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. 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 70%. The K⁺ channel activity of Na,K-ATPase is somewhat confusing. In some H,KATPase studies (19), many K⁺-like properties in the absence of K⁺ have been reported (13-15), although the dephosphorylation reaction has been 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.

In preliminary experiments De Jong (18) observed that the K⁺,Na⁺ ATPase activity was considerably increased by Na⁺. Such an effect of Na⁺ 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 0.35 M sucrose and 7% Ficoll (w/v) in 0.25 M sucrose, 50 mM Tris acetate (pH 7.0) 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 osmolality was kept constant with 0.26 M sucrose and 50 mM Tris acetate (pH 7.0).

Protein Determination—Protein was determined with the Bio-Rad Assay Kit using bovine serum albumin as a standard. All data was expressed in Lowry protein values which are 1.5 times higher than...
ATPase activities were determined with a radiochemical method. For reaction cycle turns clockwise. In the text, opposite reactions are noted by negative signs.

To 0.15 ml of the clear supernatant containing the liberated inorganic phosphate (32Pi), 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 the enzyme in the absence of KCl but in the presence of 0.1 mM SCH 28080. After incubation for 1–30 min at 37 °C the reaction was stopped by adding 500 µl of 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 (10,000 g). The amount of [32P]phosphoenzyme was determined as described (22).

Steady-state ATP Phosphorylation Level—H,K-ATPase (5–50 µm-1) was incubated at 22 °C for 3–10 s in 100 µl medium containing 20 µM [γ-32P]ATP (specific radioactivity 0.03–0.3 Ci mmol-1), Radiochemical Centre, Amersham, UK, 0.12 mM MgCl2, and 50 mM Tris-acetate (pH 7.0). The reaction was stopped by adding 5 ml of 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid. After filtration over a Schleicher & Schüll filter (type ME28, 1.2-µm pore width, Dassel, Germany) and washing with stopping solution, the a2P-protein content was diluted with nonradioactive Tris-ATP (pH 7.0). Nigericin, valinomycin (Sigma), and CCCP (Aldrich) were dissolved in ethanol and diluted to their final concentrations of maximally 100 µM in 1% ethanol. SCH 28080 (2-methyl-8-[phenylmethoxy]imidazo-[1-2-a]pyridine-3-acetonitrile) was kindly provided by Dr. B. Wallmark, Häsle, Mölndal, Sweden. All other chemicals were of analytical grade.

RESULTS

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 mM) the ATPase activity was activated at low (K0.5 = 14 mM), and inhibited at high NaCl concentrations. These observations suggest that the rate-limiting step in the reaction cycle changes at the different [Na+]i tested. From the data of Fig. 2B the turnover number of the enzyme can be calculated using the equation v = k(E-P), where v represents the rate of ATP hydrolysis and k the dephosphorylation 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 dephosphorylation 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 mM. 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+

The abbreviations used are: SCH 28080, 2-methyl-8-[phenylmethoxy]imidazo-[1-2-a]pyridine-3-acetonitrile; CCCP, carbonyl cyanide m-chlorophenylhydrazone.
**Na⁺ Acts as K⁺ Analog on Gastric H,K-ATPase**

**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/ml) was preincubated at 22 °C in the presence of 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 (A) and 0.125 mg/ml (B) 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 30 (A) or 120 (B) s the reactions were terminated and the steady-state ATP phosphorylation level (A, nmol E-P per mg protein) or the ATPase activity (B, 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. Maximal 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. 3A). In the presence of 5 µM ATP we observed a Kᵣ₅ 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, Kᵣ₅ 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 Kᵣ₅ 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. 3B). 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⁺.

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**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\textsuperscript+ to NaCl media was completely recovered, indicating that high [Na\textsuperscript+] did not disturb the K\textsuperscript+ determination. Since K\textsuperscript+ at these concentrations had hardly any effect, this finding already suggests that the Na\textsuperscript+ effect is not due to contaminating K\textsuperscript+.

Second, with the use of the K\textsuperscript+ ionophores, valinomycin and nigericin, the K\textsuperscript+ activation of ATPase activity was studied in ion-tight vesicles. In this type of H,K-ATPase vesicles the K\textsuperscript+ activation site is located intravesicularly (23). Fig. 4A shows that in these vesicles the basal (Mg-ATPase) activity was very low and that activation by extravesicular (cytosolic) K\textsuperscript+ was not possible. In the presence of nigericin, a K\textsuperscript+ for H\textsuperscript+ exchanger, the K\textsuperscript+ activation profile was nearly identical to that of a leaky H,K-ATPase preparation, in which the K\textsuperscript+ activation site is freely accessible (Fig. 3A). In the presence of the specific K\textsuperscript+ 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 protontophore CCCP. When similar experiments (Fig. 4B) were carried out in the presence of Na\textsuperscript+, activation of the ATP hydrolysis was only observed in the presence of nigericin, which ionophore can also, but to a lesser extent, exchange Na\textsuperscript+ for H\textsuperscript+ (23). Valinomycin either alone or in combination with CCCP could not induce a Na\textsuperscript+-activated P\textsubscript{i} production due to the absolute selectivity of this ionophore for K\textsuperscript+.

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

**The Effects of Na\textsuperscript+ and K\textsuperscript+ on the E\textsubscript{1}»E\textsubscript{2} Transition**—In closed vesicles no activation of the luminal K\textsuperscript+ (or Na\textsuperscript+)-site by extravesicular ligands can occur, see above and Ref. 20. At low ATP concentrations it is feasible to study the effects of these ligands on the E\textsubscript{1}»E\textsubscript{2} transition by determining the steady-state ATP phosphorylation level (19), as only the E\textsubscript{1} enzyme can be phosphorylated by ATP. Fig. 5A shows that both Na\textsuperscript+ and K\textsuperscript+, but not choline chloride, reduce the phosphorylation level at suboptimal ATP concentrations. In the presence of either 0.4 mM K\textsuperscript+ or 36 mM Na\textsuperscript+ (ratio Na\textsuperscript+/K\textsuperscript+ = 88) the amount of phosphoenzyme obtained was reduced by 50%. Other related monovalent cations such as Tl\textsuperscript+, Rb\textsuperscript+, NH\textsubscript{4}\textsuperscript+, Cs\textsuperscript+, and Li\textsuperscript+ had I\textsubscript{50} values of 0.005, 0.4, 0.5, 20, and 65 mM, respectively. This result indicates that Na\textsuperscript+, like K\textsuperscript+, and the other monovalent cations, drives the enzyme to the E\textsubscript{2} conformation.

**E\textsubscript{1}»E\textsubscript{2} Studies at pH 8.0**—When the extravesicular proton concentration in these experiments was reduced 10 times, by changing the pH to 8.0, the affinity for ATP in the steady-state ATP phosphorylation reaction decreased, K\textsubscript{d,obs} = 0.2 \textmu M (Fig. 6) compared to 0.01 \textmu M at pH 7.0 (see Fig. 2A, Ref. 19). Fig. 6 shows, in addition, that Na\textsuperscript+ did not increase the phosphoenzyme level at suboptimal ATP concentrations, demonstrating the absence of any H\textsuperscript+–like properties of Na\textsuperscript+ under these conditions. Na\textsuperscript+ only led to a decrease in the steady-state phosphorylation level (I\textsubscript{50} values for Na\textsuperscript+ were 6, 18, and 40 mM in the presence of 0.2, 2, and 20 \textmu M ATP, respectively), underlining once more the ATP/Na\textsuperscript+ antagonism.

**DISCUSSION**

In this study data is presented which clearly shows that Na\textsuperscript+ ions behave like K\textsuperscript+ ions in the H,K-ATPase reaction cycle. Na\textsuperscript+ activates the dephosphorylation reaction (steps 5 and 6, Fig. 1) in a preparation in which both cytosolic and luminal ion binding sites are accessible. In intact H,K-ATPase vesicles no enhancement of this process was observed, showing that the Na\textsuperscript+-activation site, like the K\textsuperscript+-site (19, 25), is located intravesicularly (the luminal side). In ion-tight vesicles the phosphorylation capacity was used as a measure for the relative amount of the E\textsubscript{1} form of H,K-ATPase, as only the E\textsubscript{1} form can activate the dephosphorylation reaction. With the use of ion-tight vesicles, in combination with valinomycin and CCCP it was possible to show that the site of inhibition is located at the extravesicular (cytosolic) side of the membrane. Parallel to K\textsuperscript+, the Na\textsuperscript+-activated dephosphorylation rate was also reduced in the presence of ATP (data not shown).

The Effects of Na\textsuperscript+ and K\textsuperscript+ on the Dephosphorylation Reaction—The dephosphorylation reaction was studied in leaky H,K-ATPase preparations. Fig. 5A shows that both Na\textsuperscript+ and K\textsuperscript+, but not choline chloride, reduce the phosphorylation level at suboptimal ATP concentrations. In the presence of either 0.4 mM K\textsuperscript+ or 36 mM Na\textsuperscript+ (ratio Na\textsuperscript+/K\textsuperscript+ = 88) the amount of phosphoenzyme obtained was reduced by 50%. Other related monovalent cations such as Tl\textsuperscript+, Rb\textsuperscript+, NH\textsubscript{4}\textsuperscript+, Cs\textsuperscript+, and Li\textsuperscript+ had I\textsubscript{50} values of 0.005, 0.4, 0.5, 20, and 65 mM, respectively. This result indicates that Na\textsuperscript+, like K\textsuperscript+, and the other monovalent cations, drives the enzyme to the E\textsubscript{2} conformation.

![Graph A](image1.png)

**Graph A**

**Graph B**

![Graph B](image2.png)
Na⁺ Acts as K⁺ Analog on Gastric H,K-ATPase

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 by Na⁺ in the presence of 60 mM Tris acetate (pH 8.0). In both Na,K-ATPase and H,K-ATPase, K⁺ activates the 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 conditions is due to an increase in the E₁ form of the enzyme.
circumstances actually means a shift of the $E_2\rightarrow E_1$ equilibrium to the right. Another interpretation is that the $E_2Na$ form has a higher fluorescence than the $E_1K$ form.

A comparable "H*-like" effect of Na* has also been observed by Polvani et al. (8), who measured an increased $22Na$ 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 $22Na^*$ 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 the luminal $K^*$ (normally the $K^*$-$K^*$ exchange) and not a Na,K-ATPase activity.

Our observation of an antagonism between ATP and either $K^*$ or Na* was not made by Wallmark et al. (16). The main difference between their and our approach is that they used constant (2 mM) Mg$^{2+}$ and we always used 0.1 mM Mg$^{2+}$ in excess of [ATP]. In our experience the latter combination gives maximal activities. When we also used 2 mM Mg$^{2+}$ we found the effect of ATP on the $K_0.5$ for $K^*$ activation to be reduced, e.g. at 20 $\mu$M ATP the $K_0.5$ for $K^*$ doubled from 0.16 mM to 0.32 mM Mg$^{2+}$ (our conditions) to 0.33 mM at 2 mM Mg$^{2+}$ (conditions of Wallmark et al. (16)). The maximal H,K-ATPase activity was not affected. Hence, Mg$^{2+}$ 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 of the enzyme to 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 $E_2P$ 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 1). 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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