Kinetic Properties of the K^+/H^+ Antiport of Heart Mitochondria†

Gerald P. Brierley* and Dennis W. Jung
Department of Physiological Chemistry, Ohio State University Medical Center, Columbus, Ohio 43221
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ABSTRACT: The fluorescence of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) has been used to follow a K^+-dependent alkaline shift in the matrix pH (pH_i) of isolated heart mitochondria. The K^+-dependent pH_i change has properties consistent with an inward exchange of K^+ for matrix H^+ on the K^+/H^+ antiport of the mitochondrion. The reaction is activated by depletion of matrix Mg^{2+} with A23187 and by an alkaline external pH (pH_o) and hypotonic conditions. The exchange is inhibited by quinine, dicyclohexylcarbodiimide, and exogenous Mg^{2+}, but not by Li^+. The rate of K^+/H^+ antiport measured in this way increases with increasing pH_o to a maximum near pH_o 9. The rate is a hyperbolic function of $[K^+]$ at pH_o values above 8.3 with an apparent K_m of 30 mM at pH_o 8.4 and 14 mM at pH_o 8.8. External H^+ acts as a mixed-type inhibitor of the K^+/H^+ antiport under these conditions with a K_i equivalent to pH_o 8.6–8.8. When pH_o is kept constant, the reaction is relatively insenstive to matrix pH (pH_i) in the range from 7.0 to 7.5. Above this pH_i, the K^+-dependent H^+ extrusion shows a hyperbolic dependence on $[H^+]_i$ with an apparent K_m equivalent to pH_i 8.1. The activated antiport shows an affinity sequence of $Li^+ > K^+ = Rb^+ > Cs^+$. The inward antiport of K^+ is inhibited noncompetitively by NH_4^+ and is also sensitive to benzamil and to 5-N-substituted amiloride analogues with I_{50} values near 20 μM. Both NH_4^+ and the amiloride analogues increase pH_i at constant pH_o and appear to be concentrated in the matrix under these conditions.

Isolated mitochondria contain both an overt Na^+/H^+ antiport and a latent K^+/H^+ antiport [see Brierley and Jung (1988a) or Garlid (1988a) for reviews]. Recent studies in three different laboratories have defined the kinetic properties of the Na^+/H^+ antiport in considerable detail (Kapus et al., 1988; Nath & Garlid, 1988; Brierley et al., 1989). This Na^+/H^+ antiport is specific for Na^+ (or Li^+) and shows no activity with K^+. It normally functions to extrude Na^+ from the matrix at the expense of the ΔpH component of the protonmotive force but can support the uptake of Na^+ when a source of H^+ is provided in the matrix. The uptake of Na^+ on this antiport is inhibited competitively by Li^+ (Kapus et al., 1988; Nath & Garlid, 1988; Brierley et al., 1989) and by external $[H^+]$ (Kapus et al., 1988; Brierley et al., 1989). Recent studies using the fluorescent probe 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)[1] have established that matrix H^+ acts as a simple Michaelis substrate with a K_m of pH_i 6.8 during the inward exchange of Na^+ at constant external pH (Brierley et al., 1989).

The kinetic properties of the K^+/H^+ antiport are not as well established. This antiport transports K^+ as well as Na^+ and Li^+ [see Brierley and Jung (1988b) or Garlid (1988b) for reviews]. It is activated by conditions that deplete matrix Mg^{2+} and is thought to have a regulatory site for Mg^{2+} on its matrix aspect. The K^+/H^+ antiport shows optimal activity only in hypotonic media and at alkaline pH and is inhibited by quinine and by DCCD. Martin and Garlid (1982) have reported that K^+ efflux on the K^+/H^+ antiport is inhibited by H^+ under conditions in which ΔpH is kept low. They found a linear relationship between the inverse velocity of K^+ extrusion and the square of $[H^+]$ and suggested that H^+ ions react at a regulatory site in addition to the transport site on the antiport (Garlid, 1987, 1988b; Martin & Garlid, 1982).

In a recent study from this laboratory (Brierley et al., 1989), we were able to detect little K^+-dependent extrusion of matrix H^+ using BCECF fluorescence, even when matrix Mg^{2+} was depleted with A23187. However, it was noted that the acid pH_i produced by $Mg^{2+}/2H^+$ exchange under these conditions might be expected to limit K^+/H^+ antiport on the basis of the observations of Garlid (1987, 1988b) and Martin et al. (1984). In the present study, we have defined conditions that permit K^+/H^+ antiport to be followed as a K^+-dependent alkaline shift in pH_i. This study provides a direct demonstration of H^+ movements that can be attributed to the K^+/H^+ antiport. It also establishes that $[H^+]_o$ acts as a mixed-type inhibitor of K^+ entry on this antiport and that the rate of K^+-dependent H^+ extrusion shows a hyperbolic relationship to $[H^+]_i$ with an apparent K_m near pH_i 8.1. The sensitivity of the K^+/H^+ antiport to NH_4^+ and to amiloride analogues is also documented.

Materials and Methods

Beef heart mitochondria were prepared as previously described (Brierley et al., 1984). The fluorescence of BCECF was followed with excitation at 509 and 450 nm and emission at 550 nm using a Perkin-Elmer fluorometer interfaced with a computer [see Jung et al. (1988, 1989) for details of the calibration of fluorescence in terms of pH_i and estimation of initial rates of pH change]. Mitochondrial Mg^{2+} and K^+ were determined by atomic absorption (Brierley et al., 1987) following rapid centrifugation of the mitochondria in an Eppendorf microcentrifuge. The time course of K^+ loss was monitored with a Corning monovalent cation electrode with a double-junction reference electrode and an Orion pH meter. Amiloride analogues, synthesized as previously described (Cragoe et al., 1967), were supplied by Dr. E. J. Cragoe, Jr.

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[1] Abbreviations: BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; pH_o, pH of the suspending medium; pH_i, pH of the matrix; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SMP, submitochondrial particle(s); DCCD, dicyclohexylcarbodiimide; TEA^+, tetraethylammonium ion; NEM, *N*-ethylmaleimide.

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Osmolality of the suspending media was measured in an Osmette A osmometer.

Estimation of the Rate of K^+-Dependent H^+ Extrusion. Beef heart mitochondria were loaded with BCECF as previously described (Jung et al., 1988, 1989) and incubated at 0.45 mg of protein/mL in a medium of choline chloride (50 mM) containing Tricine (20 mM, neutralized to the indicated pH_o with triethylammonium hydroxide), EGTA (30 μM), EDTA (400 μM), rotenone (3 μg/mL), and oligomycin (2 μg/mL). The temperature was maintained at 12 °C in order to keep the rate of K^+-dependent H^+ extrusion in a convenient measuring range, and also to minimize the loss of BCECF and prevent the development of K^+ uniport pathways that occurs at elevated pH_o at higher temperatures [see Brierley et al. (1984), for example]. The mitochondria were depleted of Mg^{2+} and Ca^{2+} by addition of A23187 (1 μM). After the pH_i stabilized, the rate of K^+-dependent H^+ extrusion was established by the rapid addition of KCl (usually 5–30 mM). Because the rate of K^+/H^+ antiport is profoundly affected by the osmotic strength of the medium (see below), all of the kinetic comparisons were made at a constant final osmolality. Thus, if 30 mM KCl was added, the rate in response to this addition was compared to that of 5 mM KCl added along with 25 mM choline chloride. There is no H^+ extrusion in response to addition of choline chloride alone. The kinetic data obtained were analyzed by using the GraphPAD program (ISI software) to fit a rectangular hyperbola, by double-reciprocal plots of velocity vs substrate concentration and by Hanes plots [see Dixon and Webb (1979)]. The buffering power of the mitochondria was measured as previously described (Mitchell & Moyle, 1969; Beavis, 1989; Jung et al., 1989).

Passive osmotic swelling was followed as a light-scattering change at 520 nm using a Brinkmann PC801 probe colorimeter. Heart mitochondria (0.5 mg/mL) were incubated at 24 °C in a medium of potassium acetate (50 mM) containing Tricine, oligomycin, rotenone, EGTA, and EDTA as described for the fluorescence assay above, and swelling was initiated by the addition of A23187 (1 μM).

Results

When A23187 is added to BCECF-loaded heart mitochondria suspended in hypotonic choline chloride at pH_o 8.5, the fluorescence record shows an immediate acidification of the matrix as Mg^{2+} is exchanged for external H^+ (Figure 1A). This initial acid shift is followed by a partial rebound (deflection labeled [2] in Figure 1A) and then by a slower acid shift (labeled [3]) to a steady-state value of pH_i 7.01. A parallel K^+ electrode record (Figure 2B) shows an extensive loss of matrix K^+ in conjunction with the acid shift in pH_i during phase [3]. This influx of H^+ and efflux of K^+ is inhibited by quinine (not shown), a property that is consistent with the participation of the K^+/H^+ anitport in the phase [3] flux of K^+ and H^+. Addition of nigericin after pH_i has reached a steady-state produces a further acid shift of 0.4 pH_i unit (Figure 1A) and immediate loss of 28 nmol of K^+·(mg of protein)$^{-1}$ (Figure 1B). An additional 12 nmol of $K^+ \cdot mg^{-1}$ is released when the mitochondria are lysed with Triton X-100 (Figure 1B). Addition of 30 mM KCl to the nigericin-treated mitochondria results in an extensive alkaline shift in pH_i as K^+ is exchanged for matrix H^+ (Figure 1A).

The time course of the observed changes in BCECF fluorescence, but not the steady-state value achieved, appears to be affected by the activity of the phosphate transporter. Addition of NEM to block this transporter eliminates the rebound that follows the initial acid shift when A23187 is added (Figure 1A, dashed record). It appears that a portion

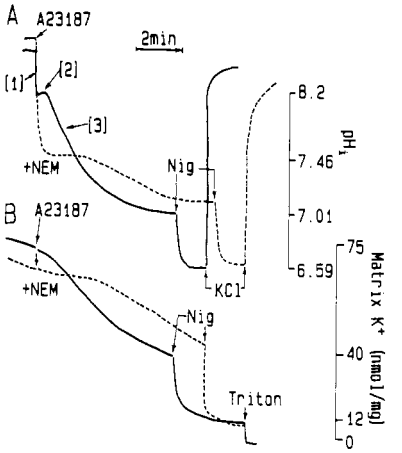

FIGURE 1: Changes in pH_i (A) and matrix K^+ (B) following the addition of A23187 to heart mitochondria. Incubation conditions are given under Materials and Methods. (A) Record of BCECF fluorescence with excitation at 509 nm and emission at 550 nm. At the indicated point, A23187 (1 μM) was added. The initial acid shift in pH_i due to $Mg^{2+}/2H^+$ exchange is designated [1]. Mg^{2+} is depleted from 27 nmol to 4 nmol/mg during this phase. The dashed record shows the response in the presence of NEM [67 nmol·(mg of protein)$^{-1}$]. The NEM-sensitive rebound in pH_i that appears to be due to the activity of the phosphate transporter is designated [2], and the slower matrix acidification that accompanies K^+ loss is designated [3]. The response to nigericin (1 μM) and KCl (30 mM) after the fluorescence record has reached a steady state is also shown. (B) Record of K^+ loss in a parallel incubation using BCECF-loaded mitochondria with other conditions identical with (A) and following K^+ with a Corning monovalent cation electrode. Where indicated, the mitochondria were lysed with Triton X-100 (0.07%). This fraction is presumed to represent a more tightly bound K^+ fraction. The matrix volume (Jung et al., 1988) averaged 1.0 μL/mg, and light-scattering records show only small changes in volume in these protocols.

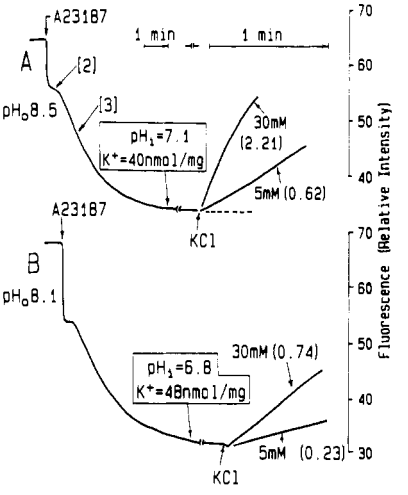

FIGURE 2: K^+-dependent H^+ extrusion as followed by BCECF fluorescence. (A) pH_o 8.53. (B) pH_o 8.12. The conditions were identical with those in Figure 1. Where indicated, the response to KCl (5 or 30 mM) is shown. The recorder speed was increased just prior to addition of KCl. When 5 mM KCl was added, 25 mM choline chloride was added simultaneously to maintain comparable osmotic conditions. Rates of K^+-dependent H^+ extrusion are given in ΔpH per minute in parentheses. Steady-state values for pH_i were estimated by the fluorescence ratio method as previously described (Jung et al., 1989).

of the H^+ exchanged for endogenous Mg^{2+} can be extruded with endogenous P_i when the phosphate transporter is active. The loss of matrix K^+ and influx of H^+ are delayed in the presence of NEM (Figure 1A,B), but both reach virtually the same steady-state levels as those found in the absence of NEM. It is now well established (Garlid, 1988b; Brierley & Jung,

Table I: Inhibitor Profile for K^+-Dependent H^+ Extrusion Corresponds to That of Mitochondrial K^+/H^+ Antiport[a]

expt	pH_o	additions	pH_i	K^+/H^+ exchange ($\Delta pH \cdot min^{-1}$)	% inhibition
1	8.9	none (EDTA, 100 μM)	7.6	3.53	
		quinine (20 μM)	7.9	0.54	85
		DCCD (40 $nmol \cdot mg^{-1}$)	7.6	1.77	50
		$MgCl_2$ (50 μM)	7.7	2.12	40
		$MgCl_2$ (70 μM)	7.8	1.47	58
2	8.6	none	7.0	1.80	
		CCCP (1.5 μM)	7.0	1.60	11
		valinomycin (1.0 μM)	6.9	1.70	6
3	8.5	none	6.9	1.50	
		choline chloride (50 mM)	7.2	0.51	66

[a] The protocol was identical with that shown in Figure 2A with pH_o as indicated and the inhibitor added just before K^+ addition. In expt 1, the EDTA concentration was 100 μM rather than the 400 μM present in expt 2 and 3. The DCCD was allowed to react for 1 min prior to KCl addition in expt 1. The standard incubation medium contained 50 mM choline chloride (Materials and Methods).

1988b) that depletion of matrix Mg^{2+} at elevated pH activates a K^+/H^+ antiport in mitochondria and in addition opens an anion-conducting channel (Garlid & Beavis, 1986). It is therefore likely that most of the available mobile anions of the matrix have been lost when pH_i reaches its steady-state after A23187 addition (Figure 1A). The ion content at this point ($[K^+]$ near 30 mM and $[H^+]$ equivalent to pH_i 7.01) reflects the Donnan distribution resulting from fixed negative charges in the mitochondrion under these conditions. The response of the fluorescence records to nigericin and KCl addition (Figure 1A) establishes that K^+/H^+ antiport in either direction can be followed by using BCECF to detect changes in matrix $[H^+]$.

Heart mitochondria treated with A23187 at pH_o 8.5 (Figure 2A) reach and maintain a steady-state pH_i of 7.1 (ΔpH 1.4). When these mitochondria are challenged with KCl, there is a rapid K^+-dependent alkaline shift in pH_i (Figure 2A) consistent with the inward exchange of K^+ for matrix H^+ on the mitochondrial K^+/H^+ antiport. The response to K^+ is immediate, and the pH change is linear for at least 10 s. The rate of H^+ extrusion increases with increasing K^+ concentration in the range from 5 to 30 mM K^+ (Figure 2A). The observed rate of 2.2 pH units/min for 30 mM K^+ can be converted to a rate of H^+ extrusion of 44 $nmol \cdot min^{-1} \cdot (mg \text{ of protein})^{-1}$ at 12 °C by using the measured buffering power of 20 nmol of H^+/pH unit. The rate of K^+-dependent H^+ extrusion is approximately doubled at 22 °C (not shown). When the protocol of Figure 2A is carried out at a lower external pH (pH_o 8.12, Figure 2B), the steady-state pH_i is somewhat lower (pH 6.8) and $[K^+]$ higher (about 35 mM). Under these conditions, the rate of K^+-dependent H^+ extrusion is considerably lower than that obtained at pH_o 8.5 (Figure 2B vs Figure 2A). When the protocol is repeated at pH_o 8.98, the steady-state pH_i is 7.5, the steady-state $[K^+]$ decreases to about 16 mM, and the rate of response to 30 mM K^+ increases to 87 nmol of $H^+ \cdot min^{-1} \cdot mg^{-1}$ (not shown). Matrix Mg^{2+} is depleted from 27 nmol/mg of protein to 4 nmol/mg in 1 min or less by A23187 at each pH_o value examined (pH_o 8.1, 8.5, 9.0).

Inhibitor Profile for K^+-Dependent H^+ Extrusion Corresponds to That of the Mitochondrial K^+/H^+ Antiport. Addition of 20 μM quinine just prior to K^+ in the protocol of Figure 2A increases the pH_i from 7.6 to 7.9 and inhibits the K^+-dependent change in pH_i by 85% (Table I). The K^+-dependent extrusion of H^+ is also strongly inhibited by DCCD (Table I) when this reagent is added after Mg^{2+} is depleted [see Martin et al. (1984)].

The experiments shown in Figures 1 and 2 were carried out in the presence of 400 μM EDTA. In the absence of chelator at pH_o 8.8, there is an immediate drop in pH_i to about 8.1 following addition of A23187, but no phase [3] pH change and virtually no K^+-dependent H^+ extrusion (ΔpH of only 0.3/min) indicative of K^+/H^+ antiport. The ratio of internal to external free $[Mg^{2+}]$ should be about 40 under these conditions (Tsien, 1983), so it is quite likely that matrix Mg^{2+} is not depleted sufficiently to activate K^+/H^+ antiport in the absence of the chelator. The EDTA concentration can be decreased to 100 μM under these conditions without effect on the rate of K^+-dependent H^+ extrusion. Addition of 50 μM Mg^{2+} in the presence of 100 μM EDTA after the pH_i has reached a steady state causes a small increase in pH_i (7.6 to 7.7) and inhibits the rate of K^+-dependent H^+ extrusion by 40% (Table I). When $[Mg^{2+}]$ is increased to 70 μM under these conditions, the pH_i increases to 7.8, and the reaction is inhibited by 55% (Table I). Higher levels of added Mg^{2+} abolish the ΔpH necessary to follow the K^+/H^+ antiport by this technique.

It is clear that an inward exchange of K^+ and efflux of H^+ on the quinine-sensitive K^+/H^+ antiport would account for the observed change in pH_i in response to K^+ (Figure 2). However, a similar K^+-dependent H^+ extrusion would also be seen if separate uniport pathways for K^+ and H^+ were present under these conditions. The rate of change in pH_i as K^+ is lost following Mg^{2+} depletion (phase [3] in Figure 1A) and the subsequent rate of K^+-dependent H^+ extrusion (Table I) are both barely affected by the addition of the uncoupler CCCP. If the exogenous K^+ uniporter valinomycin is added along with CCCP, there is a very rapid acidification of the matrix as the remainder of the endogenous K^+ is lost and a very rapid alkaline shift in response to the addition of 30 mM K^+ (as shown for nigericin in Figure 1A). The addition of valinomycin in the absence of CCCP results in the loss of some additional K^+ and a slight acid shift in matrix pH (Table I). However, the presence of valinomycin has very little effect on the rate of K^+-dependent H^+ extrusion when KCl is added (Table I). These results establish that large increases in either K^+ uniport or H^+ uniport conductivity have little effect on K^+-dependent H^+ extrusion and are consistent with an obligatory exchange of matrix H^+ for external K^+ to produce the observed change in pH_i under these conditions.

Activation of K^+/H^+ Antiport by Hypotonic Conditions. The K^+/H^+ antiport is activated by decreasing osmotic strength when measured as a respiration-dependent extrusion of K^+ (Bernardi & Azzone, 1983), by passive osmotic swelling, or by passive $^{42}K^+/K^+$ exchange [see Brierley and Jung (1988b)]. Both the phase [3] acid shift in pH_i following Mg^{2+} depletion and the subsequent K^+-dependent extrusion of matrix H^+ are increased in a 50 mM choline chloride medium compared to a medium containing 100 mM choline chloride (Table I). Decreasing the choline chloride concentration to 25 mM or omitting this salt completely produce a further small activation of K^+/H^+ antiport as measured under these conditions, but the rates are less reproducible (not shown). Quite comparable results are obtained when the osmolality of the suspending medium is adjusted with sucrose rather than choline chloride. It therefore does not seem likely that choline competes with K^+ for a site on the antiporter. It is clear that the activity of this antiport is increased significantly when the matrix swells osmotically in Mg^{2+}-depleted mitochondria at constant pH_o. Choline chloride added after Mg^{2+} depletion

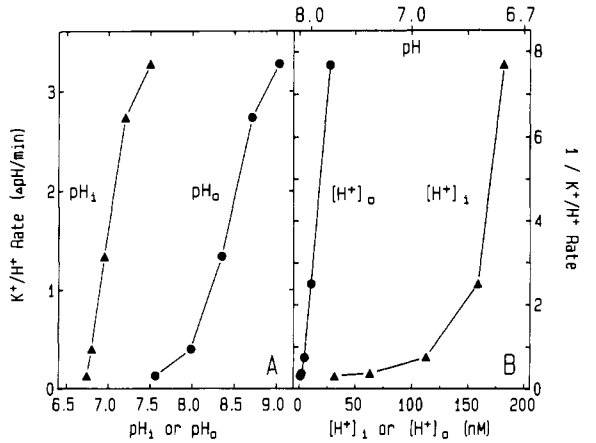

FIGURE 3: Effect of pH_o and pH_i on the rate of K^+-dependent extrusion of matrix H^+ under the conditions of Figure 2. (A) Changes in rate ($\Delta pH \cdot min^{-1}$) vs pH_o (glass electrode) and pH_i as estimated from BCECF fluorescence just prior to the addition of KCl (20 mM). (B) Dixon plots of the data from (A).

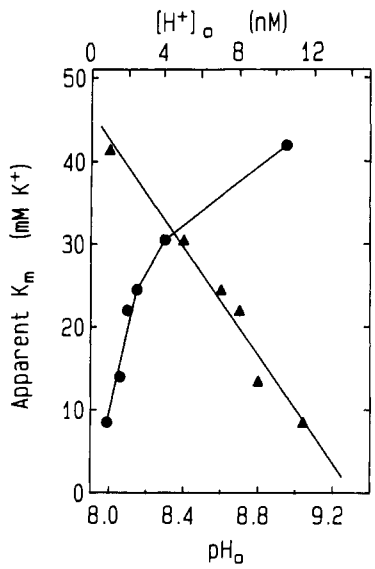

FIGURE 4: Relationship of the apparent K_m for K^+ to pH_o (triangles) and $[H^+]_o$ (circles). A total of 18 determinations of the apparent K_m for K^+ were made at the indicated pH_o values using at least 4 concentrations of K^+, and the apparent K_m was evaluated from rectangular hyperbolae or Hanes plots (Dixon & Webb, 1979). These points fit the line plotted vs pH_o with a correlation coefficient of 0.936. The points shown at pH_o 8.8 and 8.6 are the mean of five separate determinations. The pH_o intercept of this plot falls at 9.26.

and just prior to a challenge with KCl (protocol of Figure 2A) inhibits K^+/H^+ antiport with virtually the same effectiveness as when the salt is present prior to the addition of A23187 (I_{50} of 60 mM under either set of conditions).

Effects of pH on K^+/H^+ Antiport. When the protocol of Figure 2 is carried out at different values of pH_o, the rate of K^+-dependent H^+ extrusion increases as pH_o increases (Figure 3A). Rates of K^+/H^+ antiport start to decline only above pH_o 9.2–9.4 (not shown). In these protocols, the steady-state pH_i obtained after the addition of A23187 (see Figure 1A) also varies, and the maximum rate of K^+-dependent H^+ extrusion is seen at pH_i values near 7.5.

Plots of reciprocal rate vs $[H^+]_o$ (Dixon plots) are linear in the range from pH_o 7.5 to 9.0 and extrapolate to very near the origin (Figure 3B). The analogous plot vs $[H^+]_i$ on the same scale is not linear (Figure 3B) and indicates little change in rate with changing pH_i in the range from pH_i 7.0 to 7.5. This plot also indicates that the rate of K^+-dependent H^+ extrusion decreases with increasing $[H^+]_i$ in the range below pH_i 7.0 (Figure 3B).

The rate of K^+-dependent H^+ extrusion shows a hyperbolic dependence on external $[K^+]$ at values of pH_o above 8.3 but a near-linear relationship at lower pH_o. The apparent K_m for K^+ at pH_o 8.8 averages 13.5 ± 2.9 mM (n = 5; ±SD) whereas that at pH_o 8.4 averages 30 ± 1 mM (n = 3). A total of 18 reliable estimates of the apparent K_m have been generated over a period of several months, and these values show a good linear relationship (r = 0.936) to pH_o (Figure 4). This plot extrapolates to zero at pH_o 9.26 (Figure 4). The apparent K_m for K^+/H^+ antiport therefore does not show the linear relationship to $[H^+]_o$ (Figure 4) that was found for Na^+/H^+ antiport (Brierley et al., 1989). This establishes that $[H^+_o$ is not a competitive inhibitor of K^+ entry on the K^+/H^+ antiport.

Lineweaver–Burk plots at fixed pH_o values (not shown) are consistent with $[H^+]_o$ acting as a mixed-type inhibitor of inward K^+ antiport under these conditions, in that the reciprocal plots intersect to the left of the $1/v$ axis and not on the $1/S$ axis [see Dixon and Webb (1979)]. In a typical experiment, the apparent K_m for K^+ decreased from 47.6 mM at pH_o 8.16 to 21.1 mM at pH_o 8.63 and to 7.9 mM at pH_o 9.08. The corresponding apparent V_{max} (y intercept) values were 1.58, 3.41, and 5.78 $\Delta pH \cdot min^{-1}$ at the three values of pH_o. A plot of these intercepts vs $[H^+]_o$ is linear and intersects the x axis at −1.5 nM. This is equivalent to pH_o 8.8 and can be taken as the K'_i for $[H^+]_o$ as a mixed inhibitor of K^+/H^+ antiport under these conditions. This plot (not shown) extrapolates to a V_{max} at zero $[H^+]_o$ of 8.8 $\Delta pH \cdot min^{-1}$ or about 160 nmol of $H^+ \cdot min^{-1} \cdot mg^{-1}$ at 12 °C. The V_{max} is approximately doubled at 22 °C in this assay (not shown) and corresponds well to the value of 300 $nmol \cdot min^{-1} \cdot mg^{-1}$ obtained by Garlid (1988b) for K^+ extrusion on this antiport. The experiment just described was repeated a total of 3 times with essentially the same value for the apparent K_m for K^+ at a given pH_o and with the same value for K'_i for $[H^+]_o$. The V_{max} values varied with the preparation of mitochondria (5.5, 6.7, and 8.8 $\Delta pH \cdot min^{-1}$ for the three determinations).

Table II: Relative Effect of pH_o and pH_i on Kinetic Parameters for K^+-Dependent H^+ Extrusion[a]

pH_o	pH_i	app K_m (mM)	V_{max} ($\Delta pH \cdot min^{-1}$)
8.8	7.4	12	6.3
8.4	7.0	30	3.9
8.4	7.4[b]	26	3.0

[a] Incubation conditions are described under Materials and Methods. The apparent K_m and V_{max} were calculated from Hanes plots. [b] pH_i adjusted by addition of 0.3 mM Na^+.

A replot of the slopes of such double-reciprocal plots vs $[H^+]_o$ is not linear (not shown), so that a graphical evaluation of K_i for $[H^+]_o$ as a mixed inhibitor of K^+/H^+ antiport [see Dixon and Webb (1979)] is not possible. However, plots of $[K^+]/v$ vs $[H^+]_o$ for different K^+ concentrations are linear and intersect at a point near −2.3 nM $[H^+]_o$ (equivalent to pH_o 8.6). This intersection point has been used to evaluate K_i for a mixed inhibitor in other enzyme systems.

Kinetic parameters obtained at different pH_o values from protocols such as those of Figure 4 may not be strictly comparable, because pH_i as well as pH_o varies under these conditions (see Figure 3). However, the experiment summarized in Table II indicates that changes in pH_i have relatively little effect on the apparent K_m and V_{max} for any given pH_o value. It is possible to vary pH_i at constant pH_o by allowing a small amount of Na^+ to displace a portion of the matrix H^+ by Na^+/H^+ antiport [see Brierley et al. (1989)]. When this

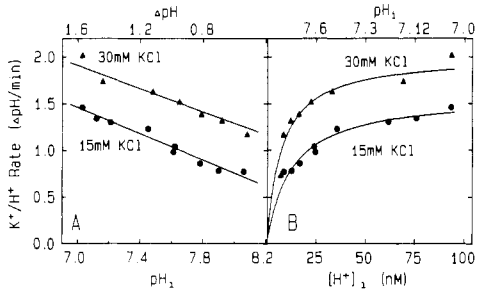

FIGURE 5: Effect of changing pH_i at constant pH_o on the rate of K^+-dependent H^+ extrusion. (A) Plot of velocity ($\Delta pH \cdot min^{-1}$) vs pH_i for two different concentrations of K^+ (15 and 30 mM) under the conditions of Figure 1 with pH_o 8.6. Internal pH was adjusted by small additions of Na^+ (0.3–2.0 mM) prior to the addition of K^+. (B) Replot of the data of (A) vs $[H^+]_i$. Hanes plots of these data give apparent K_m values equivalent to pH_i 7.9 for 15 mM K^+ and pH_i 8.2 for 30 mM K^+. The corresponding V_{max} values are 1.6 and 2.1 $\Delta pH \cdot min^{-1}$, respectively.

procedure is used to increase pH_i from 7.0 to 7.4 at pH_o 8.4, there is only a small decrease in both the apparent K_m and V_{max} of the K^+-dependent H^+ extrusion reaction (Table II). In contrast, when the pH_i is held constant at 7.4, it is clear that there is a large decrease in the apparent K_m at pH_o 8.8 compared to pH_o 8.4 and a much greater V_{max} at the higher pH_o (Table II). It should be noted that the driving force for the antiport reaction is the ΔpH in these protocols (since the steady-state matrix $[K^+]$ is near 30 mM and external $[K^+]$ varies from 5 to 30 mM) and that ΔpH decreases as pH_i is increased under the conditions of Table II.

When pH_i is increased systematically at constant external pH by addition of increasing amounts of Na^+, the rate of K^+-dependent H^+ extrusion falls off as a linear function of pH_i or ΔpH (Figure 5A). The response to 30 mM K^+ is roughly parallel to the plot produced by addition of 15 mM K^+ (Figure 5A). Plots of the velocity of K^+/H^+ antiport vs $[H^+]_i$ are hyperbolic (Figure 5B) and indicate that the rate changes very little in the region from pH_i 7.0 to about 7.5. This provides a rationale for the lack of effect of pH_i on the Hanes plots generated when $[K^+]$ is varied at different pH_o values (Table II). Hanes plots of the rate of K^+/H^+ exchange as a function of $[H^+]_i$ show nearly the same apparent K_m for $[H^+]_i$ at 30 mM and at 15 mM K^+ (pH_i 8.2 and 7.9; plots not shown).

Plots of velocity vs pH_i are also linear when the response to a fixed level of K^+ is evaluated at two different values of pH_o and pH_i is varied by Na^+ addition as just described. The plots of velocity vs $[H^+]_i$ generated by this protocol are hyperbolic, and Hanes plots of the resulting data (not shown) indicate that the apparent K_m for $[H^+]_i$ is pH_i 8.1 at pH_o 8.6 and 8.3 at pH_o 9.0. The V_{max} is considerably higher at pH_o 9.0 than at 8.6 because there is less inhibition of K^+ influx by $[H^+]_o$ at the higher external pH. Six different estimates of the apparent K_m for $[H^+]_i$ in protocols such as those just described give a mean pH_i of 8.1 ± 0.15.

Inhibition of K^+/H^+ Antiport by NH_4^+. Addition of NH_4^+ to increase pH_i at constant pH_o produces a considerably greater inhibition of K^+/H^+ antiport than that found for a comparable pH change induced by the addition of Na^+ (Figure 6) or K^+ (identical with Na^+; not shown). The inhibition of K^+-dependent extrusion of matrix H^+ by added NH_4^+ is noncompetitive with K^+ (data not shown), causing a large decrease in V_{max} with virtually no effect on the apparent K_m for K^+. The K_i is about 0.35 mM.

Inhibition of K^+/H^+ Antiport by Benzamil and Other Amiloride Analogues. The K^+-dependent extrusion of matrix H^+

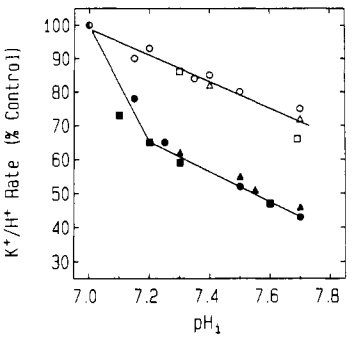

FIGURE 6: Inhibition of K^+-dependent H^+ extrusion by NH_4^+. Plot of velocity vs pH_i when pH_i was adjusted with Na^+ (open symbols) vs NH_4^+ (closed symbols). Data from three separate preparations of mitochondria are combined (○, △, □). The conditions were identical with those of Figure 1 with pH_o 8.6.

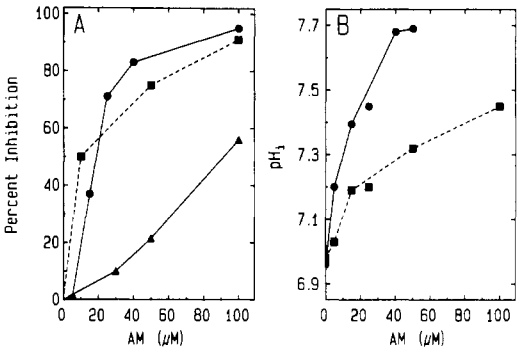

FIGURE 7: Inhibition of K^+/H^+ antiport by amiloride analogues. (A) Plot of percent inhibition by the indicated concentration of benzamil (■---■) or 5-*N*,*N*-hexamethyleneamiloride (●—●) of K^+-dependent H^+ extrusion under the conditions of Figure 1 at pH_o 8.7. The inhibition of A23187-dependent passive swelling in potassium acetate (Materials and Methods) at pH_o 7.86 is also shown (▲—▲). (B) Change in pH_i produced by the indicated level of amiloride analogues [symbols and conditions as for (A)].

is strongly inhibited by benzamil (Figure 7A) with an I_{50} of about 10 μM. Increasing concentrations of added benzamil increase the pH_i under these conditions (Figure 7B), and this can be taken as an indication that at least a portion of the drug is entering the matrix as the free amine and reacting with $[H^+]_i$. The inhibition is much greater with benzamil than the change in K^+/H^+ antiport rate that results from a comparable change in pH_i on addition of low concentrations of Na^+. For example, increasing pH_i to 7.8 by addition of Na^+ produces an inhibition of about 25% (Figure 6) whereas benzamil inhibits by 85% when it increases pH_i to about 7.7 (Figure 7B). The lipophilic amiloride analogue 5-*N*,*N*-hexamethyleneamiloride is very similar to benzamil in its ability to inhibit K^+/H^+ antiport (Figure 7A) and has an even greater effect on pH_i (Figure 7B). Passive swelling of A23187-treated mitochondria in potassium acetate is one of the accepted assays for K^+/H^+ antiport activity [see Brierley and Jung (1988b)]. This reaction is also inhibited by benzamil at pH_o 7.86 (Figure 7A, but under these conditions, the I_{50} is close to 100 μM. At this pH_o, the BCECF record (not shown) shows a transient acid shift of 0.2 pH unit following the addition of A23187 that is reequilibrated within 1 min.

Cation Specificity of the K^+/H^+ Antiport. When A23187-treated mitochondria are challenged with Na^+ under the conditions of Figure 1, the cation-dependent extrusion of H^+ is much more rapid than that seen in response to K^+ addition (Figure 8). The V_{max} for Na^+ averages over 150 nmol of $H^+ \cdot min^{-1} \cdot mg^{-1}$ as compared to 54 nmol of $H^+ \cdot min^{-1} \cdot mg^{-1}$ for K^+ in the same preparations of mitochondria. The apparent K_m of 11 mM Na^+ is lower than the 25 mM found for

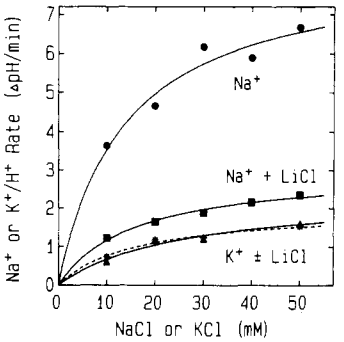

FIGURE 8: Inhibition of Na^+-dependent H^+ extrusion by Li^+ (0.4 mM) and failure of Li^+ to inhibit K^+-dependent H^+ extrusion. Conditions were identical with those of Figure 1.

Table III: Kinetic Parameters for Cation-Induced H^+ Efflux from A23187-Treated Heart Mitochondria[a]

pH_o	cation	app K_m (mM)	V_{max} $\Delta pH \cdot min^{-1}$	V_{max} nmol of $H^+ \cdot min^{-1} \cdot mg^{-1}$
8.6	Li^+	6	3	54
	Na^+	11 ± 2 (3)	8.3	150
	K^+	25 ± 3 (3)	3	54
8.8	Li^+	7	5.5	100
	K^+	19	5.0	90
	Rb^+	16	5.0	90
	Cs^+	92	6.0	108

[a] The conditions were identical with those described under Materials and Methods. Kinetic parameters were evaluated from Hanes plots.

K^+ (Table III). Because the Na^+/H^+ antiport, as well as the K^+/H^+ antiport, should be active under these conditions [see Nakashima and Garlid (1982)], it is likely that the response to Na^+ reflects a contribution of both of these transporters. It has been established that Li^+ is a competitive inhibitor of the Na^+/H^+ antiport but does not inhibit the K^+/H^+ antiport (Garlid, 1988a). The Na^+-dependent extrusion of matrix H^+ is strongly inhibited by 0.4 mM Li^+ (Figure 8), but this concentration of Li^+ has no effect on the K^+-dependent alkaline shift under these conditions (Figure 8).

Higher concentrations of Li^+ produce rapid extrusion of matrix H^+ in A23187-treated mitochondria. The V_{max} for the Li^+-dependent reaction is nearly identical with that for K^+, and the K_m is 3–4-fold lower at 7 mM Li^+ (Table III). Neither Rb^+ nor Cs^+ reacts with the mitochondrial Na^+/H^+ antiport (Garlid, 1988a), but both of these cations produce extensive H^+ efflux from A23187-treated mitochondria under the conditions of Figure 1. At pHo 8.8, the V_{max} for Li^+, K^+, Rb^+, and Cs^+ is nearly the same, and the K_m values indicate an affinity sequence of $Li^+ > K^+ = Rb^+ > Cs^+$ (Table III). It should be noted that addition of choline or TEA^+ produces no efflux of matrix H^+ under these conditions.

Discussion

The present studies establish that the activity of the mitochondrial K^+/H^+ antiport can be followed as a K^+-dependent extrusion of matrix H^+ using the fluorescence of BCECF. This reaction shows all of the properties of the K^+/H^+ antiport that have been defined by using other measuring systems (Brierley & Jung, 1988b; Garlid, 1988b). These include the requirement that matrix Mg^{2+} be depleted to activate the reaction and the need for alkaline, hypotonic conditions in order to obtain maximum activity. The K^+-dependent H^+ extrusion shows the inhibitor pattern expected for K^+/H^+ antiport in that it is inhibited by quinine, by DCCD, and by exogenous Mg^{2+}, but insensitive to Li^+. The need for an acid matrix pH (relative to pH_o) in order to demonstrate cation-dependent H^+ antiport imposes certain constraints on the study of K^+/H^+ antiport by this technique [see the discussion in Brierley et al. (1989)]. However, these protocols provide an apportunity for the direct observation of the H^+ movements promoted by the K^+/H^+ antiport and allow both pH_i and pH_o to be evaluated under conditions in which a ΔpH is maintained.

Effects of pH_o and pH_i on the Mitochondrial K^+/H^+ Antiport. The activity of the mitochondrial K^+/H^+ antiport has been shown to increase markedly as the pH of the suspending medium is increased [see Brierley and Jung (1988b) and Garlid (1988b)]. This increased activity is evident when the antiport operates in the direction of K^+ influx, as with passive swelling in potassium acetate. In such a measuring system, the effect of decreasing $[H^+$ could be rationalized in terms of decreased competition between H^+ and K^+ for a catalytic site on the antiport, as is seen with the mitochondrial Na^+/H^+ antiport. However, the activity of the K^+/H^+ antiport also increases with increasing pH when measured as net K^+ efflux (Bernardi & Azzone, 1983; Garlid, 1987) and as a passive exchange of matrix $^{42}K^+$ with external K^+ (Brierley et al., 1984). These effects have led to the concept that the turnover of the K^+/H^+ antiport is regulated by $[H^+]$ as well as by matrix $[Mg^{2+}]$ [see Garlid (1987)]. Martin and Garlid (1982) have reported that the reciprocal velocity of the K^+ efflux reaction shows a linear relationship to the square of the $[H^+]$ under conditions in which ΔpH can be presumed to be small. This relationship has led Garlid and co-workers to suggest that the K^+/H^+ antiport is inhibited allosterically by matrix H^+ and that such regulation is consistent with the role of this antiport and the anion channel in mitochondrial volume homeostasis (Garlid, 1987, 1988b).

The present studies permit a direct assessment of the relative effects of $[H^+]_o$ and $[H^+]_i$ on the antiport reaction when it operates in the direction of K^+ influx. It is clear that the inward exchange of K^+ for matrix H^+ increases with increasing pH_o (Figure 3A) and that $[H^+]_o$ fits the criteria for a mixed inhibitor of this reaction. A mixed inhibitor affects both the apparent K_m and the V_{max} of a reaction and can bring about such changes in a number of ways. The K_i for $[H^+]_o$ is very low, being evaluated as 2.5 nM (pH_o 8.6) using a plot of $[K^+]/v$ vs $[H^+]_o$. The Dixon plots ($1/v$ vs $H^+]_o$) shown in Figure 3B are linear in the range from pH_o 7.5 to 9.0 whereas plots of $1/v$ vs $[H^+]_o^2$ (not shown) are not linear. We should note that pH_i also changes when pH_o is varied in these protocols (Figure 3A), but changes in pH_i have little effect on the rate of K^+/H^+ antiport at constant pH_o (Figure 5B) and little effect on kinetic parameters in such experiments (Table II) in the range from pH_i 7 to about 7.5. When pH_i is held constant, an increase in $[H^+]_o$ increases the apparent K_m for K^+ and decreases V_{max} (Table II) as expected for a mixed inhibitor.

A plot of $1/v$ vs $[H^+]_o$ for the passive swelling of A23187-treated heart mitochondria in 50 mM potassium acetate is linear with an x intercept equivalent to pH_o 7.8 (r = 0.995; data not shown). This and the data of Figure 3B suggest that the reciprocal velocity for K^+ entry on the K^+/H^+ antiport bears a linear relationship to $[H^+]_o$, rather than $[H^+]^2$ as reported for K^+ efflux (Martin & Garlid, 1982). It is quite possible that K^+ influx on the antiport could have a different response to H^+ (either matrix or external) than does the K^+ efflux reaction. However, Bernardi and Azzone (1983) also measured the rate of K^+ extrusion as a function of pH_o, and a replot of their data as reciprocal velocity vs $[H^+]$ is more nearly linear than is the $1/v$ vs $[H^+]^2$ plot (not shown). In this study, the rates were measured in more hypertonic media

than is optimal, however, and this could account for the apparent discrepancy between these results and the report of Martin and Garlid (1982).

The present studies establish that $[H^+]_i$ acts as a Michaelis substrate for the K^+/H^+ antiport with an apparent K_m near pH_i 8.1 when pH_o is held constant and pH_i varied by displacing internal H^+ with Na^+ or K^+ (Figure 5). These studies provide no indication of a regulatory role for $[H^+]_i$. However, this method of varying pH_i alters ΔpH, the driving force of the reaction, and also may introduce factors (such as the presence of Na^+ in the matrix) that could possibly obscure such regulatory effects of $[H^+]_i$ on the reaction. This point will require further experimental clarification as will the issue of whether the kinetics of K^+ influx on the K^+/H^+ antiport differ from K^+ efflux on this antiporter.

Kinetic Properties of the Mitochondrial K^+/H^+ Antiport vs the Na^+/H^+ Antiport. The mitochondrial Na^+/H^+ antiport is inhibited competitively by $[H^+]_o$ when it operates in the direction of Na^+ influx (Nath & Garlid, 1988; Kapus et al., 1988; Brierley et al., 1989) with an apparent K_m for Na^+ near 70 mM at pH_o 7.2 and 20 mM at pH_o 7.8. These parameters and the low K_i for $[H^+]_o$ (near pH_o 8.6) would favor this antiport's activity as a Na^+-extruding device in respiring mitochondria maintaining an alkaline matrix in a neutral cytosol (Brierley et al., 1989). This antiport shows an apparent K_m for $[H^+]_i$ extrusion of 160 nM, and at pH_i 7.8, the matrix $[H^+]_i$ should be an order of magnitude less than this (16 nM). Accordingly, the extrusion of $[H^+]_i$ in exchange for external Na^+ would be limited, even if the Na^+/H^+ antiport carried out a completely symmetrical exchange of its two substrate ions. The external $[H^+]$ at pH_o 7.2 (or 63 nM) is closer to the K_m for the Na^+/H^+ antiport and would favor H^+ influx in exchange for matrix Na^+. The Na^+/H^+ antiport shows a well-defined maximum activity when pH_i is near 7.3 (Brierley et al., 1989).

In contrast, the low K_i of the K^+/H^+ antiport for $[H^+]_o$ and the fact that the inhibition is not competitive suggest that this component can have little activity when the pH_o is 7.2. If it is assumed that the K^+/H^+ antiport promotes a symmetrical exchange of K^+ and H^+ in both directions when activated, it is clear that $[K^+]$ should be well above the apparent K_m at both the matrix and the cytosol aspect of the antiport. The $[K^+]$ should be about 140 mM on each side, and the apparent K_m at pH_o 7.2 (derived by extrapolation of the plot of Figure 4) should be 70 mM whereas that at pH_i 7.8 should be 50 mM. The apparent K_m for $[H^+]$ of 8 nM (pH 8.1) should also be exceeded by both $[H^+]_i$ (16 nM or pH_i 7.8) and $[H^+]_o$ (63 nM or pH_o 7.2). Because the antiport in either direction appears to be inhibited by $[H^+]$ and the present studies suggest that it is primarily the $[H^+]_o$ component that is inhibitory, the present work raises the question as to whether this exchange component can function as an effective K^+ extrusion device under physiological conditions.

In the past, this laboratory has raised several points of concern with regard to the physiological relevance of the K^+/H^+ antiport that is unmasked by A23187 treatment [see Brierley et al. (1984) and the Discussion in Brierley and Jung (1988b)]. There can be no doubt that an antiport capable of handling K^+ as well as Na^+ and Li^+ can be activated when Mg^{2+} is depleted to low levels and pH_o elevated in a hypotonic medium. However, whether such a component would be activated under physiological conditions to regulate mitochondrial volume remains open to question. If indeed there is need to elevate pH_o, rather than pH_i, in order to activate K^+/H^+ antiport, then several additional constraints are placed on the system. The pH_i could well rise above 8 under some conditions in respiring mitochondria, but a large increase in pH_o would not be expected under physiological conditions.

Properties of the K^+ Transport Site on the K^+/H^+ Antiport. It has been reported from this laboratory (Brierley et al., 1984) that DCCD, an irreversible inhibitor of K^+/H^+ antiport (measured either as K^+ influx or as efflux), does not inhibit $^{42}K^+/K^+$ exchange. This has been confirmed for $^{86}Rb^+/Rb^+$ exchange (Nath & Garlid, 1989) and can be interpreted as meaning that separate sites for K^+ and H^+ transport are present on the antiporter (Garlid et al., 1986). The lack of competitive inhibition of K^+ influx by H^+_o seen in the present work would appear to support the presence of a separate binding site for the two substrates, K^+ and H^+.

The affinity sequence of $Li^+ > K^+ = Rb^+ > Cs^+$ derived from the present studies corresponds reasonable well to the relationship reported by Nath and Garlid (1989). As pointed out above, the place of Na^+ in the sequence is somewhat questionable due to the availability of the Na^+/H^+ antiport as a second pathway for Na^+ movement under the conditions of the present assay. The Na^+/H^+ antiport shows an apparent K_m near 7 mM at pH_o 8.6 with a V_{max} at 12 °C of 4 $\Delta pH \cdot min^{-1}$ (Brierley et al., 1989). The V_{max} for Na^+ of 8 $\Delta pH \cdot min^{-1}$ under conditions in which both the Na^+/H^+ and the K^+/H^+ antiports can be presumed to be active is in line with a nearly equal contribution of both transporters to the observed flux. The higher K_m observed under these conditions suggests that the K^+/H^+ component has a lower affinity for Na^+ than the Na^+/H^+ antiport and that its K_m is close to that for K^+. The affinity for Li^+ is clearly higher, whereas that for Cs^+ is much lower than K^+ (Table III). This is a sequence indicative of a high field strength (Diamond & Wright, 1969) interaction between the cation and its transport site.

The K^+-dependent efflux of H^+ from the matrix is inhibited noncompetitively by $[NH_4^+]$ with a low K_i. Because the addition of NH_4^+ produces an immediate increase in pH_i under these conditions, it appears that free NH_3 is entering the more acid matrix and generating NH_4^+ in that compartment. The inhibition by NH_4^+ is greater than that seen for a comparable change in pH_i generated with Na^+ or K^+ and therefore appears to result from interaction with the antiport. A similar situation prevails with benzamil and other amiloride analogues. These reagents are potent inhibitors of Na^+/H^+ antiport under similar conditions (acid matrix with elevated pH_o) and are presumed to react with sites in the matrix aspect of the antiport (Brierley et al., 1989). The competitive inhibition seen with other analogues at lower pH_o values (Kapus et al., 1988) suggests that reactive sites for these reagents may be available on the external aspect of the Na^+/H^+ antiport as well. Benzamil has been shown to inhibit both Na^+/H^+ and K^+/H^+ antiport in SMP in which the matrix aspect of the antiport should be oriented to the outside (Brierley et al., 1988). The present studies (Figure 7) indicate that these compounds are more effective inhibitors of K^+/H^+ antiport when the concentrating effect of ΔpH is present, since the I_{50} values are low for K^+-dependent H^+ extrusion (large ΔpH) compared to those for passive swelling in potassium acetate (low ΔpH). The fact that both the mitochondrial Na^+/H^+ and K^+/H^+ antiports are sensitive to amiloride analogues is of interest considering that the two transporters show different kinetic patterns. It also means that these reagents will be of limited use in distinguishing between the two components in isolation and reconstitution protocols.

ACKNOWLEDGMENTS

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Registry No. K, 7440-09-7; H, 12408-02-5; Li, 7439-93-2; Rb, 7440-17-7; Cs, 7440-46-2; NH_4, 14798-03-9.

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Effects of Potassium on Lipid–Protein Interactions in Light Sarcoplasmic Reticulum†

Barry S. Selinsky‡ and Philip L. Yeagle*
Department of Biochemistry, School of Medicine, University at Buffalo (SUNY), Buffalo, New York 14214
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ABSTRACT: This study shows the effect of K^+ on phospholipid–protein interactions in light sarcoplasmic reticulum (LSR) as measured by ^{31}P NMR. In the presence of 110 mM K^+, a substantial effect of the membrane protein on the behavior of the phospholipids was detected. Subtracting the spectrum of the LSR lipid extract from the spectrum of the intact LSR membrane produced a difference spectrum of much greater breadth than the normal phospholipid bilayer powder pattern. This powder pattern is indicative of a phospholipid domain considerably more motionally restricted than the phospholipids in a normal phospholipid bilayer. The apparent axially symmetric powder pattern is consistent with axial diffusion. In a reconstituted membrane containing the calcium pump protein at a lipid/protein ratio much less than in the light sarcoplasmic reticulum, the broad component was more prominent. The relative resonance intensity of the broad component appeared to be proportional to the lipid/protein ratio of the membrane. In 10 mM K^+, no broad powder pattern is observed in the corresponding difference spectrum. Thus, in the absence of potassium, the membrane protein has much less influence on the phospholipid of the membrane, as measured by ^{31}P NMR. In addition to the effects of K^+ on the membrane structure of the sarcoplasmic reticulum, K^+ modulated the function of the calcium pump. The rate of calcium-dependent ATP hydrolysis increased in light sarcoplasmic reticulum when [K^+] increased from 10 to 110 mM. The rate of calcium transport was also stimulated by an increase in K^+.

The effects of phospholipids upon the structure and function of integral membrane proteins have been vigorously explored. Some membrane-bound enzymes have a clearly demonstrated kinetic requirement for specific membrane lipids [reviewed by Sandermann et al. (1978)]. For example, the enzyme β-hydroxybutyrate dehydrogenase has an absolute requirement for phosphatidylcholine (Gazzotti et al., 1974), while phosphatidic acid has been recently shown to activate diacylglycerol kinase isolated from *Escherichia coli* (Russ et al., 1988). Other

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‡Present address: Department of Chemistry, Villanova University, Villanova, PA 19085.

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