

The Non-gastric H,K-ATPase Is Oligomycin-sensitive and Can Function as an H^+ , NH_4^+ -ATPase*

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We used the baculovirus/Sf9 expression system to gain new information on the mechanistic properties of the rat non-gastric H,K-ATPase, an enzyme that is implicated in potassium homeostasis. The α_2 -subunit of this enzyme (HK α_2) required a β -subunit for ATPase activity thereby showing a clear preference for NaK β_1 over NaK β_3 and gastric HK β . NH_4^+ , K^+ , and Na^+ maximally increased the activity of HK α_2 -NaK β_1 to 24.0, 14.2, and 5.0 $\mu\text{mol P}_i\cdot\text{mg}^{-1}\text{protein}\cdot\text{h}^{-1}$, respectively. The enzyme was inhibited by relatively high concentrations of ouabain and SCH 28080, whereas it was potently inhibited by oligomycin. From the phosphorylation level in the presence of oligomycin and the maximal NH_4^+ -stimulated ATPase activity, a turnover number of 20,000 min^{-1} was determined. All three cations decreased the steady-state phosphorylation level and enhanced the dephosphorylation rate, disfavoring the hypothesis that Na^+ can replace H^+ as the activating cation. The potency with which vanadate inhibited the cation-activated enzyme decreased in the order $K^+ > NH_4^+ > Na^+$, indicating that K^+ is a stronger E_2 promoter than NH_4^+ , whereas in the presence of Na^+ the enzyme is in the E_1 form. For K^+ and NH_4^+ , the E_2 to E_1 conformational equilibrium correlated with their efficacy in the ATPase reaction, indicating that here the transition from E_2 to E_1 is rate-limiting. Conversely, the low maximal ATPase activity with Na^+ is explained by a poor stimulatory effect on the dephosphorylation rate. These data show that NH_4^+ can replace K^+ with similar affinity but higher efficacy as an extracellular activating cation in rat non-gastric H,K-ATPase.

The family of X,K-ATPases is functionally characterized by K^+ transport toward the cytosol, coupled to transport of either Na^+ or H^+ toward the extracellular medium. Two family members have been investigated in great detail, Na,K-ATPase and the gastric H,K-ATPase, both consisting of a catalytic α - and an accessory β -subunit. There are four genes for the α -subunit of Na,K-ATPase and three for its β -subunit (1). In contrast, there is only one gene for the α -subunit of gastric H,K-ATPase and one for its β -subunit (2). The amino acid sequence of the α_1 -subunit of Na,K-ATPase is 60–65% identical to that of the α -subunit of gastric H,K-ATPase (α_1). Species differences within each subfamily are small; not more than 1–2% of the amino acids is different. Members of the Na,K-ATPase subfamily are inhibited by ouabain and other cardiac glycosides, whereas the gastric H,K-ATPase is inhibited by SCH 28080.

There is a third family member that is called the non-gastric H,K-ATPase (3). This ATPase is predominantly expressed in epithelia of colon and the distal part of the nephron (4). Only the α -subunit (coined α_2) has been found, and there is no evidence for the existence of a specific β -subunit. On the contrary, it is assumed that this ATPase uses the β -subunit of one of the other X,K-ATPases (5). Most studies support the idea that the β_1 subunit of Na,K-ATPase is the most likely candidate (6–8), but in expression studies the β -subunit of gastric H,K-ATPase (9) and the β_3 -subunit of Na,K-ATPase, previously referred to as HK β (10), have also been successfully used.

The amino acid sequence of the α_2 -subunit of non-gastric H,K-ATPase differs from that of Na,K-ATPase and gastric H,K-ATPase by some 35% so that the evolutionary distance between the three members of this subfamily is rather similar. Strikingly, the interspecies difference in amino acid sequence of the α_2 -subunit of non-gastric H,K-ATPase is about 15%, whereas that of Na,K-ATPase and gastric H,K-ATPase is maximally 2%. There has even been discussion whether the catalytic α_2 -subunits of the various species have the same genetic origin (3).

As the name suggests, the non-gastric H,K-ATPase is assumed to exchange H^+ ions for K^+ ions. Although this transport is electroneutral (11), the amount of outwardly transported H^+ ions is much smaller than that of inward transported K^+ ions (12). This has led to the idea that the non-gastric H,K-ATPase can also transport Na^+ ions in addition to H^+ ions (13, 14). Thus far, however, evidence that Na^+ can stimulate phosphorylation of the enzyme by ATP is lacking. Moreover, one should realize that these studies were performed in intact cells, leaving the possibility of other transporters being involved and thus complicating correct interpretation of the results. In contrast to the above idea, Na^+ was shown to compete with K^+ for transport via the non-gastric H,K-ATPase in the rabbit collecting duct (15), whereas in the rat collecting duct Na^+ was found to increase the activity of this enzyme in the absence of K^+ (16). Finally, there are indications that this enzyme might be involved in NH_4^+ transport (17–19), although the direction of this transport is controversial.

One of the intriguing findings is that during K^+ depletion the non-gastric H,K-ATPase is up-regulated in renal medulla but not in distal colon, suggesting a role in K^+ conservation in the kidney (6, 20–22). Initially, the properties of non-gastric H,K-ATPase were investigated *in situ* or in crude membranes from rat distal colon (16, 18, 23). More recently, this enzyme has also been studied in several expression systems including *Xenopus laevis* oocytes (6, 24–27), HEK 293 cells (14, 28), and the baculovirus expression system (9, 29). The results of these studies are, however, rather confusing, in particular regarding the sensitivity toward ouabain and SCH 28080. This might be due to the use of different species, β -subunits, and/or expression systems. Moreover, many basic properties of the enzyme such as dephosphorylation rate and turnover number have not yet been reported.

Most physiological studies concerning the non-gastric H,K-ATPase have been carried out in the rat. For this species the existence of two

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splice variants of the α_2 -subunit has been described (30). To study the properties of these two splice variants, we expressed them with three different β -subunits in Sf9 cells. These cells have a low endogenous ATPase activity and are therefore highly suitable for these studies. The results obtained do not support the idea that other cations can replace protons as co-activating ions. Moreover, this study surprisingly shows that the enzyme is more active in the presence of NH_4^+ ions than in the presence of K^+ ions.

EXPERIMENTAL PROCEDURES

Recombinant H,K-ATPase—The cDNA of the non-gastric rat H,K-ATPase α_2 -subunit, a gift of Dr. H. Binder (31), was cloned with BamHI behind the polyhedrin promoter in the pFastbacDual (pFD) vector (Invitrogen). Upon sequencing we found a Gly on position 315 and not the published Asp (SID³¹⁵II). Because a Gly is present on this position in nearly all members of the 2C subfamily of P-type ATPases, we assumed that the published Asp is due to a sequence error (GAC instead of GGC). To prepare the HK α_2 b-subunit, we deleted the first 324 nucleotides of the N terminus of the α_2 -subunit (30). For this purpose, the pFD-HK α_2 a construct was digested with ApaI/XhoI and subcloned into the pTLN vector (32). The HK α_2 b cDNA was then cut with HindIII (blunted) and XhoI and ligated into the empty pFD vector (EcoRI (blunted) and SalI (treated) resulting in the pFD-HK α_2 b construct. The cDNA of the non-gastric rat Na,K-ATPase (β_3 -subunit (NaK β_3 , also called HKc β), a gift of Dr. H. Binder (31), was cloned with KpnI and BamHI into the pFD vector (BbsI and KpnI sites) behind the P10 promoter. The generation of pFD vectors containing the β_1 -subunit of rat Na,K-ATPase (NaK β_1) and the β -subunit of rat gastric H,K-ATPase (HK β) has been reported previously (33, 34). Finally, the pFD vectors were combined to yield pFD-HK α_2 a, pFD-HK α_2 a-NaK β_1 , pFD-HK α_2 a-NaK β_3 , pFD-HK α_2 a-HK β , pFD-HK α_2 b, pFD-HK α_2 b-NaK β_1 , pFD-HK α_2 b-NaK β_3 , and pFD-HK α_2 b-HK β . All DNA manipulations were carried out according to standard molecular biology techniques described by Sambrook *et al.* (35), and the modifications were controlled by sequence analysis.

Generation of Recombinant Viruses—The pFD vectors containing the different cDNAs were transformed to competent DH10bac *Escherichia coli* cells (Invitrogen) harboring the baculovirus genome (bacmid) and a transposition helper vector. Upon transposition between the Tn7 sites in the transfer vector and the bacmid, recombinant bacmids were selected and isolated (36). Subsequently, insect Sf9 cells were transfected with recombinant bacmids using the Cellfectin reagent (Invitrogen). After a 3-day incubation period, recombinant baculoviruses were isolated and used to infect Sf9 cells at a multiplicity of infection of 0.1. Four days after infection, the amplified viruses were harvested.

Preparation of Sf9 Membranes—Sf9 cells were grown at 27 °C in 100-ml spinner flask cultures as described by Klaassen *et al.* (33). For production of H,K-ATPase, $1.5 \cdot 10^6$ cells \cdot ml⁻¹ were infected at a multiplicity of infection of 1–3 in the presence of 1% (v/v) ethanol (37) and 0.1% (w/v) pluronic F-68 (ICN, Aurora, OH) in Xpress medium (Bio-Whittaker, Walkersville, MD) as described previously (38). After 3 days, Sf9 cells were harvested by centrifugation at $2,000 \times g$ for 5 min. The cells were washed once at 0 °C with 0.25 M sucrose, 2 mM EDTA, and 25 mM Hepes/Tris (pH 7.0), resuspended in sucrose/EDTA/Tris buffer, and sonicated at 60 watts (Branson Power Co., Danbury, CT) for 30 s at 0 °C. After centrifugation for 30 min at $10,000 \times g$ the supernatant was collected and recentrifuged for 60 min at $100,000 \times g$ at 4 °C. The pelleted membranes were resuspended in the above mentioned buffer and stored at -20 °C.

Protein Determination—The protein concentrations were quantified with the modified Lowry method according to Peterson (39) using bovine serum albumin as a standard.

Generation of Antibodies—Antibodies were raised against part of the α -subunit of rat non-gastric H,K-ATPase (Glu⁵²⁸–Met⁵⁵⁴), rat Na,K-ATPase β_1 -subunit (Gln⁸⁷–Lys¹⁵⁷), and rat Na,K-ATPase β_3 -subunit (Pro⁸⁷–Ala¹⁴³). These domains were cloned into the pGex-3x vector. The obtained glutathione S-transferase fusion proteins were purified and used for production of rabbit polyclonal antibodies against HK $\alpha_{2\text{rat}}$ (C384–M79), NaK $\beta_{1\text{rat}}$ (C385–M77), and NaK $\beta_{3\text{rat}}$ (C386–M92).

Western Blotting—Protein samples from the membrane fraction were solubilized in SDS-PAGE sample buffer and separated on SDS gels containing 10% acrylamide according to Laemmli (40). For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). The expressed subunits were visualized with the antibodies C384–M79 (HK α_2), 2G11 (HK β), C385–M77 (NaK β_1), and C386–M82 (NaK β_3).

ATPase Assay—The ATPase activity was determined using a radiochemical method (41). For this purpose, 0.6–15 μ g of Sf9 membranes were added to 100 μ l of medium containing 10–2000 μ M Mg[γ -³²P]ATP, 0.8 mM MgCl₂, 0.1 mM EGTA, 0.2 mM EDTA, 1 mM Tris-N₃, 50 mM Tris-HCl (pH 7.0) and various concentrations of activating cations as indicated. Ionic strength was kept constant with choline chloride. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 μ l of 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid, and after 10 min at 0 °C the mixture was centrifuged for 10 s at $10,000 \times g$. To 0.15 ml of the clear supernatant containing the liberated inorganic phosphate (³²P_i), 3 ml of OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added, and the mixture was analyzed by liquid scintillation analysis. In general, blanks were prepared by incubating in the absence of membranes. ATPase activity is presented as the difference in activity between membranes of HK α_2 -expressing cells and mock-infected cells.

ATP-dependent Phosphorylation—ATP-dependent phosphorylation was determined as described previously (37, 38, 42). Sf9 membranes (1–6 μ g) were incubated at 0 or 21 °C in 50 mM Hepes-Tris (pH 6.0) containing 1.3 mM MgCl₂ and other ions and drugs as indicated in a volume of 80 μ l. After 30–60 min, 20 μ l of 0.5 μ M [γ -³²P]ATP was added, and the mixture was incubated for 10 s at the same temperature as before. The reaction was stopped by adding ice-cold 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid, and the phosphorylated protein was collected by filtration over a 0.8- μ m membrane filter (Schleicher & Schuell). After washing twice with 5 ml of stop solution, the filters were analyzed by liquid scintillation analysis. Data are corrected for the levels of phosphorylated protein obtained with mock-infected membranes.

Dephosphorylation Studies—After ATP-dependent phosphorylation the reaction mixture was diluted from 100 to 500 μ l with non-radioactive ATP (final concentration 1 mM; to prevent rephosphorylation with radioactive ATP) in 50 mM Hepes-Tris (pH 6.0) and varying concentrations of cations or ADP as indicated. The mixture was incubated for another 3 s at 0 °C. The reaction was stopped as described above, and the levels of phosphorylated protein were determined.

Potassium Determinations—The K⁺ concentrations of the enzyme-containing assay media, to which no K⁺ was added, were determined by flame photometry (FCM 6343, Eppendorf, Hamburg, Germany) and varied between 0.5 and 2.3 μ M.

Chemicals—Cellfectin, competent DH10bac *E. coli* cells, and all enzymes used for DNA cloning were purchased from Invitrogen. [γ -³²P]ATP (3000 Ci \cdot mmol⁻¹) was obtained from Amersham Bio-sciences. Oligomycin was obtained from Sigma. SCH 28080 was kindly provided by Dr. C. D. Strader (Schering-Plough, Kenilworth, NJ). The

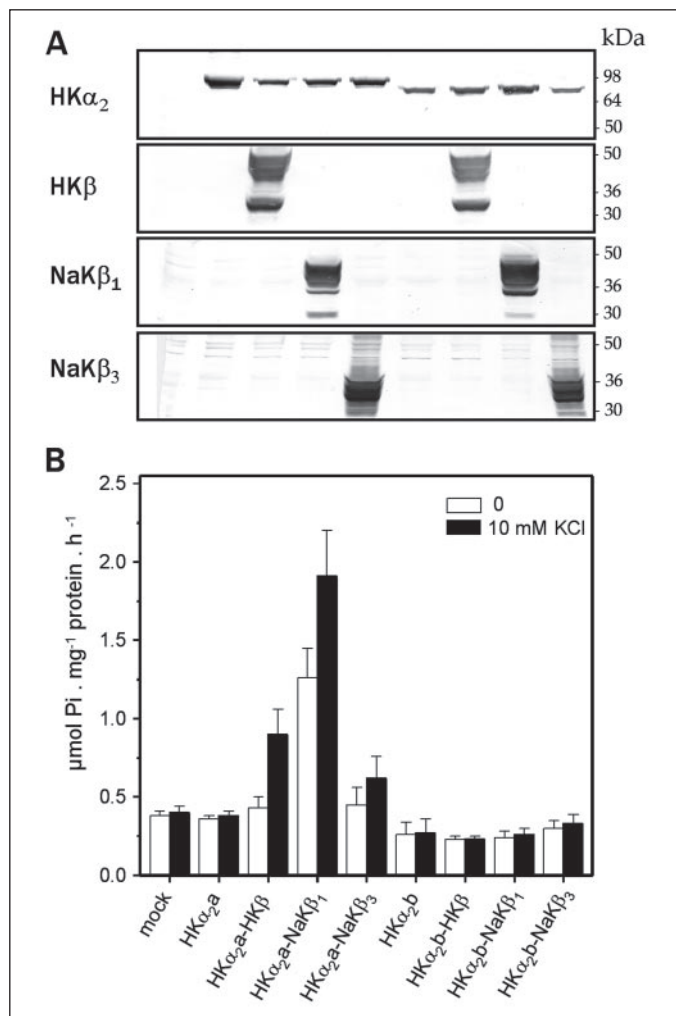


FIGURE 1. Expression of non-gastric H,K-ATPase in Sf-9 cells. A, Western blot ($\pm 10 \mu\text{g}$ membranes) of the different non-gastric H,K-ATPase preparation probed with anti-HK α_2 (C384–M79), anti-HK β (2G11), anti-NaK β_1 (C385–M77), and anti-NaK β_3 (C386–M92). B, ATPase activity of the membrane preparations measured in the presence of 0.1 mM MgATP, 0.1 mM EGTA, 0.2 mM EDTA, 0.5 mM MgCl₂, 1 mM Tris-N₃, 50 mM Tris-HCl (pH 7.0) with and without 10 mM KCl as indicated under “Experimental Procedures.” Average with S.E. of three to nine expression experiments.

antibody 2G11 was a gift from Dr. J. Forte (University of California, Berkeley).

Analysis of Data—All data are presented as mean values for two to five individual preparations with standard error of the mean. Differences were tested for significance by means of Student's *t* test. IC_{50} and $K_{0.5}$ values were determined by analyzing the plots using the non-linear curve fitting program (Hill equation function) of Origin 6.1 (OriginLab Corp., Northampton, MA).

RESULTS

Subunit Expression in Sf9 Cells—Both the a- and b-variant of the α_2 -subunit of rat non-gastric H,K-ATPase, referred to as HK α_2 a and HK α_2 b, respectively, were expressed in Sf9 cells, alone or together with either the β_1 - or β_3 -subunit of rat Na,K-ATPase or the β -subunit of rat gastric H,K-ATPase. After culture of the Sf9 cells for 36h at 27 °C, total membranes were isolated and analyzed for proper expression of the relevant subunits. The apparent molecular weight of the b-variant of the α -subunit was slightly lower than that of the a-variant, which is in agreement with its lower molecular mass (Fig. 1A). All three β -subunits were present in the non-glycosylated and core-glycosylated form. As usual for Sf9 cells hardly any highly glycosylated β -subunit was found. The

amount of β -subunit and its degree of glycosylation did not depend on the type of α_2 -subunit that was used.

ATPase Activity of Various Subunit Combinations—In previous studies we showed that the activity of any ATPase overexpressed in Sf9 cells can be easily measured at low ATP concentrations in a membrane preparation of these cells because of the low endogenous ATPase activity under these conditions (43). When measured at 0.1 mM ATP, neither the ion-independent nor the K⁺-dependent ATPase activity differed between membranes of mock-infected cells or cells expressing HK α_2 a alone (Fig. 1B). However, after co-expression with either one of the β -subunits, the K⁺-stimulated ATPase activity of HK α_2 a was significantly increased. By far the highest activity was measured in the presence of the β_1 -subunit of Na,K-ATPase. With this combination, there was already a significant activity (three times the background value) in the absence of added K⁺. In contrast to HK α_2 a, HK α_2 b remained inactive under all conditions tested. All further experiments were therefore carried out with membranes of Sf9 cells co-expressing the a-variant of the α_2 -subunit of rat non-gastric H,K-ATPase and the β_1 -subunit of rat Na,K-ATPase (HK α_2 a-NaK β_1). The ATPase activity of this combination was so high that it could be measured easily at the optimal ATP concentration of 2 mM, without being hampered by the endogenous activity, which was about 1 $\mu\text{mol Pi} \cdot \text{mg}^{-1} \text{protein} \cdot \text{h}^{-1}$ at this ATP concentration.

Fig. 2A depicts the cation dependence of the activity of HK α_2 a-NaK β_1 at 2 mM ATP and pH 7.0. In the absence of any added cation, the mock-corrected ATPase activity was $1.4 \pm 0.6 \mu\text{mol Pi} \cdot \text{mg}^{-1} \text{protein} \cdot \text{h}^{-1}$. This activity was maximally increased by NH₄⁺, K⁺, and Na⁺ to 24.0 ± 4.0 , 14.2 ± 2.0 , and $5.0 \pm 1.2 \mu\text{mol Pi} \cdot \text{mg}^{-1} \text{protein} \cdot \text{h}^{-1}$, respectively (average from four experiments \pm S.E.). The apparent affinities for NH₄⁺ and K⁺ were of the same order of magnitude (~ 1 mM), whereas that for Na⁺ was considerably lower. Fig. 2B shows that Na⁺ did not change the activity of the K⁺-activated enzyme (10 mM), whereas NH₄⁺ caused a further increase. In the latter case, however, the maximal activity of the enzyme was clearly lower than in the presence of NH₄⁺ alone. In agreement with this, K⁺ decreased the activity of the NH₄⁺-activated enzyme (10 mM) in a concentration-dependent manner (Fig. 2C). Again, Na⁺ did not change the activity of the NH₄⁺-activated enzyme (10 mM). In agreement with the above findings, 100 mM Na⁺ did not affect the K⁺ and NH₄⁺ activation curves (Fig. 2D). These experiments indicate that K⁺ and NH₄⁺ compete for the same binding site(s).

Activation of the enzyme by NH₄⁺ (10 mM) and K⁺ (10 mM) depended strongly on the ATP concentration, and half-maximal activities were obtained at 0.3 and 0.5 mM ATP, respectively (Fig. 3A). In contrast, ATP only slightly increased the activity of the Na⁺-activated enzyme. At ATP concentrations at or below 0.1 mM, 100 mM Na⁺ caused a higher increase in activity than 10 mM K⁺, whereas at ATP concentrations below 0.05 mM, 100 mM Na⁺ increased the activity of the enzyme to the same extent as 10 mM NH₄⁺. The ion-independent ATPase activity only slightly increased with increases in the ATP concentration.

Vanadate potentially inhibits P-type ATPases provided that the enzyme is in the *E*₂ conformation (44). Thus, the less vanadate is needed for inhibition of the activity of the enzyme, the more enzyme molecules are in the *E*₂ conformation. To determine the sensitivity for this inhibitor, vanadate inhibition curves were made at various cation concentrations. Fig. 3B shows that the IC_{50} value for vanadate inhibition decreased with increasing K⁺ or NH₄⁺ concentration, indicating an increase in sensitivity for vanadate. Fig. 3B shows that about 10 times more NH₄⁺ than K⁺ was required to obtain the same level of vanadate inhibition. These findings indicate that at higher NH₄⁺ concentrations and (in particular) higher K⁺ concentrations considerably more enzyme molecules are in the *E*₂ form (see also Fig. 8). In the presence of 100 mM Na⁺, the IC_{50}

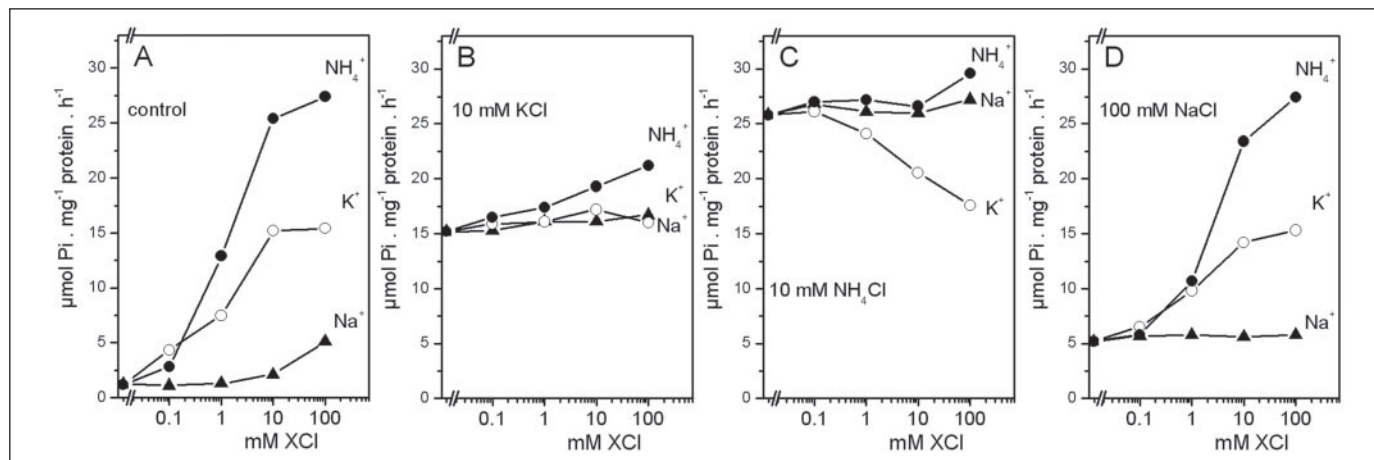
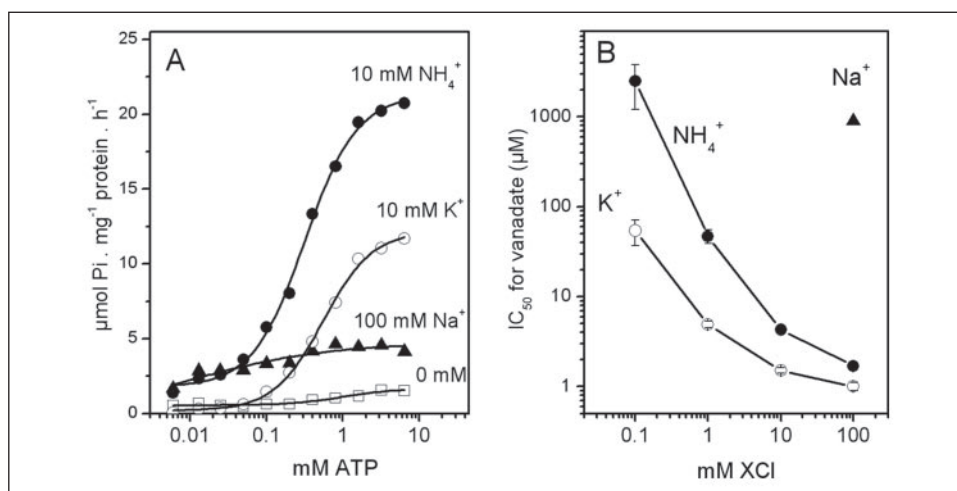


FIGURE 2. Effect of various cations on the ATPase activity of $\alpha_2a\text{-NaK}\beta_1$. The ATPase activity of the membrane preparations was measured in the presence of 2.0 mM MgATP, 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM MgCl_2 , 1 mM Tris- N_3 , at pH 7.0 (50 mM Tris acetate buffer) at the indicated KCl (K^+) (\circ), NaCl (Na^+) (\blacktriangle), and NH_4Cl (NH_4^+) (\bullet) concentrations (A–D). In B, 10 mM KCl, in C, 10 mM NH_4Cl , and in D, 100 mM NaCl was additionally added. The ionic strength was kept constant with choline chloride. All ATPase activities were corrected for those of mock-infected membranes. The results are representative of four experiments. XCl, KCl or NaCl or NH_4Cl .

FIGURE 3. The dependence of the cation-activated ATPase activity of $\alpha_2a\text{-NaK}\beta_1$ on ATP and vanadate. A, ATPase activity of the membrane preparations measured in the presence of different MgATP concentrations as indicated, 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM MgCl_2 , 1 mM Tris- N_3 , 50 mM Tris-acetate, pH 7.0, and either no added cations (\square) or 100 mM NaCl (Na^+) (\blacktriangle) or 10 mM KCl (K^+) (\circ) or 10 mM NH_4Cl (NH_4^+) (\bullet). Ionic strength was kept constant with choline chloride. B, ATPase activity was measured similarly as in A but only at 2 mM ATP and with various vanadate and cation concentrations. At each cation concentration the IC_{50} value for vanadate was calculated as described under "Experimental Procedures" and plotted as a function of the cation concentration used. All ATPase activities were corrected for those of mock-infected membranes. XCl, KCl or NaCl or NH_4Cl .



value for vanadate was about 1 mM, suggesting that the enzyme under these conditions is in the E_1 form. When vanadate inhibition measurements were carried out at higher pH values, less vanadate was needed to inhibit the ATPase activity (not shown). This is in agreement with an E_1 -promoting effect of H^+ (see also Fig. 9).

Other Inhibitors—Fig. 4A shows the effect of ouabain on the increase in enzyme activity caused by either 10 mM NH_4^+ , 10 mM K^+ , or 100 mM Na^+ at 2 mM ATP and pH 7.0. Ouabain inhibited the Na^+ -activated enzyme much more potently than the NH_4^+ - or K^+ -activated enzyme (apparent IC_{50} values of 0.05 ± 0.01 , 0.20 ± 0.03 , and 4.0 ± 0.4 mM, respectively). SCH 28080 gradually inhibited the enzyme activity, but even at 1 mM SCH 28080, the residual activity was still higher than 50% of the initial value with either 10 mM K^+ or NH_4^+ as activating ion. A slightly larger inhibition was obtained for the Na^+ -activated enzyme. Calculation of IC_{50} values for this drug is rather ambiguous (Fig. 4B). Finally, Fig. 4C shows that all activities were equally sensitive to inhibition by oligomycin (apparent IC_{50} values of 3 ± 1 μM). The ion-independent activity of the enzyme was insensitive to these drugs.

ATP Phosphorylation—P-type ATPases are called as such because of the formation of a phosphorylated intermediate during the catalytic cycle. Fig. 5A depicts an SDS-PAGE electrophoretogram of the K^+ -sensitive phosphorylated intermediate of the catalytic α_2 -subunit of non-gastric H,K-ATPase (lanes 5 and 6, without and with K^+ , respectively). The apparent molecular mass of this intermediate was higher than that

of the recombinant α_1 -subunit of gastric H,K-ATPase (Fig. 5A, lanes 3 and 4, without and with K^+ , respectively). As reported previously (43) all lanes contained a phosphorylated product that originated from the Sf9 cells (Fig. 5A, lanes 1 and 2). Membranes of cells expressing non-gastric H,K-ATPase showed an increase in the level of phosphorylated intermediate that was dependent on the ATP concentration when incubated in the absence of any added cation and at a pH of 6.0 (Fig. 5B, open symbols). Scatchard plot analysis of these mock-corrected phosphorylation values revealed a high apparent affinity for ATP ($K_d = 75 \pm 9$ nM; $n = 6$), whereas extrapolation to infinite ATP concentrations indicated a maximal phosphorylation level of 11.2 ± 1.2 pmol P·mg $^{-1}$ protein (Fig. 5C). These phosphorylation experiments were carried out at the lowest possible protein concentrations so that the endogenous K^+ concentration was minimal (≤ 1 μM). When the phosphorylation experiment was carried out in the presence of 100 μM oligomycin the maximal phosphorylation level was about twice as high (20.0 ± 2.2 pmol P·mg $^{-1}$ protein), whereas the apparent K_d decreased about five times to 14 ± 1 nM (Fig. 5, B and C, closed symbols).

The steady-state phosphorylation level in the absence of oligomycin at pH 6.0 was about three times higher than at pH 8.5 (Fig. 6). This is in agreement with a stimulatory role of H^+ on the phosphorylation reaction. At pH 6.0, all three cations decreased the phosphorylation level in a concentration-dependent manner. The apparent IC_{50} value for K^+ was 6 ± 1 μM , which is very low (Fig. 6A). Here, however, one has to

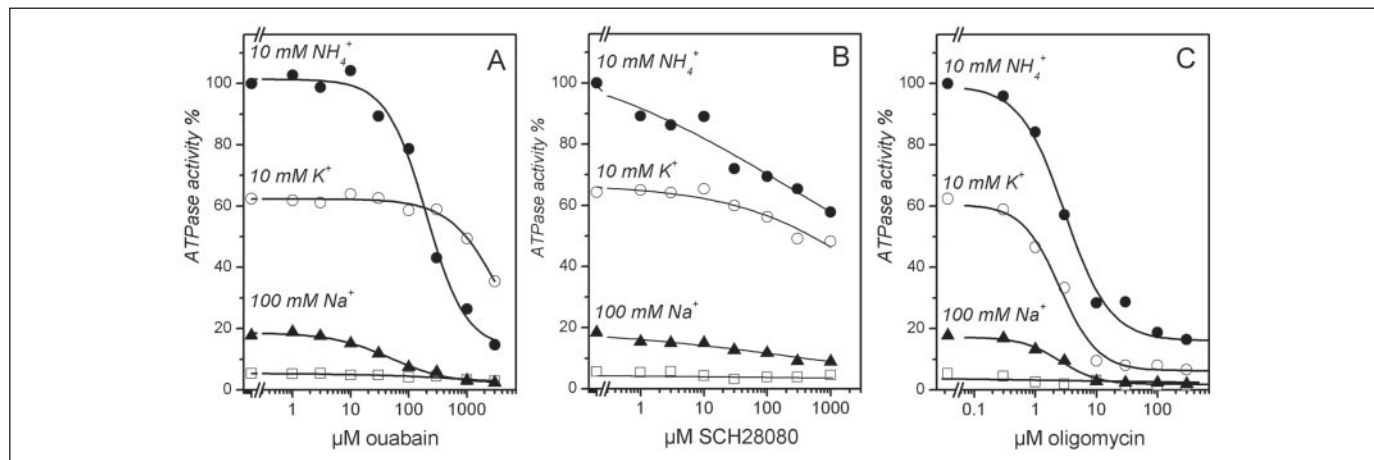
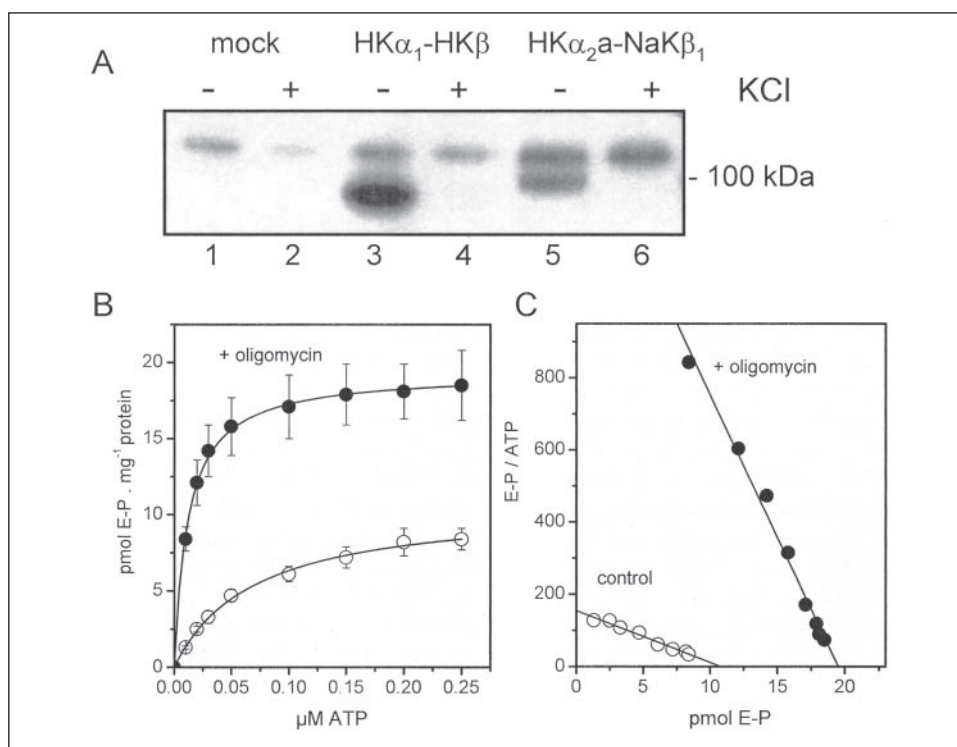


FIGURE 4. The effects of ouabain (A), SCH 28080 (B), and oligomycin (C) on the ATPase activity of HK α_2 a-NaK β_1 . ATPase activity of the membrane preparations measured in the presence of 2.0 mM MgATP, 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM MgCl $_2$, 1 mM Tris-N $_3$, and 50 mM Tris acetate, pH 7.0, in the absence of cations (\square) or 10 mM KCl (K $^+$) (\circ) or 100 mM NaCl (Na $^+$) (\blacktriangle) or 10 mM NH $_4$ Cl (NH $_4^+$) (\bullet) and the indicated inhibitor concentrations. The ionic strength was kept constant with choline chloride. All ATPase activities were corrected for those of mock-infected membranes. The activity in the presence of 10 mM NH $_4$ Cl without inhibitor was set at 100%.

FIGURE 5. The ATP dependence of the ATP phosphorylation level of HK α_2 a-NaK β_1 . A, SDS-PAGE electrophoretogram of phosphorylated membranes of mock-infected cells (lanes 1 and 2), gastric H,K-ATPase (lanes 3 and 4), and non-gastric H,K-ATPase (lanes 5 and 6). In the lanes with the even numbers the membranes were treated with 10 mM KCl for 30 min before phosphorylation. SDS gel electrophoresis was carried out as described previously (46). B, membranes of HK α_2 a-NaK β_1 were treated with (\bullet) and without (\circ) 100 μ M oligomycin for 30 min at 0°C after which they were incubated in the presence of the indicated ATP concentrations and 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM MgCl $_2$, 1 mM Tris-N $_3$, and 50 mM Tris acetate, pH 6.0, for another 10 s. The phosphorylation level was determined as described under "Experimental Procedures" and corrected for the activity of mock-infected cells. C, Scatchard plot of the data from B. Control, K_d apparent = 75 ± 9 nM, $E-P_{max}$ = 11.2 ± 1.2 pmol P-mg $^{-1}$ protein. With oligomycin, K_d apparent = 14 ± 1 nM, $E-P_{max}$ = 20.0 ± 2.2 pmol P-mg $^{-1}$ protein.



realize that in the absence of any added K $^+$, there is still about 1 μ M K $^+$ present in the reaction medium so that the phosphorylation level in the complete absence of K $^+$ is somewhat higher. Consequently, the calculated IC $_{50}$ value is slightly underestimated. The apparent IC $_{50}$ values for NH $_4^+$ (Fig. 6B) and Na $^+$ (Fig. 6C) were 4.7 and 13,700 times higher than that for K $^+$. Upon increasing the pH to 8.5, the phosphorylation level was reduced to about one third of the value at pH 6.0. Also at this pH value, both K $^+$ and NH $_4^+$ decreased the phosphorylation level in a concentration-dependent manner. Na $^+$ exerted its inhibitory effect at elevated pH only at concentrations beyond 30 mM. The decrease in phosphorylation level observed with Na $^+$ is probably mainly because of a stimulatory effect on the dephosphorylation process. The lowering effect of K $^+$ and NH $_4^+$ on the phosphorylation level, however, must be attributed to both their stimulatory effects on the dephosphorylation rate and their inhibitory effects on the $E_2 \rightarrow E_1$ conversion, which can be counteracted by high ATP concentrations. Importantly, even in the

presence of oligomycin Na $^+$ did not increase the steady-state phosphorylation level at pH 8.5, demonstrating that this cation does not stimulate the phosphorylation reaction (data not shown).

The properties of the phosphorylated intermediate(s) generated in the absence (Fig. 7A) or presence (Fig. 7B) of oligomycin were tested by first phosphorylating the enzyme in the absence or presence of this inhibitor followed by a 3-s chase in the absence or presence of either 2 mM ADP or 1.0 mM K $^+$. Fig. 7A shows that in the control situation, already $76 \pm 5\%$ of the phosphorylated intermediate was hydrolyzed within 3 s of incubation in the absence of any added cation. For the oligomycin condition, this value amounted to $55 \pm 2\%$ (Fig. 7B). ADP slightly increased the dephosphorylation rate in the control situation, whereas it dramatically increased this rate in the oligomycin condition (Fig. 7B). Conversely, K $^+$ did not alter the dephosphorylation rate in the oligomycin condition, whereas this cation stimulated the dephosphorylation step in the control situation. These findings indicate that the

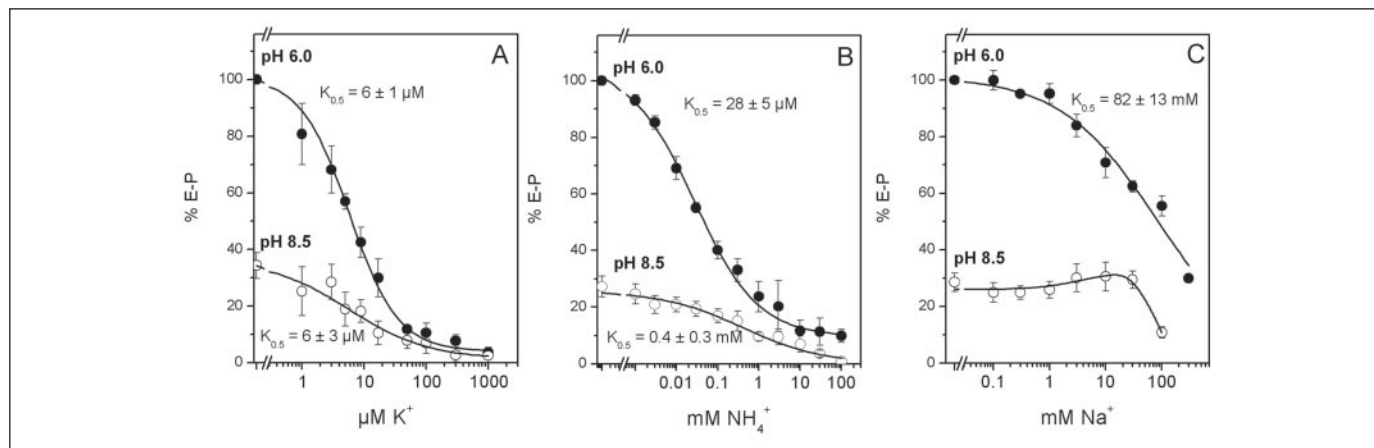


FIGURE 6. The effect of cations on the steady-state ATP phosphorylation level of HK α_2 a-NaK β_1 . The membranes of HK α_2 a-NaK β_1 were incubated with 0.1 μ M ATP, 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM MgCl $_2$, 1 mM Tris-N $_3$, and 50 mM Tris-acetate, pH 6.0 (●) or pH 8.5 (○), and the indicated KCl (K $^+$) (A), NH $_4$ Cl (NH $_4^+$) (B), or NaCl (Na $^+$) (C) concentrations for 10 s at 0 °C. Data were corrected for those of mock-infected cell membranes, and the phosphorylation level in the absence of added cation at pH 6.0 was set at 100%.

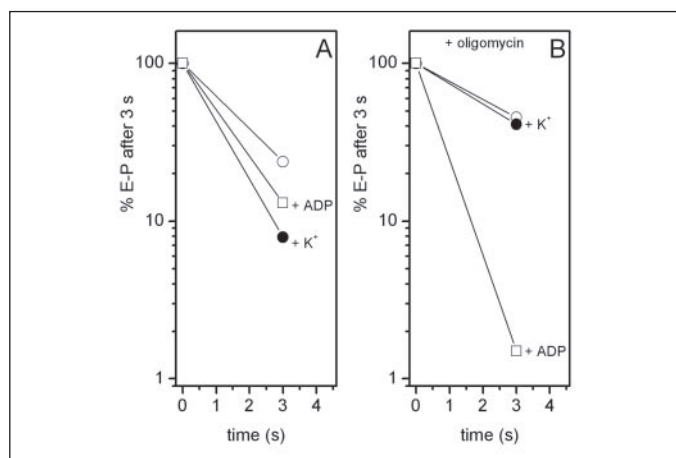


FIGURE 7. The effect of K $^+$ and ADP on the dephosphorylation of phosphorylated intermediates of HK α_2 a-NaK β_1 generated in the absence and presence of oligomycin. The membranes of HK α_2 a-NaK β_1 were incubated with 0.1 μ M ATP, 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM MgCl $_2$, 1 mM Tris-N $_3$, and 50 mM Tris acetate, pH 6.0, without (A) or with (B) 100 μ M oligomycin for 10 s at 0 °C. Next, the mixture was 8.3 times diluted with 1 mM non-radioactive ATP without (○) and with either 1 mM KCl (●) or 2 mM ADP (□) after which the incubation was continued for another 3 s. The residual phosphorylation level corrected for that of mock-infected cell membranes was expressed as a percentage of the level at $t = 0$.

phosphorylated intermediate generated in the presence of oligomycin is nearly completely in the E $_1$ -P conformation, whereas the phosphorylated intermediate generated in the absence of this inhibitor is largely in the E $_2$ -P form. The cation specificity of the dephosphorylation process was further studied in the absence of oligomycin (Fig. 8). All three of the cations dose-dependently increased the dephosphorylation rate. Close inspection of Fig. 8 suggests a somewhat higher affinity for NH $_4^+$ (B) than for K $^+$ (A), whereas the affinity for Na $^+$ (C) was clearly very low.

DISCUSSION

In this study, we performed a detailed analysis of the characteristics of the rat non-gastric H,K-ATPase. Most importantly, we show that this enzyme is activated by NH $_4^+$ with approximately the same affinity and even higher efficacy than those observed for K $^+$, whereas Na $^+$ activates the enzyme with very low affinity and poor efficacy. Moreover, we show that the activity of this enzyme is potently inhibited by oligomycin, with an IC $_{50}$ value that is independent of the activating cation. Finally, we show that this enzyme is phosphorylated by ATP, that the amount of phosphorylated intermediate increases when the pH is lowered, and that this amount is doubled in the presence of oligomycin. In Na,K-

ATPase, oligomycin inhibits the E $_1$ -P to E $_2$ -P conversion (Fig. 9, step 3), a step that is not directly influenced by the applied cations (48). This is apparently also the case with the non-gastric H,K-ATPase, because only in the presence of oligomycin is the dephosphorylation rate increased by ADP.

When the phosphorylation reaction was performed in the presence of oligomycin the apparent K_d value for ATP was 5 times lower than when this reaction was performed in the absence of this inhibitor. Because the phosphorylated intermediate obtained in the presence of oligomycin is in the E $_1$ -P conformation, it is likely that the K_d value measured in the presence of this inhibitor represents the affinity for ATP in the phosphorylation reaction. The increased K_d value measured in the absence of oligomycin is probably because of the presence of small amounts of K $^+$ (± 1 μ M), which stimulate the dephosphorylation reaction (Fig. 9, step 4) and inhibit the E $_2$ -K to E $_1$ conversion (Fig. 9, steps 5 and 1). However, as more ATP is added, the negative effect of these small amounts of K $^+$ on the E $_2$ -K to E $_1$ conversion becomes less, and the steady-state phosphorylation level (mainly E $_2$ -P) increases.

The higher phosphorylation level obtained in the presence of oligomycin is obviously a better measure for the actual amount of phosphorylation sites and should therefore be used to calculate the turnover number of the enzyme. From the maximal ATPase activity of 24.0 ± 4.0 μ mol Pi \cdot mg $^{-1}$ protein \cdot h $^{-1}$ (measured in the presence of NH $_4^+$) and the maximal steady-state phosphorylation level in the same six preparations of 20.0 ± 2.2 pmol P \cdot mg $^{-1}$ protein, obtained after extrapolation to infinite ATP concentrations (Fig. 5B), a turnover number of $20,000 \pm 4,000$ min $^{-1}$ was calculated. This value is still twice as high as that of Na,K-ATPase (45) and about 15 times as high as that of gastric H,K-ATPase (46).

Kone and Higham (30) reported the presence of a splice variant of the non-gastric H,K-ATPase that was truncated by 108 amino acids at its N terminus. Expression of this splice variant with the β -subunit of gastric H,K-ATPase resulted in increased 86 Rb $^+$ uptake in HEK 293 cells. This uptake was relatively SCH 28080-resistant and partially inhibited by 1 mM ouabain. In the present study, however, no enzyme activity was observed following expression of this splice variant with any of the β -subunits despite the fact that all subunits were expressed. The reason for this discrepancy is presently unclear.

The data presented show that co-expression of any of the β -subunits was required for the full-length α_2 -subunit to display cation-dependent ATPase activity in Sf9 cells. This is in contrast with a previous study showing that single expression of the full-length α_2 -subunit in Sf9 cells was sufficient for K $^+$ -stimulated ATPase activity (29). However, the

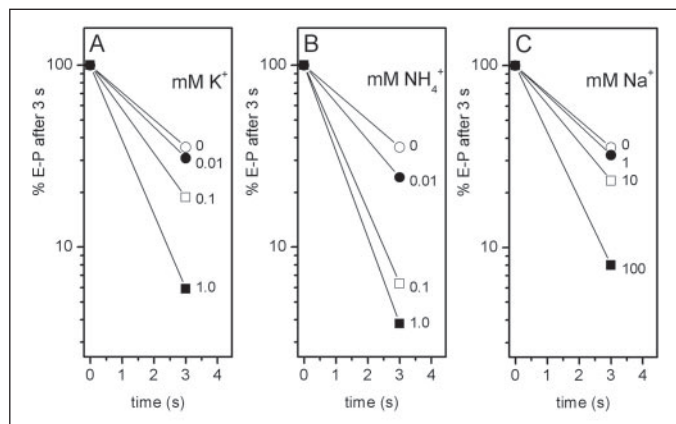


FIGURE 8. The effect of cations on the dephosphorylation of phosphorylated intermediates of HK α_2 a-NaK β_1 . The membranes of HK α_2 a-NaK β_1 were incubated at 0 °C for 10 s with 0.1 μ M ATP, 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM MgCl₂, 1 mM Tris-N₃, and 50 mM Tris acetate, pH 6.0. Next, the mixture was 8.3 times diluted with 1 mM non-radioactive ATP and the indicated KCl (K⁺) (A), NH₄Cl (NH₄⁺) (B), or NaCl (Na⁺) (C) concentrations, and the incubation was continued for 3 s. The residual phosphorylation level corrected for that of mock-infected cell membranes was expressed as a percentage of the level at $t = 0$.

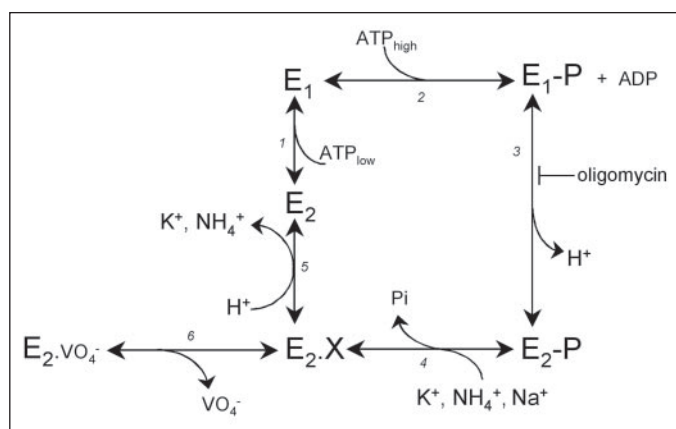


FIGURE 9. Post-Albers reaction scheme for H,K-type ATPases. The simplified scheme includes the site of vanadate (VO₄³⁻) and oligomycin inhibition.

specific enzyme activity reported by Lee *et al.* (29) was less than 10% of that measured in the present study. Nearly all studies performed with P-type ATPases of the 2C class have demonstrated the necessity of a β -subunit. In our studies with gastric H,K-ATPase (43) and Na,K-ATPase (34), we never observed any β -subunit-independent ATPase activity.

By far the highest K⁺-stimulated activity of rat non-gastric H,K-ATPase was measured following co-expression with the β_1 -subunit of rat Na,K-ATPase. However, with the β_1 -subunit of rat gastric H,K-ATPase and the β_3 -subunit of rat Na,K-ATPase considerable K⁺-stimulated ATPase activities were measured. This lack of specificity is in agreement with most of the other studies (25, 26, 47). Our findings are in line with results from co-immunoprecipitation studies, suggesting that the β_1 -subunit of Na,K-ATPase is most likely the physiological β -subunit of non-gastric H,K-ATPase (6–8).

The present study shows that non-gastric H,K-ATPase, like all other P-type ATPases, is phosphorylated by ATP. The steady-state phosphorylation level was considerably higher at pH 6.0 than at pH 8.5, which is in agreement with a stimulatory effect of protons. In some expression systems, the non-gastric H,K-ATPase has been suggested to transport Na⁺ in addition to H⁺. Here we show, however, that Na⁺ had virtually no effect on the steady-state phosphorylation level at pH 8.5. It has also been suggested that the non-gastric H,K-ATPase might be involved in

NH₄⁺ secretion to the urine compartment (17). However, this idea is disfavored by the present finding that NH₄⁺ did not increase the steady-state phosphorylation level. The data presented show that all studied cations stimulated dephosphorylation of the phosphorylated intermediate (Fig. 9, step 4). The experiments presented in Fig. 7 revealed that the affinities for NH₄⁺ and K⁺ were much higher than for Na⁺, which is in agreement with the effects of these ions on the ATPase activity (Fig. 2A). The competition experiments support the conclusion that NH₄⁺ and K⁺ have approximately similar affinities and that the affinity for Na⁺ is much lower.

However, why is the maximal activity obtained with the various cations so different? The lower maximal activity obtained with 100 mM K⁺ as compared with 100 mM NH₄⁺ can be explained by a more prominent inhibitory effect of K⁺ on the E₂-K to E₁ transition (Fig. 9, steps 5 and 1). Here, it is important to realize that this transition actually consists of two steps, de-occlusion of the cation (Fig. 9, step 5) and ATP-driven conversion to the E₁ form (Fig. 9, step 1). We assume that at low ATP concentrations the latter step is rate-limiting, whereas the former step is so at saturating ATP concentrations. In view of this, the lower efficacy of K⁺ at saturating ATP concentrations favors the idea that the de-occlusion step is slower for K⁺ than for NH₄⁺. This conclusion is supported by the observation that the K⁺-activated ATPase was inhibited by lower vanadate concentrations than the NH₄⁺-activated enzyme, suggesting that during the catalytic cycle, the former enzyme stays longer in the E₂ conformation than the latter enzyme. In sharp contrast, very high vanadate concentrations were required for inhibition of the Na⁺-activated ATPase, suggesting that the E₂ conformation of this enzyme is only very short-lived. With Na⁺ as activating ion, the dephosphorylation reaction (Fig. 9, step 4) is most probably rate-limiting.

There is quite some uncertainty in the literature regarding the inhibition of non-gastric H,K-ATPase by ouabain and SCH 28080. The present study shows that this enzyme was hardly sensitive to SCH 28080, whereas relatively high concentrations were required for inhibition by ouabain. In the latter case, the degree of inhibition clearly depended on the concentration and type of cation that was present. Ouabain binds preferentially to the E₂-P form of Na,K-ATPase, and its inhibitory effect is effectively antagonized by K⁺ ions that stimulate the dephosphorylation reaction. Fig. 4A shows that the apparent affinity for ouabain decreases in the order Na⁺ < NH₄⁺ < K⁺. If one assumes that the inhibition mechanism for ouabain is similar in non-gastric H,K-ATPase to that in Na,K-ATPase, one can conclude from Fig. 4A that the relative amount of E₂-P during the catalytic cycle is the lowest with K⁺ followed by NH₄⁺ and Na⁺. Because the amount of E₂-P is decreased by dephosphorylation and Na⁺ is much less potent than K⁺ and NH₄⁺, this hypothesis fits for Na⁺. K⁺ and NH₄⁺, on the other hand, are equally potent in the dephosphorylation reaction so that one would expect equal apparent affinities for ouabain when the ATPase is activated either by K⁺ or by NH₄⁺. However, K⁺ more strongly antagonizes ouabain inhibition than NH₄⁺. One has to realize, however, that the circumstances of the dephosphorylation experiments (time and temperature) are so different from those of the ATPase measurements that their results can only be used in a semiquantitative way.

In contrast to the present study, Na⁺ did not increase the activity of human non-gastric H,K-ATPase when co-expressed with the β -subunit of gastric H,K-ATPase in Sf21 cells, whereas it inhibited the K⁺-stimulated enzyme (9). The maximal ATPase activity reported by Adams *et al.* (9) was only 5–10% of that measured in the present study. Adams *et al.* (9), however, included 6 μ M oligomycin in their assay media, whereas we show here that this drug inhibits rat non-gastric H,K-ATPase with an IC₅₀ of 3 μ M. It is not clear whether the different results are because

of species differences, a different β -subunit, and/or methodical differences.

The most used natural sources of non-gastric H,K-ATPase are isolated apical membranes of distal colon. Gustin and Goodman (49) showed some 20 years ago the presence of a K^+ -activated ATPase in these membranes. They also showed that this ATPase could be phosphorylated by ATP and that the phosphorylation level decreased in the presence of K^+ . This ATPase was further characterized by Codina *et al.* (18). They found that K^+ as activating cation could be replaced by NH_4^+ . However, in that study the additional presence of Na^+ was required both for this cation-activated ATPase activity and the inhibitory effect of ouabain. In our study no Na^+ was required, and Na^+ even inhibited the K^+ -activated ATPase. A possible reason for this discrepancy might be that in the colon preparation, an additional factor is present that makes the ATPase sensitive to Na^+ .

Non-gastric H,K-ATPase has been demonstrated to be present in both the apical membrane of colon cells and in cells of the distal convoluted tubule in the renal medulla. Our findings indicate that this enzyme not only exchanges K^+ for H^+ , but also NH_4^+ for H^+ and, under special conditions, even Na^+ for H^+ . There is no indication from the present study that the role of H^+ can be taken over by NH_4^+ . A role in NH_4^+ back transport, however, is very difficult to reconcile with studies showing that chronic hypokalemia that results in an up-regulation of the non-gastric H,K-ATPase in the kidney also results in increased ammonia secretion (17).

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