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The effects of Na⁺ on gastric H,K-ATPase were investigated using leaky and ion-tight H,K-ATPase vesicles. Na⁺ activated the total ATPase activity in the absence of K⁺, reaching levels of 15% relative to those in the presence of K⁺. The Na⁺ activation, which takes place at the luminal side of the membrane, depended on the ATP concentration and the type of buffer used. The steady-state ATP phosphorylation level, studied with leaky vesicles, was reduced by Na⁺ due to both activation of the dephosphorylation reaction and a shift to E₂ in the E₁↔E₂ equilibrium. By studying this equilibrium in ion-tight H,K-ATPase vesicles, it was found that Na⁺ drives the enzyme via a cytosolic site to the nonphosphorylating E₂ conformation. No H⁺-like properties of cytosolic Na⁺ could be detected. 

We therefore conclude that Na⁺ behaves like K⁺ rather than like H⁺ in the H,K-ATPase reaction.

H,K-ATPase is an intrinsic membrane protein complex, which is located in the secretory vesicles of the gastric parietal cell and is able to generate a proton gradient of 10⁶ across the membrane in exchange for potassium. Na,K-ATPase, present in plasma membranes of all mammalian cells, is responsible for the maintenance of the intracellular levels of potassium and sodium and can generate a Na⁺ gradient of about 10¹² (1, 2). The catalytic α-subunits of both ATPases have been cloned from several species and have a molecular mass of 112–114 kDa (1, 2). The homology between these two ATPases is higher than (3). Both Na,K-ATPase and H,K-ATPase belong to the P-type ATPases as (i) the catalytic subunits can be phosphorylated at an aspartyl residue, (ii) they are inhibited by submicromolar vanadate concentrations, and (iii) two different enzyme conformations (E₁ and E₂) can be distinguished. The E₁ form has high-affinity cation binding sites at the cytosolic side, whereas the E₂ form has high affinity cation binding sites at the luminal side of the membrane. The Post-Albers scheme, which is based on the alternate formation of these two conformations, is often used to explain the reaction mechanisms of both Na,K-ATPase and H,K-ATPase. In the overall H,K-ATPase reaction cycle (7, 8), where H⁺ is transported across the cytosol to the lumen in exchange for K⁺ (steps 1–7, Fig. 1), different partial reactions can be distinguished such as (i) the steady-state ATP phosphorylation reaction (steps 2, 3, and 4), (ii) the dephosphorylation reaction (steps 5 and 6), and (iii) the E₁↔E₂ transition (steps 7 and 1).

Due to the common characteristics of the two ion transporting enzymes have been studied intensively. Proton-like effects of sodium on H,K-ATPase (a "Na,K"-ATPase activity) (9) and sodium-like effects of protons on Na,K-ATPase (a "H,K"-ATPase activity) have been claimed (10), although the latter effects were not found when ATP phosphorylation was studied (11). In Na,K-ATPase, Na⁺ shows, besides effects of its own, K⁺-like properties in the absence of K⁺. This Na,Na-ATPase or Na-ATPase activity is the result of activation of both the ATP phosphorylation, and the dephosphorylation reaction by Na⁺ (12). The data regarding the effects of Na⁺ on H,K-ATPase is somewhat confusing. In some H,K-ATPase studies an identical activation by Na⁺ of the ATP hydrolysis reaction, a H,Na-ATPase activity, has been observed (13). Similar K⁺-like effects of Na⁺ have been found on the rate of ATP phosphorylation (13–15), but the dephosphorylation reaction has been claimed to be either activated (16), or insensitive toward Na⁺ (15). Furthermore, two studies (9, 17) indicate that Na⁺ behaves more like H⁺ and drives the enzyme to an E₁ conformation.

In preliminary experiments De Jong (18) observed that the K₀.₅ for ATP in the phosphorylation reaction was considerably increased by Na⁺. A similar Na⁺-like effect cannot easily be explained when Na⁺ behaves like H⁺. It could be explained, however, when Na⁺ behaves as a K⁺ analog. In that case the ion activates the dephosphorylation reaction and drives the enzyme into the E₂ conformation. With the use of ion-tight H,K-ATPase vesicles, where no activation of the dephosphorylation process by extravesicular cations can occur (19), and by comparing their properties with those of leaky vesicles, where such activation does occur, the effects of Na⁺ on the total ATPase reaction, the steady-state ATP phosphorylation level, the dephosphorylation reaction, and the E₁↔E₂ transition were investigated. The results show that Na⁺ displays K⁺-like actions under those reaction conditions, thus activating the dephosphorylation process at the luminal side of the membrane and driving the enzyme into an E₂ conformation by interacting at the cytosolic side.

**MATERIALS AND METHODS**

**H,K-ATPase Preparations**—Gastric H,K-ATPase was purified from pig gastric mucosa as reported previously (20). 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 and stored at 4 °C. Leaky vesicles were prepared by diluting these ion-tight vesicles in 20 mM Tris acetate (pH 7.0) followed by centrifugation (100,000 × g), resuspension in water and freeze drying. This preparation was stored at −20 °C in 0.25 M sucrose, 50 mM Tris acetate (pH 7.0). In the experiments in which ion-tight H,K-ATPase vesicles were used the osmolarity was kept constant with 0.25 M sucrose and 50 mM Tris acetate (pH 7.0).

**Protein Determination**—Protein was determined with the Bio-Rad protein assay (21) using bovine serum albumin as a standard. All data was expressed in Lowry protein values which are 1.5 times higher than...
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The Effect of NaCl on the Steady-state ATP Phosphorylation Level—Fig. 2A shows the combined effects of ATP and NaCl on the steady-state ATP phosphorylation level of gastric H,K-ATPase. At 22 °C and pH 7.0, in the absence of NaCl the ATP affinity was very high, the K0.5 being about 0.01 μM (19). Upon increasing the [Na+]i, the ATP affinity decreased. The maximal phosphorylation level tended to be slightly reduced at higher [Na+], indicating that the inhibition is not simply a competition between Na+ and ATP. If the effect of Na+ on the steady-state ATP phosphorylation was compared with its effect on the overall ATP hydrolysis rate, at 37 °C, and in the presence of 20 μM ATP (Fig. 2B), it was observed that, whereas the ATP phosphorylation decreased (I50 = 150 μM) the ATPase activity was activated at low (K0.5 = 14 μM), and inhibited at high NaCl concentrations. These observations suggest that the rate-limiting step in the reaction cycle changes at the different [Na+] tested. From the data of Fig. 2B the turnover number of the enzyme can be calculated using the equation \( v = k \cdot [E-P] \), where \( v \) represents the rate of ATP hydrolysis and \( k \) the phosphorylation rate constant. NaCl increased this turnover number up to a value of 350 min⁻¹ in the presence of 60 mM NaCl. This value is about 15% of the maximal activity obtained in the presence of 1 mM ATP and 10 mM K⁺ (19). At high concentrations of NaCl (>100 mM) the turnover number decreased again. The stimulatory effects of Na+ suggest that this ion, like K+, increases the phosphorylation rate, which is the rate-limiting step in the H,K-ATPase reaction cycle under normal conditions.

Comparison of the Na*- and K+ -activated ATPase Activity of H,K-ATPase—The properties of the overall H,K-ATPase activity depend on the conditions in which the assay is performed (19). Fig. 3A shows that the maximal H,K-ATPase activity and the degree of K⁺ activation of the ATP hydrolysis depend on the ATP concentration. At low ATP concentration (5 μM), K⁺ activation occurred with a K0.5 value of 0.04 μM. Either in the presence of 1 mM KCl instead of 50 mM NaCl, or in the presence of both NaCl and KCl the I50 value for SCH 28080 increased to 0.2 μM, probably due the antagonism between SCH 28080 and K⁺. Although ouabain (1 mM) did not change the inhibition profile of SCH 28080, it was included in most experiments to ensure that any contaminating Na,K-ATPase activity was blocked. Corrections for the basal Mg-ATPase activity, which is the activity in the absence of K⁺ or Na⁺ in the presence of 0.1 mM SCH 28080, were also made.

Results

**Comparison of the Na⁺ and K⁺-activated ATPase Activity of H,K-ATPase**—The properties of the overall H,K-ATPase activity depend on the conditions in which the assay is performed (19). Fig. 3A shows that the maximal H,K-ATPase activity and the degree of K⁺ activation of the ATP hydrolysis depend on the ATP concentration. At low ATP concentration (5 μM), K⁺ activation occurred with a K0.5 value of 0.04 μM. The activity obtained with optimal K⁺ concentrations (0.4 mM) was about 20% of the maximal activity obtained with 0.5 mM ATP and 5 mM K⁺. At an ATP concentration of 5 mM the K0.5 value for K⁺
**Fig. 2.** The effect of Na⁺ on the steady-state ATP phosphorylation level and on the ATPase activity. A, the combined effect of Na⁺ and ATP on the steady-state ATP phosphorylation level. A leaky H,K-ATPase preparation (0.012–200 μg/80 μl) was preincubated at 22 °C in the presence 50 mM Tris acetate (pH 7.0), 0.1 mM MgCl₂, 0.2 mM ouabain, and 12.5, 25, 62.5, 125, and 250 mM NaCl. After 20 min the steady-state ATP phosphorylation level was determined by incubating for 5 s with 0.006–80 μM [γ-³²P]ATP (20 μl). The ATP phosphorylation level (nmol of E-P per mg of protein) at the different [NaCl] and [ATP] present during the phosphorylation period is plotted. B, comparison between the effects of Na⁺ on the steady-state ATP phosphorylation level and the ATP hydrolysis rate. H,K-ATPase (0.01 mg/ml (●) and 0.125 mg/ml (◆) was incubated at 37 °C in the presence of 20 μM [γ-³²P]ATP, 0.12 mM MgCl₂, 50 mM Tris acetate (pH 7.0) and NaCl as indicated. After 3 (●) or 120 (◆) s the reactions were terminated and the steady-state ATP phosphorylation level (●, nmol of E-P per mg protein) or the ATPase activity (◆, μmol of ATP hydrolyzed per mg of protein/h) were determined as described under "Materials and Methods."

**Fig. 3.** The effect of KCl and NaCl on the H,K-ATPase activity at varying concentrations of ATP. A leaky H,K-ATPase vesicle preparation (1–400 μg/ml) was incubated for 1–10 min at 37 °C in the presence of 30 mM Tris-HCl (pH 7.0), 5–5000 μM [γ-³²P]MgATP, 0.1 mM MgCl₂, 0.1 mM ouabain, and the KCl (A) or NaCl (B) concentrations as indicated. Maximally 30% of the ATP was converted. Activity is given as μmol of ATP hydrolyzed per mg of protein/h.

was 1.0 mM, which is 25 times the value at 5 μM ATP. High concentrations of K⁺ inhibited the ATP hydrolysis rate, the inhibition occurring at lower [K⁺] when less ATP was used.

The activation of the ATPase activity by Na⁺ depended, like the K⁺-activation, on the ATP concentration (Fig. 3B). In the presence of 5 μM ATP we observed a $K_{0.5}$ for Na⁺ of 10–20 mM while in the presence of 5 mM ATP this value increased to about 100 mM. The maximal Na⁺-ATPase activity reached levels of about 15–25 μmol of ATP hydrolyzed/mg of protein per h, which is about 15% of the activity obtained in the presence of K⁺.

The Affinity for ATP in the Na⁺-activated ATPase Reaction—The data of Fig. 3B show that the maximal "Na⁺"-ATPase activity hardly changed with the concentrations of ATP used. This indicates that the affinity for ATP is far below 5 μM. In the presence of 20 mM NaCl only one (high) affinity ATP site could be detected, $K_{0.5}$ for ATP = 0.25 μM. High concentrations of ATP did not increase the Na⁺-ATPase activity further as they did in the presence of KCl. The inhibitory action at high cation concentrations apparently overruled the activation process.

**Effect of the K⁺-antagonist Imidazole on the Na⁺-ATPase Activity—**Imidazole, a tertiary amine, is an antagonist of the activation by K⁺ of the H,K-ATPase activity (19). In the presence of 20 μM ATP, 0.12 mM Mg²⁺ and 50 mM imidazole/acetate (pH 7.0), a $K_{0.5}$ of 80 mM for Na⁺ was determined. This value is about five times higher than in the presence of 50 mM Tris acetate (see Fig. 2B). So, there is an antagonism between tertiary amines and Na⁺, which is similar to the antagonism between tertiary amines and K⁺.

**The Specificity of the Na⁺ Activation—**In order to test whether the activating effect of Na⁺ is due to Na⁺ itself or to a contamination by K⁺, two different types of experiments were carried out. First, the amount of K⁺ in the different media was determined with a flame photometer. The 125 mM NaCl stock solution used for most experiments contained only 4.8 μM K⁺.
The extra addition of K⁺ to NaCl media was completely recovered, indicating that high [Na⁺] did not disturb the K⁺ determination. Since K⁺ at these concentrations had hardly any effect, this finding already suggests that the Na⁺ effect is not due to contaminating K⁺.

Second, with the use of the K⁺ ionophores, valinomycin and nigericin, the K⁺ activation of ATPase activity was studied in ion-tight vesicles. In this type of H,K-ATPase vesicles the K⁺ activation site is located intravesicularly (20). Fig. 4A shows that in these vesicles the basal (Mg-ATPase) activity was very low and that activation by extravesicular (cytosolic) K⁺ was not possible. In the presence of nigericin, a K⁺-like ionophore, the K⁺ activation profile was nearly identical to that of a leaky H,K-ATPase preparation, in which the K⁺ activation site is freely accessible (Fig. 3A). In the presence of the specific K⁺ ionophore valinomycin, however, there was only a slight activation, probably due to the ionophore-induced voltage difference across the vesicle membrane (23). The lack of activation could partially be overcome by the extra addition of the protophosphorylating ionophore CCCP. When similar experiments (Fig. 4B) were carried out in the presence of Na⁺, activation of the ATP hydrolysis was only observed in the presence of nigericin, which ionophore can also, but to a lesser extent, exchange Na⁺ for K⁺ (23). Valinomycin either alone or in combination with CCCP could not induce a Na⁺-activated Pi production due to the absolute selectivity of this ionophore for K⁺.

These observations indicate that the activation of the ATP hydrolysis by Na⁺ is not due to a contamination by K⁺, but that Na⁺ itself activates the dephosphorylation process at the luminal (intravesicular) side of the membrane.

The Effects of Na⁺ and K⁺ on the Dephosphorylation Reaction—The dephosphorylation reaction was studied in leaky H,K-ATPase preparations. Fig. 5A shows that both Na⁺ and K⁺ enhance its rate, with an apparent 50% enhancement of this process was observed, showing that the Na⁺-activation site, like the K⁺-site (19, 25), is located intravesicularly (the luminal side). In ion-tight vesicles the phosphophorylation capacity was used as a measure for the relative amount of the E₁ form of H,K-ATPase, as only the E₁ form can...
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Fig. 5. The effects of NaCl, KCl, and choline chloride on the dephosphorylation reaction and the E₁→E₂ transition. A, cation specificity of the dephosphorylation reaction. Leaky H,K-ATPase vesicles (2 μg/50 μl) were incubated in the presence of 1 mM [γ-32P]ATP, 0.1 mM MgCl₂, and 20 mM Tris acetate (pH 7.0). After 10 s, 0.45 ml of dephosphorylation mixture was added and incubated for another 3 s as described under "Materials and Methods." Final concentration of ATP was 20 mM. % E-P hydrolysed is the percentage E-P hydrolyzed during 3 s incubation. B, cation specificity in the E₁→E₂ transition. Ion-tight H,K-ATPase vesicles (1 μg in 180 μl) were preincubated for 5 s at room temperature, under iso-osmotic conditions with 20 mM Tris acetate (pH 7.0) and different chlorides (XCl) at the concentrations as indicated. The steady-state ATP phosphorylation was determined during a 3-s incubation with 50 mM [γ-32P]ATP and 0.1 mM MgCl₂. The percentage of phosphoenzyme is plotted as function of the cation concentration present during the phosphorylation reaction (200 mM). The osmolarity was kept constant with sucrose.

Fig. 6. The effect of Na⁺ on the E₁→E₂ transition at pH 8.0. Ion-tight H,K-ATPase vesicles (2 μg) were incubated at room temperature, under iso-osmotic conditions (corrected with choline chloride) in the presence of 50 mM Tris acetate (pH 8.0), 0–100 mM NaCl, 0.2, 2.0, and 20 mM [γ-32P]MgATP, and 0.1 mM MgCl₂. After 5 s the amount of phosphoenzyme (nmol of P/mg of protein) was determined as described under "Materials and Methods" and plotted as function of the NaCl concentration present during the phosphorylation reaction.

be phosphorylated and the dephosphorylation reaction cannot be activated by the ligands Na⁺ or K⁺. It was found that Na⁺, like K⁺ (19), reduces the phosphorylation capacity of the enzyme, meaning that Na⁺ shifts the equilibrium to the E₂ form. Even at low Na⁺ concentrations no increase in the steady-state ATP phosphorylation level, measured at suboptimal conditions, could be observed, excluding H⁺-like properties of Na⁺. The observation that cytosolic Na⁺ has K⁺-like properties, driving the enzyme to the E₂ form, is in line with the inhibition of the ATP phosphorylation rate (13–15) and the reduction of the H⁺-transport rate (15, 25, 28) by this ion.

Variable effects of Na⁺, in the absence of K⁺, on the overall H,K-ATPase activity (steps 1–7) have been reported (13, 15). These variations might be due to differences in assay conditions, since we demonstrate that high concentrations of ATP (Fig. 3B), Mg²⁺, and imidazole have marked effects on the Na⁺ (and the K⁺ (19)) affinity in the overall ATPase reaction. Moreover, high [Na⁺] inhibits the latter activity. In the overall ATPase experiments we were able to show that Na⁺, like K⁺, at relative low concentrations activated the hydrolysis of ATP, via the dephosphorylation reaction (steps 5 and 6), and inhibited the ATPase reaction at high concentrations by driving the E₁→E₂ equilibrium to the right (steps −1 and −7). The combination of both effects explains the increasing effect of Na⁺ on the K₉,5 for ATP in the phosphorylation reaction (Fig. 1A).

In both Na,K-ATPase and H,K-ATPase, K⁺ activates the dephosphorylation reaction. The role of K⁺ can be performed in both enzymes by Na⁺ (Refs. 27 and 28 and this study), although the affinity for Na⁺ is much lower than that of K⁺. In both enzymes K⁺ also drives the equilibrium E₁→E₂ to the E₂ form, whereas Na⁺ (for Na,K-ATPase) and H⁺ (for H,K-ATPase) shifts the equilibrium to the E₁ form. The present study shows that with H,K-ATPase Na⁺ can perform the latter role of K⁺, but not that of H⁺. With Na,K-ATPase there is no indication for an E₂ promoting effect of Na⁺ in the absence of K⁺. The ion specificity of Na⁺ and H⁺ as E₁ promoters in Na,K-ATPase and H,K-ATPase, respectively, is much more prominent. Neither an effect of H⁺ on the steady-state phosphorylation level of Na,K-ATPase (11) nor of Na⁺ on this parameter of H,K-ATPase (this study) was observed.

The data seems to conflict with studies by Rabon et al. (17), who used a fluorescein isothiocyanate-labeled H,K-ATPase preparation to test the effects of Na⁺. The fluorescence of this modified enzyme, incapable of being phosphorylated by ATP, increased in the presence of Na⁺. Although an antagonism between H⁺ and Na⁺ was observed, the increase in fluorescence was interpreted as an increase in the E₁ form of the enzyme, analogous to that with Na,K-ATPase. It has not been proven, however, that an increase in fluorescence under these
circumstances actually means a shift of the $E_{1}\rightarrow E_{2}$ equilibrium to the right. Another interpretation is that the $E_{2}Na$ form has a higher fluorescence than the $E_{1}K$ form.

A comparable “H⁺-like” effect of Na⁺ has also been observed by Polvani et al. (9), who measured an increased $^{22}Na^{+}$ uptake in H,K-ATPase vesicles, under special conditions of low [H⁺] (pH > 8.0), [Na⁺] between 2 and 5 mM, and the presence of intravesicular K⁺. The $^{22}Na^{+}$ uptake and the ATP hydrolysis rates were in a 1:1 ratio activated by luminal K⁺. Cytosolic Na⁺ failed to increase the hydrolytic rate of ATP, what should have been the case if Na⁺ has H⁺-like properties. Therefore, it is most likely that they measured H⁺- and K⁺-activated ATP hydrolysis together with an exchange of cytosolic 22Na⁺ for Na⁺ has H⁺-like properties. Therefore, it is most likely that they measured H⁺- and K⁺-activated ATP hydrolysis together with an exchange of cytosolic 22Na⁺ for Na⁺. Hence, Mg²⁺ (our conditions) to 0.33 mM does not affect the effect of ATP on the maximal activities. When we also used 2 mM Mg²⁺ we found an excess of [ATP]. In our experience the latter combination gives a higher fluorescence than the conditions of (De Pont, J. J. H. H., ed) pp. 27-55, Elsevier Science Publishers, Amsterdam.

The maximal H,K-ATPase activity was not affected. Hence, Mg²⁺ decreases the affinity of the enzyme for K⁺ and consequently the K⁺/ATP antagonism.

Another fundamental question is: if Na⁺ can substitute for K⁺ as activating cation for H,K-ATPase, why then is the maximal activity only 15% of that with K⁺? The most likely explanation is the difference between the affinities at the luminal and the cytosolic K⁺ sites. Since Na⁺ has a higher affinity for the cytosolic site than for the luminal site (affinity ratios of 88 versus 500), the inhibitory action on the ATP hydrolysis overrules the stimulatory effect. We cannot exclude that an additional reason for the lower activity of H,K-ATPase in the presence of Na⁺ is due to a decreased maximal rate of dephosphorylation in the presence of the latter ion.

In the Post-Albers model (Fig. 1) the activating K⁺ site (and/or Na⁺ site) is located at the luminal side of the membrane. Upon K⁺ binding to the $E_{2}P$ complex the enzyme changes to a conformation with “low” affinity for K⁺ at the cytosolic side. Our findings indicate that the ligands (K⁺ or Na⁺) which are promoters of this “on reaction” (steps 5 and 6) are, at the same time inhibitors of the “off reaction” (steps 7 and 8). Inhibitors of the on reaction, like H⁺ (15, 25), ATP (24), and tertiary amines (19), are in parallel, promoters of the off reaction.

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