated as described in Fig. 2, but with 2 mM MgATP in the presence of 50 mM Tris-acetate (□), 50 mM imidazole-acetate (○) and 50 mM Hepes-Tris (*) at the indicated pH. The $K_{0.5}$ values were calculated by Lineweaver-Burk analysis and plotted as function of pH. (B) Dixon plot of the $K_{0.5}$ values for K^+, obtained by Scatchard analysis using 0–10 mM K^+, as function of different concentrations of Tris-acetate (□), imidazole acetate (○) and Hepes-Tris (*) at pH 7.0. Further conditions as in (A). (C) Dixon plot of the $K_{0.5}$ values for K^+ at pH 7.0, obtained by Scatchard analysis using 0–10 mM KCl, as function of the triallylamine concentration. Further conditions as in (A).

the presence of imidazole the $K_{0.5}$ value increased with decreasing pH to a value of 13.2 mM at pH 6.0 (Fig. 3A). A similar, but quantitatively much smaller pH dependence for the K^+ affinity, was found with Hepes-Tris and Tris-acetate as buffers.

Fig. 3B shows that in the presence of Hepes or Tris the concentration of the buffer had virtually no effect on the $K_{0.5}$ for K^+ in the H,K-ATPase reaction. The $K_{0.5}$ value varied from 0.8 to 1.3 mM. In the presence of imidazole, however, the $K_{0.5}$ value linearly in-

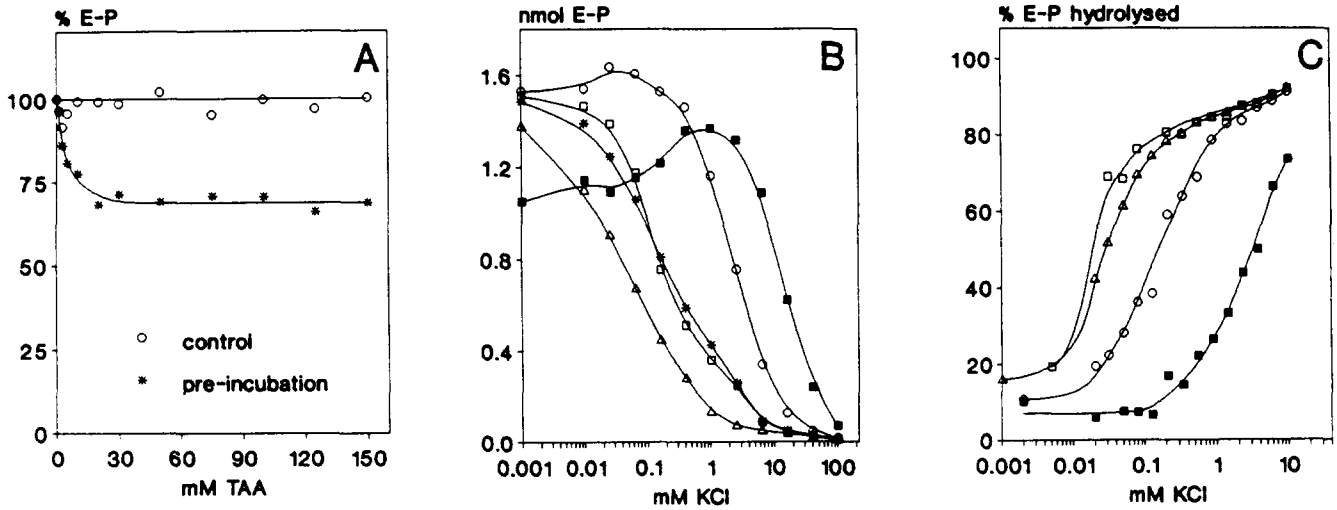

Fig. 4. Effect of K^+ and buffers on the steady-state ATP-phosphorylation level and dephosphorylation rate. (A) Effect of triallylamine on the steady-state ATP-phoshorylation level. Leaky H,K-ATPase vesicles (50 μg/ml) were preincubated at 22°C for 0 (○) or 15 min (*) with triallylamine at the concentrations indicated, in the presence of 20 mM Tris-acetate (pH 7.0). The ATP-phosphorylation level (E-P) was determined during 5 s incubation with 1 μM [γ-^{32}P]ATP and 0.1 mM $MgCl_2$ and expressed as percentage of the control (no TAA). (B) Effect of K^+ on the steady-state ATP-phosphorylation level. Leaky H,K-ATPase vesicles (50 μg/ml) were preincubated at 22°C for 15 min with K^+ at concentrations (not corrected for ionic strength), as indicated, in the presence of 100 mM Hepes-Tris (*), 100 mM Tris-acetate (□), 100 mM imidazole-acetate (○), 5 mM Hepes-Tris (△) and 100 mM triallylamine + 5 mM Hepes-Tris (■). The steady-state phosphorylation level was determined during 5 s incubation with 2 μM [γ-^{32}P]ATP and 0.1 mM $MgCl_2$. (nmol E-P = nmol P/mg protein). (C) Effect of K^+ on the dephosphorylation rate. Leaky H,K-ATPase vesicles (100 μg/ml) were phosphorylated at pH 7.0 and 22°C for 10 s with 2 μM [γ-^{32}P]ATP and 0.1 mM $MgCl_2$ in the presence of 100 mM imidazole-acetate (○), 100 mM Tris-acetate (□), 10 mM Tris-acetate plus 100 mM triallylamine (■) and 10 mM Tris-acetate plus 100 mM choline chloride (△). Dephosphorylation was studied by the addition of 10 volumes 20 μM non-labelled ATP at the same ligand concentrations as used for phosphorylation with increasing concentrations of KCl. The amount of E-P hydrolysed in 3 s is plotted.

creased with increasing concentrations of this buffer. This indicates that imidazole acts as a K^+-antagonist in the ATPase reaction. From these results a K_i value of 13 mM for imidazole on the K^+ activation of H,K-ATPase at pH 7.0 was calculated.

Comparable effects of imidazole were observed when we studied the reaction mechanism of Na,K-ATPase [18,24]. In those studies we demonstrated that the effect of imidazole was also found with other tertiary amines, with triallylamine as the most potent compound. We therefore also tested the effect of triallylamine on the H,K-ATPase activity. The latter compound also decreased competitively the K^+-affinity in the overall H,K-ATPase reaction and a K_i value of 1.6 mM for triallylamine was determined at pH 7.0 (Fig. 3C). This value is eight-times lower than that of imidazole.

The affinity of the enzyme for ATP, as studied in the H,K-ATPase activity test in the presence of 10 mM KCl using 20 mM Tris-acetate as buffer, was 56 μM. When 20 mM triallylamine was also present the ATP affinity increased to 17 μM (data not shown). This is probably due to a reduction in the antagonism between ATP and K^+, as the affinity for K^+ is decreased by triallylamine.

3.2. The effect of triallylamine on the ATP-phosphorylation level

The steady-state ATP-phosphorylation level was not influenced by either imidazole or Hepes or Tris but was about 30% inhibited by triallylamine when the enzyme was preincubated with 30–150 mM of this ligand (Fig. 4A). This inactivating effect, also observed with Na,K-ATPase [24], could partly be abolished with low concentrations of KCl, see Fig. 4B.

It is known that K^+ reduces the steady-state ATP-phosphorylation level of H,K-ATPase [12,25,26]. Fig. 4B shows that this reduction of the phosphorylation level depended on the type and concentration of the buffer used. Preincubation of H,K-ATPase at pH 7.0 with increasing concentrations of KCl in a slightly buffered medium (5 mM Hepes-Tris) resulted in an I_{50} value for K^+ of 0.05 mM. In the presence of either 100 mM Tris or Hepes the I_{50} value was slightly higher (0.2 mM), while with the tertiary amines, imidazole and triallylamine, the I_{50} value increased to 2.5 and 11 mM, respectively. Again a strong K^+-tertiary amine antagonism was observed. This decrease in K^+ sensitivity can be either due to changes in the dephosphorylation rate, or in the $E_1 \cdot K^+$ to $E_1 \cdot H^+$ transition or a combination of both effects.

3.3. The effect of triallylamine on the K^+-dependence of the dephosphorylation reaction

The dephosphorylation studies were carried out at pH 7.0 and at a low ATP concentration (20 μM) to eliminate the inhibitory action of this ligand on the dephosphorylation reaction [27]. Fig. 4C shows that the ligands Tris, imidazole and triallylamine had nearly similar effects on the K^+-activation of the dephosphorylation reaction as on the ATP-phosphorylation level (compare Fig. 4C and Fig. 4B). Either at low buffer

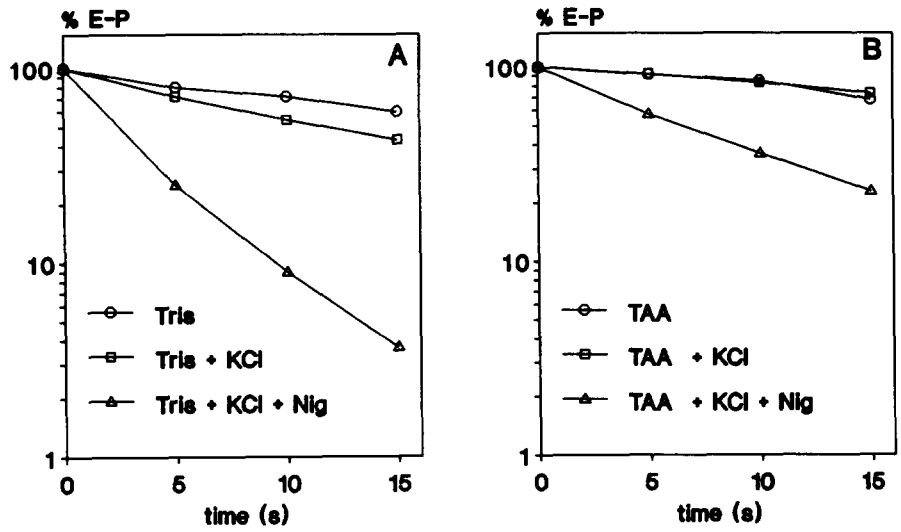

Fig. 5. The effect of triallylamine on the rate of dephosphorylation of the phosphoenzyme formed in ion-tight vesicles. Under iso-osmotic conditions, H,K-ATPase vesicles (57 μg/ml) were preincubated at room temperature for 10 s in the presence of 50 mM Tris-acetate (pH 7.0) (A) or with 50 mM triallylamine (B). Phosphorylation was started by the addition of 1 μM [γ-^{32}P]-ATP and 0.1 mM $MgCl_2$. After another 10 s dephosphorylation was studied by diluting the reaction medium 10 times with non-radioactive ATP (0.1 mM) in the absence (○) and presence of 1 mM KCl (□, △), with (△) and without (○, □) 10 μM nigericin (Nig). The amount of phosphoenzyme left, (% of original,% E-P) is plotted as function of the incubation time.

concentrations or with 100 mM choline chloride the K^+ activation of the dephosphorylation reaction already occurred in the micromolair range ($K_{0.5} = 25$ μM) while in the presence of 100 mM imidazole or 100 mM triallylamine the $K_{0.5}$ was found to be 0.15 and 3 mM, respectively. Thus tertiary amines reduce the K^+ affinity of the phospho-enzyme and thus the E_2-P to $E_2 \cdot K$ transition.

3.4. Dephosphorylation studies in ion-tight vesicles

Fig. 5A shows that the phosphoenzyme formed in ion-tight vesicles reacted only with K^+ when nigericin was also present, indicating that K^+ stimulated at the luminal side. If the enzyme was phosphorylated in the presence of 50 mM triallylamine at room temperature, the basal dephosphorylation rate decreased from 0.04 (Fig. 5A) to 0.02 s^{-1} (Fig. 5B). In order to stimulate the K^+-activated dephosphorylation reaction, the presence of nigericin was also necessary, but the activating effect of 1 mM KCl on the dephosphorylation rate was quite lower than in the absence of triallylamine (0.11 vs. 0.28 s^{-1}). This means that triallylamine antagonises the K^+-stimulated dephosphorylation process in both leaky (Fig. 4C) and ion-tight vesicles.

3.5. The ATP-phosphorylation level in ion-tight vesicles

The reduction of the steady-state ATP-phosphorylation level by K^+, observed in leaky vesicles (Fig. 4B), must at least in part be due to a stimulation of the dephosphorylation reaction (E_2-P to $E_2 \cdot K$). However, a shift in the $E_1 \cdot H$ to $E_1 \cdot K$ equilibrium can also contribute to this effect. In ion-tight vesicles where no activation by luminal K^+ can occur (see above) it was possible to study the latter possibility. In the absence of K^+ the affinity of ATP for the enzyme was very high ($K_m = 0.01$ μM) (Fig. 6A). When the K^+ concentration during the 5-s phosphorylation period was increased the affinity for ATP concentration-dependently decreased, indicating that K^+ shifts the $E_1 \cdot H$ to $E_1 \cdot K$ equilibrium to the right. When the enzyme was preincubated with triallylamine the decrease of the ATP affinity by K^+ was counteracted (Fig. 6B). So there is also a K^+-triallylamine antagonism in the $E_1 \cdot H$ to $E_1 \cdot K$ transition.

3.6. The effect of KCl and triallylamine on the turnover of H,K-ATPase

From the steady-state ATP-phosphorylation level [E-P] and the ATP hydrolysis (v) data the apparent rate constant for P_i loss from E-P (the dephosphorylation rate) can be calculated [28] using the equation $v = k_{app} \cdot [E\text{-}P]$. Since, in the experiments described above different conditions were used, both the ATP-phosphorylation level and the H,K-ATPase activity were next determined at 37°C in the presence of 1 mM ATP and 2 mM $MgCl_2$. Under these conditions K^+ reduced the E-P level with an I_{50} of 25 mM, which is about 100-times higher than in the presence of 2 μM ATP (Fig. 4B). Under the same conditions the $K_{0.5}$ for K^+ in the H,K-ATPase reaction was 1.0 mM, see also Fig. 2. The calculated apparent dephosphorylation rate

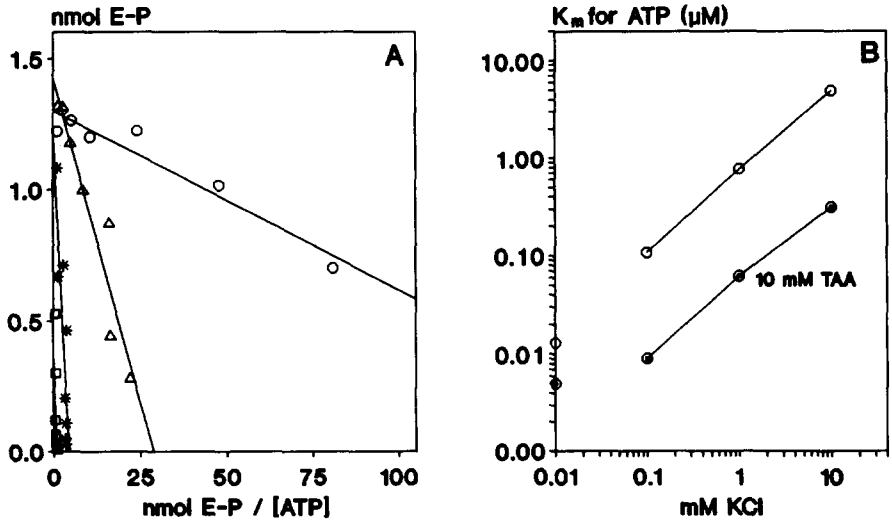

Fig. 6. The effect of K^+ and a combined effect of K^+ and triallylamine on the ATP affinity of the steady-state phosphorylation level in ion-tight H,K-ATPase vesicles. (A) Under iso-osmotic conditions ion tight H,K-ATPase vesicles (10 μg/ml) were incubated at room temperature for 5 s with 0 (○), 0.1 (△), 1 (*) and 10 mM KCl (□), 0.1 mM $MgCl_2$ and varying concentrations of [γ-^{32}P]ATP (0.008–1 μM) in 20 mM Tris-HCl (pH 7.0)/0.25 M sucrose. The lines in the Woolf-Augstinsson-Hofstee plot are calculated via linear regression analysis. (B) Dixon plot of the K_m values for ATP obtained from (A) (control, ○) and the K_m values after pretreatment (15 min) of these H,K-ATPase vesicles with 10 mM triallylamine (●).

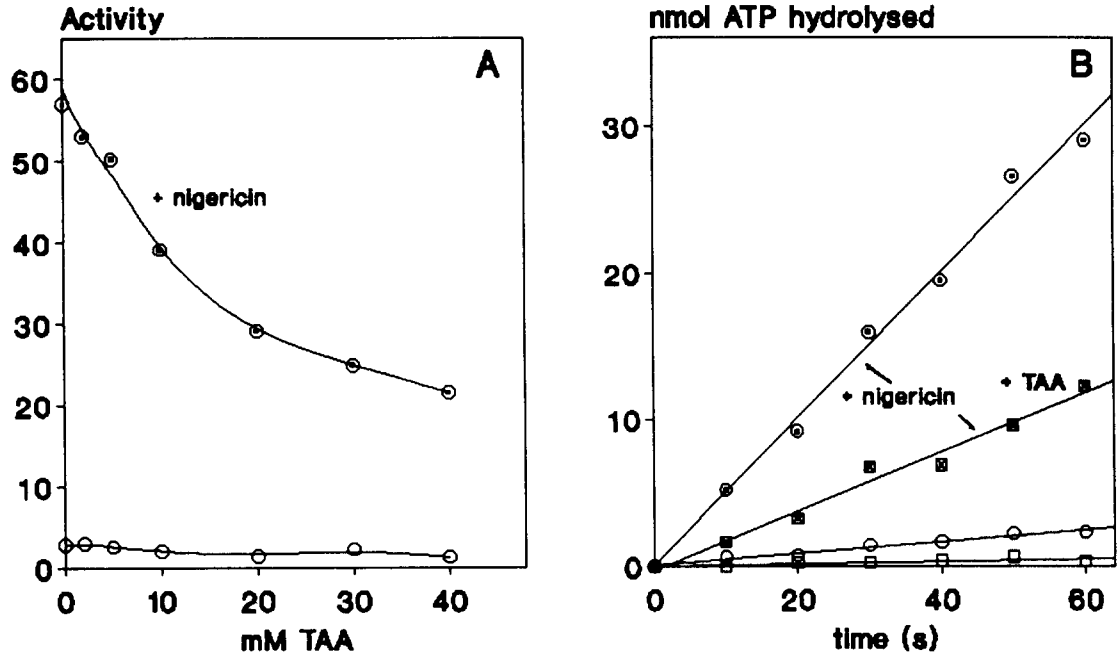

Fig. 7. The effect of triallylamine on the H,K-ATPase activity in freshly prepared H,K-ATPase vesicles. (A) Freshly prepared H,K-ATPase vesicles (275 μg/ml) were incubated for 30 s at 37°C under iso-osmotic conditions (290 mosM): 2 mM MgATP, 10 mM KCl, 0.1 mM ouabain, 0.25 M sucrose and 20 mM Tris-HCl (pH 7.0), with varying concentrations of triallylamine as indicated, with (●) and without (○) 10 μM nigericin. H,K-ATPase activity is expressed in μmol ATP hydrolysed per mg protein per h. (B) Time dependence of the K^+-activated hydrolysis of ATP in ion-tight H,K-ATPase vesicles (275 μg/ml) at 37°C in the absence (○, ●) and presence of 20 mM triallylamine (□, ■), with (●, ■) and without (○, □) 10 μM nigericin. K^+ (10 mM), triallylamine and nigericin were added at time zero to the reaction mixture.

constant increased gradually from 34 min^{-1} to 6548 min^{-1} using 0.05 to 100 mM KCl. Above 100 mM this rate constant further increased, while the H,K-ATPase activity decreased. This suggests that under conditions of high K^+ the K^+-stimulated dephosphorylation step is no longer rate-limiting for the H,K-ATPase reaction and that either the E-P formation or the E_2-E_1 transition is rate-limiting. In the presence of 20 mM triallylamine the I_{50} value for K^+ on the steady-state phosphorylation increased from 25 to 60 mM, while the $K_{0.5}$ of the H,K-ATPase reaction increased from 1.0 to 8.0 mM KCl which results in a shift in the K^+ sensitivity of the calculated dephosphorylation rate (k_{app}). This is another prove that triallylamine reduced the K^+-activated dephosphorylation rate.

3.7. The sidedness of the triallylamine inhibition

In freshly prepared ion-tight H,K-ATPase vesicles, where the cytosolic side faces the extravesicular medium [19], it was possible to study the effects of tertiary amines exclusively at the cytosolic side of the membrane.

The stimulatory K^+-site for *p*-nitrophenylphosphatase activity is located at the cytosolic side of the membrane [12,29]. When the effect of triallylamine on the K^+-activation of this activity was studied with ion-tight vesicles, a mixed-type inhibition was observed. In the presence of 20 mM triallylamine the apparent K_m for K^+ increased from 1.1 to 4.5 mM, while the V_{max} decreased to 78%. This observation suggests that the site of inhibition of triallylamine is located at the outside of the vesicles.

3.8. The H,K-ATPase activity in ion-tight vesicles

In the experiment of Fig. 7A the effect of triallylamine on the H,K-ATPase activity in ion-tight vesicles was compared with its effect in leaky vesicles. In ion-tight vesicles the H,K-ATPase activity was low (5–10% of maximum activity), and could be stimulated by the K^+ ionophore nigericin (the stimulatory K^+-site is located intra-vesicularly). Triallylamine inhibited this activation by 10 mM KCl, and an I_{50} value of 20 mM for triallylamine was observed, which was only twice that in leaky membrane preparations ($I_{50} = 10$ mM). Fig. 7B shows that the rate of ATP hydrolysis in these vesicles, at pH 7.0, is linear with time between 0 and 60 s. Similar results were obtained when the reaction was carried out at pH 6.0. At these pH values only 5% and 0.5% of total triallylamine are present in the uncharged form, respectively. Since only the uncharged form of triallyamine can penetrate the vesicle membrane it is likely that the compound acts at the cytosolic side, unless complete equilibrium is reached within a few seconds.

These results indicate that triallylamine reacts immediately with the ATPase, probably at the cytosolic

side, and thereby reduces both the affinity of the cytosolic (*p*-nitrophenylphosphatase) and the luminal activating K^+-site (H,K-ATPase).

4. Discussion

In this study we show that imidazole, a commonly used buffer, competitively decreased the K^+-affinity for H,K-ATPase, while other buffers like Hepes and Tris had no effect. By using leaky and ion-tight gastric H,K-ATPase vesicles we demonstrated that tertiary amines, responsible for the decrease of affinity of the luminal and cytosolic K^+ site, react at the cytosolic side of the enzyme. The inhibitory mechanism will be discussed and compared with those of other H,K-ATPase inhibitors.

The Albers-Post scheme, originally postulated for Na,K-ATPase, is the basis for understanding the reaction mechanism of gastric H,K-ATPase [4,5]. In Fig. 1 the basic principle is given by reaction 1–7, where E_1 and E_2 represent conformations of the enzyme with high affinity cation binding sites facing the cytoplasm (E_1) and lumen (E_2), respectively. In the E_1 conformation 2 H^+ are bound to the enzyme and 2 K^+ ions are displaced (step 1). When ATP is bound (step 2) and micromolair Mg^{2+} is present H,K-ATPase is phosphorylated (step 3). The ADP sensitive phosphointermediate is rapidly converted to the low energy K^+-sensitive phosphointermediate (step 4). During this conversion 2 H^+ are transported and released at the luminal side by subsequent binding of 2 K^+ (step 5) whereby the enzyme is rapidly dephosphorylated to the $E_2 \cdot K^+$ conformation (step 6). Finally the 2 K^+-ions are transported to the cytosol via the $E_2 \cdot K^+$ to $E_1 \cdot K^+$ transition (step 7), which process is driven by low affinity ATP binding [25].

The K^+ activation (step 5) of the total ATPase reaction (step 1–7), which is antagonized by H^+ [12], is competitively, and therefore reversibly inhibited by the tertiary amines triallylamine and imidazole. In ion-tight vesicles this K^+ activation site is located intravesicularly. As triallylamine with a relatively high pK_a value of 8.3 inhibited the ATP hydrolysis instantly in these vesicles (Fig. 7B) this compound probably interacted at the extravesicular, i.e., cytosolic, side of the enzyme. Direct analysis of the dephosphorylation reaction confirms that triallylamine decreases the K^+-affinity in reaction 5.

In the steady-state ATP-phosphorylation experiments, carried out with open membranes preparations, reactions 2–4 take place, provided no K^+ is present. When ion-tight H,K-ATPase vesicles are used the activation of reaction 5 by luminal K^+ is blocked and the equilibrium shifts to the rather stable $E_2\text{-}P \cdot H^+$ form. The affinity of ATP for the enzyme is very high (0.01 μM) and is reduced by K^+. This means that K^+ drives the enzyme from the $E_1 \cdot H^+$ to the $E_1 \cdot K$ form (step -1). In the presence of triallylamine this reducing effect of K^+ is antagonised. Together with the mixed type inhibition of the K^+-activated *p*-nitrophenylphosphatase, where the K^+-activation site is located at the cytosolic side [12,29], this indicates that triallylamine inhibits the $E_1 \cdot H$ to $E_1 \cdot K$ transition (step -1) as well.

Many inhibitors of gastric H,K-ATPase have been described so far: both specific and non-specific inhibitors. The specific H,K-ATPase inhibitors which all act extracellularly can be divided into two groups: (i) substituted benzimidazoles like omeprazole [30] reacting irreversibly with luminally located sulfhydryl groups. (ii) substituted imidazo[1,2*a*] pyridines, with SCH 28080 as most important inhibitor, act reversibly and are competitive towards K^+ [15]. SCH 28080 reacts with the luminal K^+-site [14] and reduces the steady-state ATP-phosphorylation level [9,31]. The tertiary amines described in the present study thus inhibit by a mechanism different from the two classes of specific inhibitors. Tertiary amines act at the cytosolic side. In addition they are reversible inhibitors in contrast to those of the first group and do not lower the ATP phosphorylation level in contrast to SCH 28080.

A group of tertiary amines, like 8-(*N,N*-diethylamino)octyl-3,4,5,-trimethoxybenzoate (TMB-8), trifluoperazine and verapamil have been reported [16] to interact with the luminal K^+-site too, but these drugs decreased the ATP-phosphorylation level as well. More closely related to the effects of triallylamine and imidazole are those of nolinium bromide, a quaternary amine, which blocks the K^+ entry at $E_2\text{-}P \cdot H^+$ (the dephosphorylation step 5) and does not affect the steady-state ATP-phosphorylation level [32].

In our Na,K-ATPase studies we showed that imidazole and triallylamine are inhibitors of the K^+-activated dephosphorylation step [17,18,24] and simultaneously promoters of the E_1 conformation [33]. Together with the above findings on H,K-ATPase this indicates that tertiary amines interact with K^+ activating sites of both enzymes. Although tertiary amines must be classified as non-specific H,K-ATPase inhibitors, the results indicate that they are specific for K^+-activated ATPases as a group.

In view of these findings it might be preferable to avoid the use of either imidazole or related compounds as buffers in studies on the reaction mechanism of both Na,K-ATPase and H,K-ATPase.

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