Protons as Substitutes for Sodium and Potassium in the Sodium Pump Reaction*

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The role of protons as substitutes for Na\(^+\) and/or K\(^+\) in the sodium pump reaction was examined using inside-out membrane vesicles derived from human red cells. Na\(^+\)-like effects of protons suggested previously (Blostein, R. (1985) J. Biol. Chem. 260, 829–833) were substantiated by the following observations: (i) in the absence of extravesicular (cytoplasmic) Na\(^+\), an increase in cytoplasmic [H\(^+\)] increased both strophanthidin-sensitive ATP hydrolysis \((\varphi)\) and the steady-state level of phosphoenzyme, \(EP\), and (ii) as [H\(^+\)] is increased, the Na\(^+\)/ATP coupling ratio is decreased. K\(^+\)-like effects of protons were evidenced in the following results: (i) an increase in \(v\), decrease in \(EP\), and hence increase in \(E_{pump}\) turnover \((v/EP)\) occur when intravesicular (extracellular) \([H^+]\) is increased; (ii) an increase in the rate of Na\(^+\) influx into K\(^+\)(Rb\(^+\))-free inside-out vesicles and (iii) a decrease in Rb\(^+\)/ATP coupling occur when \([H^+]\) is increased. Direct evidence for H\(^+\) being translocated in place of cytoplasmic Na\(^+\) and extracellular K\(^+\) was obtained by monitoring pH changes using fluorescein isothiocyanate-dextran-filled vesicles derived from 4',4'-disothiocyano-2',2'-stilbene disulfonate-treated cells. With the initial pH, \(pH_i\), \(\approx 6.2\), a strophanthidin-sensitive decrease in pH, \(pH_f\), was observed following addition of ATP provided the vesicles contained K\(^+\). This pH gradient was abolished following addition of Na\(^+\). With alkali cation-free inside-out vesicles, a strophanthidin-sensitive increase in pH was observed upon addition of both ATP and Na\(^+\). The foregoing changes in pH were not affected by the addition of tetrabutylammonium to dissipate any membrane potential and were not observed at pH 6.8. These ATP-dependent cardiac glycoside-sensitive proton movements indicate Na,K-ATPase mediated Na\(^+\)/H\(^+\) exchange in the absence of extracellular K\(^+\) as well as H\(^+\)/K\(^+\) exchange in the absence of cytoplasmic Na\(^+\).

The Na,K-ATPase exhibits maximal activity around physiological pH. Like most enzymes, it is inhibited as the pH is either decreased or increased (for example, see Refs. 1 and 2). Recently, Breitweiser et al. (21) have shown that this behavior reflects ionization of groups accessible at the cytoplasmic face of the enzyme, presumably in the vicinity of the active center. Under optimal conditions with respect to ligand concentrations, the pH-dependent changes in activity reflect changes in \(V_{max}\) (2). Another important feature of proton interactions with Na,K-ATPase is the associated shift in conformation of the enzyme, from the \(E_1\), Na\(^+\) form, to the \(E_0\), K\(^+\) form (3, 4).

An intriguing question that remains is whether protons have a role distinct from the aforementioned titrations, namely whether they, themselves, can substitute for Na\(^+\) at cytoplasmic sites and/or K\(^+\) at extracellular sites. Although evidence of pH effects at extracellular sites is not apparent under optimal conditions in which the pump catalyzes "normal" Na/K exchange and associated ATP hydrolysis (1, 2), it remains plausible that extracellular K\(^+\) and, indeed, cytoplasmic Na\(^+\) obscure interaction(s) of protons at translocation sites. In fact, evidence in support of protons acting as Na\(^+\) was obtained from experiments with inside-out red cell membrane vesicles (IOV) showing that, in the absence of Na\(^+\) and at low pH, the sodium pump catalyzes proton-activated Rb\(^+\) (presumably K\(^+\) as well) transport from the extracellular to the cytoplasmic side of the membrane (5). In subsequent reports, Hara et al. (6) provided evidence for protons having a Na\(^+\)-like effect on the Na\(^+\) sites of Na,K-ATPase and for H\(^+\) uptake into K\(^+\)-loaded Na,K-ATPase proteoliposomes (7); in the latter report, H\(^+\)-activated K\(^+\) efflux was not determined.

In this paper, we describe results of experiments aimed to evaluate the possible role of protons as substitutes for both Na\(^+\) and K\(^+\). Thus, to substantiate the existence of H\(^+\)/K\(^+\) exchange whereby H\(^+\) act as Na\(^+\) substitutes, pump-mediated Rb\(^+\) efflux and H\(^+\) influx as well as the side specificity of H\(^+\) effects on ATP hydrolysis have been assessed under similar conditions using inside-out membrane vesicles derived from human red cells. We have also addressed the question of whether protons can substitute for K\(^+\). Our results show that, in contrast to Na/K exchange which is inhibited by lowering the pH, pump-mediated Na\(^+\) transport measured in the absence of extracellular K\(^+\) is stimulated by a decrease in pH and that this effect is due to H\(^+\) acting at extracellular K\(^+\) sites. Finally, direct evidence for pump-mediated H\(^+\) translocation was obtained by monitoring pH changes in FITC-dextran-filled IOV.

For simplicity, we refer to pump behavior in the presence of cytoplasmic (extravesicular) Na\(^+\) and extracellular (intra-vesicular) K\(^+\) or Rb\(^+\) as the Na\(^+\)/K\(^+\) mode, that in the presence of extracellular Rb\(^+\) or K\(^+\) and absence of cytoplasmic Na\(^+\) as the O/K\(^+\) mode, and that in the presence of cytoplasmic Na\(^+\)

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and absence of extracellular K+ as the Na+/O mode. As in previous reports (5, 8), cytoplasmic Na+ is designated Na\textsubscript{cyt} and extracellular Rb+ or K+ as Rb\textsubscript{ext} or K\textsubscript{ext}, respectively.

**EXPERIMENTAL PROCEDURES**

Fresh human blood was obtained from healthy volunteers, and the red cells were collected by centrifugation and washed three times with ice-cold 154 mM choline chloride. IOV were prepared as described earlier (9, 10). In certain experiments, namely those aimed to measure intravesicular pH changes or to maintain pH gradients for short periods, the cells were treated with DIDS to minimize anion permeability (11). In addition, choline bitrate replaced choline chloride. For the DIDS treatment, the washed cells were incubated for 20-55 min at room temperature with 2 volumes of 50 mM DIDS in 154 mM choline chloride, 10 mM glucose, 10 mM sucrose, 10 mM Tris-glycylglycine, pH 7.4 (solution A). They were then centrifuged and washed four times with solution A containing 1% bovine serum albumin followed by three more washes with solution A without albumin.

For measurements of intravesicular pH changes, the pH-sensitive probe FITC dextran (average M, 17,500; see Ref. 12) was incorporated inside the vesicles by adding 2 mg/ml FITC-dextran to the vesication buffer. Unincorporated FITC-dextran was removed completely during the three subsequent washes of the IOV with 10 mM Tris-glycylglycine (glycylglycine titrated with Tris to pH 7.4) containing 0.2 mM MgSO\textsubscript{4}. Vesicles were stored for up to 6 days at a concentration of 1-3 mg/ml in either 10 or 20 mM Tris-glycylglycine containing 0.2 mM MgSO\textsubscript{4}, pH 7.4. For fluorescence measurements, vesicles were washed and equilibrated with 50 mM mannitol containing 1 mM MgSO\textsubscript{4}, 0.2 mM KCl, 0.2 mM NaCl, and 5 mM choline bitrate titrated with Tris to the desired pH. For experiments aimed to measure intravesicular buffering capacity by NH\textsubscript{4}Cl titration (13), additional buffer (5 mM Mes-Tris) was present to minimize changes in extravesicular pH upon addition of NH\textsubscript{4}Cl. Fluorescent measurements were carried out at 37 °C using a Perkin-Elmer model SL-5 fluorescence spectrophotometer equipped with a constant magnetic stirring device. For each assay, 0.02-ml vesicles were added to 2.7 ml of medium. Activity was initiated by adding ATP (K+-filled IOV) or ATP plus Na\textsubscript{2}SO\textsubscript{4} (K+-free IOV). Emission of FITC-dextran was measured at 520 nm using an excitation wavelength of 496 nm.

**RESULTS**

**Effects of pH on the Na\textsuperscript{+}/Rb\textsuperscript{+}:ATP Coupling Ratios**—In view of the appearance of an O/K+ mode of pump behavior at acidic pH (5), we reasoned that if protons were replacing Na\textsuperscript{+} and exchanging with Rb\textsuperscript{+} (K+) the number of Na\textsuperscript{+} ions translocated per ATP molecule hydrolyzed should be decreased by increasing the proton concentration. Similarly, the possibility that protons may substitute for K\textsuperscript{+} should be evidenced in a decreased Rb\textsuperscript{+} (or K\textsuperscript{+})-ATP coupling. To test these possibilities, the rates of Rb\textsuperscript{+} loss, 22Na\textsuperscript{+} gain, and strophanthidin-sensitive ATP hydrolysis were measured concurrently under conditions which were adjusted with respect to time and ATP concentration so that close to initial rates prevailed (cf. Ref. 14). As shown in Table I, with vesicles carrying out "normal" Na\textsuperscript{+}/Rb\textsuperscript{+} exchange, the Na\textsuperscript{+}/ATP ratio was reduced from 1.71 to 0.81 as the pH was lowered from 7.6 to 6.2. Similarly, in the absence of intravesicular (extracellu- lar) alkali cations (Na\textsuperscript{+} mode), the ratio was decreased from 1.55 to 0.76. It is likely that the relatively low value for the Na\textsuperscript{+}/ATP ratio obtained at higher pH values in some experiments such as Experiment A is due to the hydrolytic activity of permeable vesicles.

As shown in Table I, the Rb\textsuperscript{+}/ATP coupling ratio measured during Na\textsuperscript{+}/K\textsuperscript{+} exchange was also decreased when the pH was lowered, i.e. from a ratio of 0.65 at pH 7.95 to 0.29 at pH 6.2. Moreover, a low ratio observed at pH 6.2 in the presence of Na\textsuperscript{+} (Na\textsubscript{2}SO\textsubscript{4}/Rb\textsuperscript{+} mode) was observed also in the absence of Na\textsuperscript{+} (O/Rb\textsuperscript{+} mode).

**Effects of Protons on 22Na\textsuperscript{+} Influx**—In view of the decreased Rb\textsuperscript{+}/ATP coupling effected by acidification, we tested whether H\textsuperscript{+} can substitute for K\textsuperscript{+} in promoting ATP-dependent Na\textsuperscript{+} influx into IOV (normal efflux). The rate of 22Na\textsuperscript{+} influx was measured at pH 7.4 and 6.2 using IOV which were either K\textsuperscript{+}-free or loaded with K\textsuperscript{+}, i.e. under conditions which support Na\textsuperscript{+}/Na\textsuperscript{+}/K\textsuperscript{+} fluxes, respectively. As shown in Table II, decreasing the pH stimulated the Na+/O flux but inhibited markedly (~50%) the Na\textsuperscript{+}/K\textsuperscript{+} flux. These ATP-dependent fluxes were all strophanthidin-sensitive (not shown).

**Strophanthidin-sensitive ATPase Activity**—The enzymic correlates of the proton-stimulated ATP-dependent Na\textsuperscript{+} in-
flux into alkali cation-free IOV described above and Rb⁺ (K⁺) efflux into alkali cation-free medium as reported earlier (5) were also examined. Thus, the rate of strophanthidin-sensitive ATP hydrolysis was measured in the absence of alkali cations (O/O conditions) as well as under Na⁺/K⁺ and Na⁺/O conditions. Hydrolysis under O/O and Na⁺/O conditions was assayed with sufficiently low ATP concentration to allow detection of the relatively low ATP hydrolysis activity; hydrolysis under Na⁺/K⁺ conditions was carried out with IOV equilibrated with K⁺ (0.2 mM) in the presence of valinomycin. The results depicted in Fig. 1 show that as pH is decreased, ATP hydrolysis is stimulated under Na⁺/O conditions but inhibited under Na⁺/K⁺ conditions. Stimulation by protons was also observed in the absence of alkali cations (O/O conditions) consistent with the possibility that protons replace Na⁺ at the cytoplasmic surface and K⁺ at the extracellular surface (see below).

Sidedness of Proton Effects—To obtain information regarding the sidedness of proton effects we carried out the following experiments. With Na⁺, present, strophanthidin-sensitive hydrolytic activity was measured for short periods using vesicles equilibrated at either low pH (6.0) or high pH (7.8) and assayed in medium at either high or low pH, respectively. The activities observed were compared with those obtained in the absence of a pH gradient. The intravesicular pH values reported in Fig. 2 and Table III are those prevailing initially, namely those of the equilibrated vesicles prior to dilution into assay medium; the extravesicular pH values are those attained following dilution with assay medium and, because of the relatively small intravesicular volume (≤0.5% of the total volume), remain unaltered. To minimize dissipation of the imposed pH gradients, vesicles were prepared from red cells pretreated with DIDS (11) and assayed for only short periods (5 s).

As shown in Fig. 2, ATP hydrolysis was stimulated when intravesicular pH was lowered. This is observed irrespective of whether the extravesicular (cytoplasmic) pH is high (pH 7.7) or low pH (pH 6.5). As shown in Table III, when the steady-state level of phosphoenzyme intermediate, EP, was measured under the same conditions, intravesicular (extracellular) acidification caused a decrease in EP and thus an increase in its apparent turnover, namely the ratio of hydrolytic rate, ν, to EP. These effects are clearly similar to those observed with K⁺ (15, 16).

The possibility that protons act at Na⁺ sites is evidenced in the following results. In the absence of Na⁺ (O/O condi-

Fig. 1. Effects of pH on strophanthidin-sensitive ATP hydrolysis. Vesicles were diluted 20-fold with 50 mM choline chloride, 1 mM MgSO₄, 0.8 mM EDTA, 0.2 mM EGTA, 2 mM Mes titrated with Tris to the desired pH, concentrated by centrifugation, and equilibrated for 30 min at 37°C as described in Table I. Reactions were started by adding 1 volume of vesicles to 9 volumes of reaction medium of the same composition except for differences in pH as indicated and containing also 2 mM NaCl and 0.2 μM [γ-32P]ATP (specific activity ≈20,000 dpm/pmol). Reactions were carried out for 5 s at 37°C. All assays were carried out in two sets of quadruplicates, one without and one with strophanthidin-treated vesicles as described in Fig. 1. Isosmotic solutions at various pHs were prepared as follows: in addition to 1 mM MgSO₄, 0.8 mM EDTA, and 0.2 mM EGTA, solution A (pH 6.0) comprised 60 mM choline bitartrate and 60 mM Tris, and solution B (pH 7.8) comprised 54 mM choline bitartrate and 69 mM Tris. Solutions at other pHs were obtained by mixing solutions A and B. These included :9 mixtures of solutions A and B and of solutions B and A to obtain controls whereby extravesicular pHs were the same as obtained by adding 1 volume of vesicles equilibrated in solution A to 9 volumes of B (pH 7.7) or 1 volume of vesicles equilibrated in solution B to 9 volumes of A (pH 6.5).

Table III
Sidedness of pH effects on Na⁺-ATPase and phosphoenzyme intermediate

Assays were carried out as described in Fig. 1 except that 0.8 part of the trichloroacetic acid-treated suspension was used for the assay of EP and 0.2 part for P; release as described (10).

| pH | Extracellular | Cytoplasmic | ATP hydrolysis (ν) | EP | r/EP |
|----|--------------|-------------|------------------|----|-----|
|    | pmol/mg/min  | pmol/mg/min | pmol/mg/min       |    |     |
| 7.7| 55.4 ± 5.8   | 55.4 ± 5.8  | 0.56 ± 0.02       | 99 |     |
| 6.0| 132.2 ± 10.1 | 132.2 ± 10.1| 0.25 ± 0.01       | 527|     |
| 7.8| 24.6 ± 11.1  | 24.6 ± 11.1 | 0.70 ± 0.02       | 35 |     |
| 6.5| 74.4 ± 4.7   | 74.4 ± 4.7  | 0.21 ± 0.01       | 354|     |
tions, Table IV) extravesicular (cytoplasmic) acidification results in an increase in hydrolytic activity and an increase in the steady-state level of EP (Table II, compare rows 1 and 3). EP formed in the absence of Na⁺ is sensitive to K₉₀₀ (not shown). It should be noted also that protons have Kₛₙ₉₀₀-like effects in the absence of Naₛ₉₀₀ (O/O conditions, Table IV) as well as the presence of Naₛ₉₀₀ (Na⁺/O conditions, Table III), namely they increase v and decrease EP.

Pump-mediated Proton Translocation—To obtain direct evidence for protons acting as Na⁺ and/or K⁺ substitutes, measurements of intravesicular pH changes were monitored in FITC-dextran-loaded IOV prepared from red cells rendered anion-impermeable by pretreatment with DIDS. The response of this probe to changes in pH was linear in the pH range studied (pH 6.0–8.0); under the conditions used, a 44% change in fluorescence corresponded to a pH change of 1 unit (not shown). Intravesicular pH changes corresponding to O/K⁺ fluxes are shown in Fig. 3. The reactions were initiated by adding ATP. As shown, when the initial pH is 6.2 (pHₕ = pH₉₀₀), a rapid decrease in fluorescence corresponding to a decrease in pH was observed, reaching a plateau within 1 min. With strophanthidin-treated vesicles or when the pH was elevated to 6.8, a pH change could not be detected.

With vesicles free of alkali cations and incubated at pH 6.2, intravesicular alkalinization was observed when both ATP and Na⁺ were added (Na⁺/O mode, Fig. 4); neither ligand alone was effective. Fluorescence reached a plateau within 1 min. A much smaller but detectable alkalinization was observed with K⁺ present inside (Na⁺/K⁺ exchange conditions, Fig. 4), but in this case the behavior was distinct from that observed under O/K⁺ and Na⁺/O conditions (see below). When assays under Na⁺/O conditions were carried out with strophanthidin-treated vesicles or if the pH was higher (pH 6.8), changes in fluorescence could not be detected.

It was then examined whether the strophanthidin-sensitive pH changes are secondary changes, reflecting the redistribution of protons or hydroxyl ions in response to a membrane potential (Δψ) elicited by the so-called O/K⁺, Na⁺/O, and Na⁺/K⁺ fluxes, rather than primary events reflecting pump-driven proton movements. To test these possibilities, the lipophilic cation TBA was used to dissipate Δψ (cf. Ref. 7). As shown in Fig. 5, under conditions which support Na⁺/O

| pH | ATP hydrolysis (+) | EP v/EP |
|----|-------------------|--------|
| 7.7 | 29.9 ± 1.9 | 0.20 ± 0.01 | 116 |
| 6.0 | 52.8 ± 3.7 | 0.10 ± 0.08 | 513 |
| 7.8 | 55.7 ± 3.7 | 0.34 ± 0.05 | 165 |
| 6.5 | 80.2 ± 3.6 | 0.13 ± 0.02 | 642 |

Fig. 3. K⁺- and ATP-dependent intravesicular pH changes: H⁺/K⁺ mode. FITC-dextran-filled vesicles derived from DIDS-treated red cells were diluted 20-fold in 50 mM mannitol, 1 mM MgSO₄, 0.8 mM EDTA, 0.2 mM EGTA, 0.5 mM K₂SO₄, and 5 mM choline bitartrate titrated with Tris to either pH 6.2 or 6.8 as indicated and then concentrated by centrifugation and equilibrated as described in Table I. Twenty µl were then added to a fluorescence cuvette containing 2.7 ml of the same solution except with K₂SO₄ omitted. ATP (0.5 mM, Na⁺/free, titrated with Tris to pH 6.0) and Na₂SO₄ (2 mM) were added as indicated. Vesicles were preincubated with strophanthidin (0.02 mM) during the last 5 min of preincubation as indicated.

Fig. 4. Na⁺- and ATP-dependent intravesicular pH changes: Na⁺/H⁺ mode. Assays were carried out as described in Fig. 3 except that the vesicles were free of alkali cations and the final concentrations of ATP and Na₂SO₄ were 0.2 and 2.5 mM, respectively.

Fig. 5. Tetrabutylammonium effects on pump-mediated intravesicular pH changes. Assays were carried out as described in Figs. 3 and 4. TBA (5 mM) was added as indicated.
and O/K+ pump modes, the fluorescent changes were unaffected by TBA, irrespective of whether it was added before or after ATP. In contrast, with Na+ and ATP added to K+-filled IOV (Na+/K+ mode), the small increase in fluorescence was rapidly dissipated by TBA (Fig. 5) or TPP (not shown).

In the experiments shown in Fig. 6 the effect of ATP concentration on the pump-mediated net pH changes was examined. In the case of the O/K+ mode, the half-maximal change in fluorescence was obtained with 0.2 mM ATP; for the Na+/O mode, half-maximal change was observed with only ~3 μM ATP. Although these results are only estimates of apparent affinities for ATP, initial rates being technically difficult to measure accurately, they have interesting implications. First, the value obtained for the O/K+ mode is in agreement with the apparent Km for ATP obtained for proton-activated Rb+ transport from IOV (5), for pump-mediated activation of K+-filled Na,K-ATPase proteoliposomes (6), and, in general, the existence of a low affinity ATP site involved in K+ translocation (17). The high affinity for ATP observed in the absence of K+, (Na+/O mode) is similar to that observed for Na-ATPase (18, 19) and the Na+/O flux mode (18).

Estimate of the Initial Rates of Net Proton Fluxes—The buffering capacity of the IOV was assessed by an NH4Cl titration method (13) so that rates of H+ uptake and extrusion could be estimated from the initial rates of fluorescence changes. Accordingly, varying amounts of NH4Cl were added to dilute suspensions of FITC-dextran-filled IOV (see "Experimental Procedures"). Under the conditions used the amounts of NH4Cl added (8-80 μM) did not alter extravascular pH but caused a transient intravesicular alkalinization. This was evidenced in an abrupt rise in fluorescence followed immediately by a slower decrease (Fig. 7, inset A). As shown in Fig. 7, the heights of the peaks of fluorescence (plotted as percent change in fluorescence) were directly proportioned to the amounts of NH4Cl added. These fluorescent changes reflect, presumably, the rapid inward diffusion of NH3 molecules and their subsequent protonation. Assuming NH3 has equilibrated rapidly and that the pK of NH3 is 9.21, the intravesicular buffering capacity was calculated as Δ[NH3]/ΔpH, and used to estimate rates of H+ uptake and extrusion (15). From the typical experiment shown in Fig. 7 (insets B and C), the rates of pump-mediated H+ uptake under O/Rb+ conditions and H+ extrusion under Na+/O conditions were both estimated to be 20 μM/min. The rates of Rb+ efflux and Na+ influx obtained under the same conditions were 21 and 31 μM/min, respectively (not shown). Under the conditions used, namely additional buffer compared to the experiments shown in Figs. 4-6, the intravesicular pH changes were small and reached a plateau rapidly (~5 s). Thus, as evident from the typical experiment shown in Fig. 7, these values may only be regarded as approximations.

**DISCUSSION**

Several lines of evidence support the role of protons as substitutes for cytoplasmic Na+ and for extracellular K+. It was shown earlier that in the absence of Na+, protons activate strophanthidin-sensitive Rb+ efflux from IOV derived from human red cells (5). In the present study, we have delineated the enzymic correlates of this transport mode. Thus, in nominally Na+-free medium, lowering the pH (for example, from physiological levels to ~pH 6.2) increases strophanthidin-sensitive ATP hydrolysis; from an analysis of the sidedness of the proton effects it is evident that in the absence of Na+ and K+, cytoplasmic protons, like Na+, increase both the hydrolytic activity and the steady-state level of phosphoenzyme intermediate. The question as to whether protons can be transported as Na+ was addressed first in experiments which showed that a reduction in pH results in a decrease in the Na+/ATP coupling ratio irrespective of whether or not Rb+ (K+) is present at the extracellular side. Finally, the most direct evidence for H+ acting as Na+ is the observed strophanthidin-sensitive acidification that occurs upon addition of ATP to FITC-dextran-filled IOV containing K+ and derived from relatively anion-impermeable DIDS-treated red cells. This result is similar to that obtained with FITC-dextran-filled kidney Na,K-ATPase proteoliposomes (7).

We also show that protons can substitute for K+ in the Na,K-ATPase pump mechanism. Thus, under Na+/O condi-
tions but not Na⁺/K⁺ conditions, acidification stimulates Na⁺ uptake into IOV and increases staphrohanthidin-sensitive ATP hydrolysis. Furthermore, it is extracellular rather than cytoplasmic protons which, like K⁺, increase the rate of hydrolysis and decrease the level of EP and thus increase the apparent turnover of the phosphoenzyme. Another effect of acidification is that the reduced Rb⁺/ATP coupling consistent with protons replacing Rb⁺ (K⁺). As in the case of R² substituting for Na⁺, the most direct evidence for K⁺-like effects of protons is the staphrohanthidin-sensitive alkalinization observed upon addition of both Na⁺ and ATP to K⁺-free IOV derived from DIDS-treated cells. Little, if any, of the alkalinization observed under Na⁺/O conditions or the acidification which occurs under O/K⁺ conditions reflects H⁺ translocation in response to the development of a membrane potential, as evidenced in the lack of effect of the lipophilic cations TBA or TPP under these conditions. In contrast, the intravesicular alkalinization observed under Na⁺/K⁺ exchange conditions is abolished when TBA is present, consistent with collapse of the potential (positive inside) effected by the exchange of (presumably) 3Na⁺ for 2K⁺.

Pump-mediated Na⁺ efflux from human red cells into alkali cation-free medium was originally characterized as an uncoupled Na⁺ efflux (18, 20). More recently, Dissing and Hoffman (21) showed that this flux is electroneutral and coupled to anions, at least at physiological pH. It appears from the present study that at acidic pH, an ATP-dependent staphrohanthidin-sensitive Na⁺/H⁺ exchange also exists; its apparent affinity for ATP, albeit estimated from steady-state levels of vesicle alkalinization (Fig. 7), is similar to that reported for the “uncoupled” Na⁺ efflux (18). It remains to be determined whether the Na⁺ anion cotransport mode, like Na⁺/K⁺ exchange, decreases as pH is decreased so that at pH 6.2 it accounts for only a small fraction, if any, of the Na⁺/O flux.

The proton-mediated increases in pump-mediated Na⁺ flux and K⁺ or Rb⁺ flux in the absence of extracellular K⁺ and cytoplasmic Na⁺, respectively, appear to be effects distinct from those causing a decrease in overall activity. Thus, overall activity as evidenced in pump-mediated Na⁺/K⁺ exchange and Na⁺ + K⁺-dependent ATP hydrolysis decreases as pH is decreased, reflecting, presumably, titration of groups at or near the active center. The possibility that acidification increases activity by increasing affinities for K⁺ (Rb⁺) or Na⁺ present under so-called Na⁺/O and O/K⁺ conditions, respectively, is unlikely for the following reasons. First, as reported previously (5), the levels of Na⁺ in the nominally Na⁺-free system are too low (<5 μM) to allow for even the minimal (one-for-one) exchange with K⁺ or Rb⁺; similarly, K⁺ is virtually undetectable in the vesicle preparations (not shown). Second, the possibility that these Na⁺/O and O/K⁺ modes occur by a mechanism other than Na⁺/H⁺ and H⁺/K⁺ exchanges, respectively, requires that the associated intravesicular pH exchanges are due to electrogenic Na⁺ and K⁺ fluxes. This is not the case for either exchange as mentioned above. It remains possible, however, that the putative Na⁺/H⁺ and H⁺/K⁺ fluxes are not electroneutral (cf. normal electrogenic three Na⁺ for two K⁺ exchange) and that the electrogenic components were not detected in our experiments.

Although it has not been technically feasible to quantify accurately the net rates of proton movements, our results do show that they are, to a first approximation, of similar magnitude as the rates of movement of Na⁺ and Rb⁺ under Na⁺/O and O/Rb⁺ (presumably O/K⁺ as well) conditions, respectively. Moreover, even if accurate values could be obtained, their significance would be difficult to interpret. It is unlikely that they would reflect rates of unidirectional proton fluxes since protons may be transported in both directions concurrently, in the absence as well as presence of both Na⁺/O and K⁺/Rb⁺. This is evidenced in (i) reduced Na⁺/ATP and Rb⁺/ATP coupling ratios not only under Na⁺/O and O/K⁺ conditions, respectively, but also when the pump mediates Na⁺/Rb⁺ exchange at acidic pH (see Table I) and (ii) the increase in staphrohanthidin-sensitive ATP hydrolysis observed in the absence of alkali cations when the pH is lowered as well as the greater increase in Na⁺-ATPase relative to the increase in Na⁺ influx when the pH is lowered under Na⁺/O conditions.

The finding that the estimates of initial rates of proton fluxes approximate those of Na⁺ and Rb⁺ attests to the likelihood that the staphrohanthidin-sensitive intravesicular pH changes reflect pump-mediated H⁺/K⁺ and Na⁺/H⁺ exchanges. Thus, it becomes unlikely that the changes in intravesicular pH reflect protonation-deprotonation of the Na⁺/K⁺-ATPase that accompanies the E₁-E₂ transitions (3, 4). First, such proton-enzyme interactions are thought to occur at the cytoplasmic (extravesicular) rather than extracellular surface (2); the pH changes reported here occur inside the IOV, i.e., in the extracellular milieu. Second, since the maximal amount of pump enzyme in red cell membranes is ~1 pmol/mg protein (15), if one assumes that the intravesicular space is 10−15 μl/mg (22), the maximal change in intravesicular [H⁺] would be approximately 0.1 μM. This value is an order of magnitude smaller than the net change in intravesicular [H⁺] calculated from Fig. 7 (1−2 μM) yet represents the upper bound characteristic of a virtually complete shift from a steady state with E₁ forms predominating to one with E₂ forms predominating, or vice versa.

Since the H⁺/K⁺ exchange mode is virtually undetectable at neutral pH, it cannot explain the markedly reduced Na⁺/Rb⁺ stoichiometry and reduced Na⁺/ATP coupling evident at physiological pH when the Na⁺ concentration is reduced to very low levels (23). One explanation for reduced stoichiometry and coupling at low cytoplasmic Na⁺ at physiological pH is that offered previously (9), namely that at very low Na⁺, the normal hydrolysis pathway is aborted; hydrolysis of E₁P rather than E₁P as depicted in the Post-Albers model prevails. It is also plausible that with suboptimal Na⁺ ions bound, the hydrolytic pathway operates through the normal sequence but with less than the maximal number, presumably three, Na⁺ ions bound (23).

Considering the fact that transport ATPases involving phosphoenzyme intermediates such as Na⁺,K⁺-ATPase, sarcolemmal reticulum Ca⁺-ATPase, and gastric H⁺,K⁺-ATPase are remarkably homologous with respect to their primary sequences (24−26), it is not surprising that a number of their enzymic functions are also similar (for a concise review, see Ref. 27). Nor is it surprising that the Na⁺,K⁺-ATPase like the H⁺,K⁺-ATPase has a remarkably high selectivity for protons at the cytoplasmic (Na⁺) sites such that H⁺/K⁺ exchange becomes detectable when the proton concentration is as low as 10−6 M. It will be important to consider whether the gastric pump can transport Na⁺ in place of H⁺, or the Ca⁺⁺ pump, protons in place of Ca⁺⁺. Insights into these questions are relevant to understanding the structural basis for apparent differences in cation affinities and/or selectivities among the different transport ATPases.

Although it appears difficult to reconcile alternative uses of such physicochemically different ions as H⁺ and Na⁺ or H⁺ and K⁺, it is possible that the H⁺/K⁺ and Na⁺/H⁺ exchanges reflect titrations by H⁺ of negatively charged cytoplasmic Na⁺ and extracellular K⁺ binding sites, respectively. In accordance with the Post-Albers model for the Na⁺,K⁺-ATPase reaction sequence (for a concise overview, see Ref. 28) and with the...
proviso that (i) differences in the pKs of these sites attend the distinct conformational states of both the unphosphorylated and phosphorylated enzyme and that (ii) alternations in the sidedness of exposure of the sites are associated with the enzyme's conformational changes, it is plausible that protons bound at one side are released from the opposite side of the membrane during the course of the hydrolytic reaction.

Alternatively, it is relevant to consider the well documented evidence that although the selectivity of the Na,K-ATPase for inorganic cations is high, it is not absolute. Thus, in studies of the ability of cytoplasmic cations to effect K⁺ influx or extracellular cations, Na⁺ efflux, it is observed that Li⁺ which is considerably different from both Na⁺ and K⁺ can substitute for Na⁺ at the cytoplasmic side (29-31). At the extracellular side, the specificity is rather broad and includes not only Li⁺ but also Na⁺ among the variety of cations which act as K⁺ congeners (see Ref. 32). Of particular relevance to the foregoing is the versatility of certain cotransport systems, for example the intestinal Na⁺- or H⁺-coupled sugar transporter (33) and the melibiose transporter of Escherichia coli which utilizes Na⁺ or H⁺ for cotransport with melibiose (34). These examples of alternative uses of H⁺ and Na⁺ in bioenergetics have been discussed by Boyer (35) in his recent insightful monograph concerning the possibility that protons are transported as hydronium ions. He cites earlier studies showing that a cyclic polyether such as dicyclohexyl-18-crown-6 can form stable complexes with H₂O⁺, Na⁺, K⁺, and several other cations. Thus, complexes of H₂O⁺, Na⁺, K⁺, or even Ca²⁺ with a cluster of coordinating oxygen or even nitrogen atoms would be generally similar; differences in the number and/or arrangement of the atoms in the cluster would allow for differences or changes in affinities/selectivities. In the context of the present study, the apparent ability of H⁺ to substitute not only for Na⁺ as suggested earlier (5-7), but also K⁺, makes this possibility particularly attractive.

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