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

Gerald P. Brierley,* Michael H. Davis,‡ Edward J. Cragoe, Jr.,§ and Dennis W. Jung
Department of Physiological Chemistry, Ohio State University Medical Center, Columbus, Ohio 43210
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ABSTRACT: The fluorescence of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) has been used to follow the Na^+/H^+ antiport activity of isolated heart mitochondria as a Na^+-dependent extrusion of matrix H^+. The antiport activity measured in this way shows a hyperbolic dependence on external Na^+ or Li^+ concentration when the external pH (pH_o) is 7.2 or higher. The apparent K_m for Na^+ decreases with increasing pH_o to a limit of 4.6 mM. The K_i for external H^+ as a competitive inhibitor of Na^+/H^+ antiport averages 3.0 nM (pH_o 8.6). The V_{max} at 24 °C is 160 ng ion of H^+ min^{-1} (mg of protein)$^{-1}$ and does not vary with pH_o. Li^+ reacts with the antiporter with higher affinity, but much lower V_{max}, and is a competitive inhibitor of Na^+/H^+ antiport. The rate of Na^+/H^+ antiport is optimal when the pH_i is near 7.2. When pH_o is maintained constant, Na^+-dependent extrusion of matrix H^+ shows a hyperbolic dependence on $[H^+]_i$ with an apparent K_m corresponding to a pH_i of 6.8. The Na^+/H^+ antiport is inhibited by benzamil and by 5-N-substituted amiloride analogues with I_{50} values in the range from 50 to 100 μM. The pH profile for this inhibition seems consistent with the availability of a matrix binding site for the amiloride analogues. The mitochondrial Na^+/H^+ antiport resembles the antiport found in the plasma membrane of mammalian cells in that Na^+, Li^+, and external H^+ appear to compete for a common external binding site and both exchanges are inhibited by amiloride analogues. However, there are significant differences in the sensitivity of the two antiports to these inhibitors, and the mitochondrial exchanger appears to operate in a more alkaline region than the plasmalemmal component. The increased affinity of the antiport for Na^+ with increasing pH is in line with the putative role of this exchanger as a device for extruding Na^+ from the alkaline matrix of respiring mitochondria.

Isolated heart mitochondria can be loaded with the fluorescent pH indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)[1] by procedures quite analogous to those used with intact cells (Davis et al., 1987a; Jung et al., 1988). Studies from this laboratory have established that changes in matrix pH (pH_i) reported by BCECF fluorescence are in close agreement with values obtained by the distribution of acetate or DMO (Jung et al., 1988) and that these procedures provide a convenient and continuous readout of pH_i with changing metabolic conditions (Davis et al., 1987a). Estimation of pH_i by BCECF fluorescence appears to be free of the binding artifacts that affect the distribution of weak base pH probes, such as methylamine (Jung et al., 1988), and with a pK_a near 7.0 (Rink et al., 1982), BCECF can effectively report pH_i over a range from 6.0 to 8.0.

In the present study, the fluorescence of BCECF is used to monitor the changes in pH_i that result from monovalent cation/H^+ antiport reactions across the inner membrane of the mitochondrion. Mitochondria appear to contain both an

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‡ Present address: Aqua Tech Environmental Consultants Inc., Marion, OH 43302-0436.
§ Present address: 2211 Oak Terrace Dr., Lansdale, PA 19446.

[1] Abbreviations: SMP, submitochondrial particle(s); BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; pH_o, pH of mitochondrial suspending medium; pH_i, pH of matrix compartment as reported by BCECF fluorescence; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TEA^+, tetraethylammonium ion; DMO, 5,5′-dimethyl-2,4-oxazolidinedione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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overt Na^+/H^+ antiport and a latent K^+/H^+ exchange component [see Brierley (1983), Brierley and Jung (1988a,b), and Garlid (1988a,b) for recent reviews]. The activity of the Na^+/H^+ antiport has been followed by the passive osmotic swelling of mitochondria in acetate salts (Mitchell & Moyle, 1969b; Brierley et al., 1978; Nakashima & Garlid, 1982), by glass electrode records of Na^+-dependent changes in the pH of the suspending medium of either mitochondria (Mitchell & Moyle, 1967; Crompton & Heid, 1978) or SMP (Papa et al., 1973), and by changes in quinacrine fluorescence in energized SMP (Rosen & Futai, 1980; Brierley et al., 1984). The respiration-dependent uptake of Na^+ by SMP has also been attributed to this antiport (Douglas & Cockrell, 1973), as has respiration-dependent extrusion of accumulated Na^+ from intact mitochondria (Brierley et al., 1977). These studies indicate that the Na^+/H^+ antiport is specific for Na^+ and Li^+ (Brierley et al., 1978; Rosen & Futai, 1980), has a pH_o optimum near 7.3 in acetate (Brierley et al., 1978), and is insensitive to quinine, divalent cations, and DCCD (Nakashima & Garlid, 1982; Garlid, 1988a). These properties permit the Na^+/H^+ antiport to be distinquished from the K^+/H^+ exchanger, an antiporter that becomes apparent in swollen or Mg^{2+}-depleted mitochondria (Brierley & Jung, 1988a,b; Garlid, 1988a) and which appears capable of transporting Na^+ and Li^+, as well as K^+.

The present studies establish that BCECF fluorescence can be used to monitor Na^+/H^+ antiport as a Na^+- or Li^+-dependent alkaline shift in pH_i. The extrusion of matrix H^+ is accompanied by the uptake of Na^+. The Na^+/H^+ antiport measured in this way is inhibited competitively by Li^+, a result that agrees with the recent reports of Garlid (1988b), Nath and Garlid (1988), and Kapus et al. (1988). The present studies also show that the mitochondrial antiport, like the Na^+/H^+ antiport of the plasma membrane (Aronson et al., 1983), is inhibited competitively by external H^+ and is sensitive to a number of amiloride analogues. The two exchangers show marked differences in K_i for external H^+ as well as differences in I_{50} values for effective amiloride analogues. It is concluded that the mitochondrial Na^+/H^+ antiport resembles both the plasma membrane component [see Mahnensmith and Aronson (1985) for a review] and the corresponding antiport of microbial membranes (Krulwich, 1983) in many respects, but shows significant differences in others.

Materials and Methods

BCECF Loading. Beef heart mitochondria were prepared as previously described (Brierley et al., 1984). Mitochondria (25 mg of protein/mL) suspended in sucrose (0.25 M) containing Tris buffer (2 mM, pH 7.4) were treated with 5 μg/mL BCECF acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR; dissolved in dimethyl sulfoxide). After 10 min at 23 °C, the suspension was diluted 4-fold in the Tris–sucrose medium, and the mitochondria were reisolated by centrifugation (14000*g* for 10 min). This pellet was resuspended in 4 mL of Tris–sucrose, incubated for 5 min at 30 °C, and reisolated by centrifugation.

Fluorescence Measurements. The BCECF-loaded mitochondria were resuspended at 12.5 mg/mL in Tris–sucrose for use. Fluorescence was measured in stirred, temperature-regulated cuvettes using a Perkin-Elmer LS-5B fluorometer with emission at 530 nm and excitation at 500 nm. At intervals, the excitation was switched to 450 nm so that pH_i could be calculated by using a calibration plot of the fluorescence ratio (500/450-nm excitation) vs pH as previously described (Jung et al., 1988). In practice, there is virtually no change in the fluorescence with excitation at 450 nm in the short-term protocols reported, and a record of fluorescence at 500_{ex} can be used as a direct readout of changes in internal pH with time. Initial rates of the Na^+-dependent change in pH_i were calculated directly from the fluorometer recorder plots of the fluorescence vs time or by first feeding the fluorometer output into a IBM Model 60 computer and calculating initial rates.

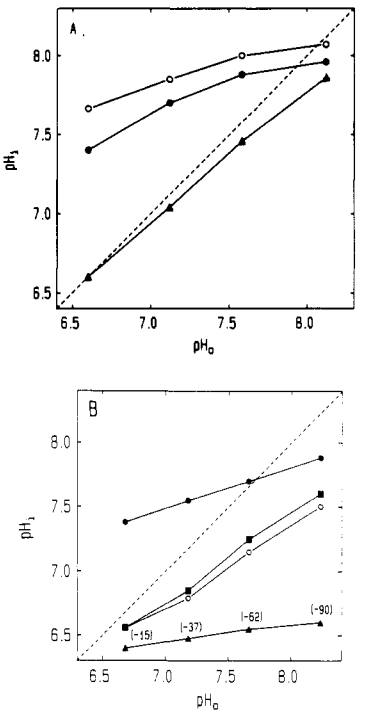

FIGURE 1: Relationship between pH_i as reported by BCECF fluorescence and pH_o. (A) Mitochondria suspended at 12 °C in KCl (100 mM) containing TES buffer (30 mM neutralized to the indicated pH with tetraethylammonium hydroxide), EGTA (30 μM), rotenone (3 μg/mL), and oligomycin (2 μg/mL). The concentrationof BCECF-loaded mitochondria (see Materials and Methods) was 0.4 mg/mL. The pH_i without further addition (●) was calculated from the fluorescence as described under Materials and Methods, as is the pH_i following the addition of 4 mM succinate (○) or 1.5 mM CCCP (▲). The dashed line indicates equilibration of pH_i with pH_o. (B) Mitochondria suspended in choline chloride (100 mM) containing the same additions as in (A). The initial pH_i was recorded (●), followed by that produced by the addition of CCCP (○). Valinomycin (1.0 μM) was then added and the further acid shift in pH_i recorded (▲). The mitochondria were then treated with 50 mM NaCl, and the alkaline shift in pH_i was quantitated (■). The dashed line indicates equilibration of pH_i with pH_o. The Donnan potential ($\Delta\psi$) calculated by assuming no protonmotive force and $\Delta\psi = 57\ \Delta pH$ [see Jung et al. (1988)] is given in parenthesis. The time course of these changes in pH_i is shown in Figure 2B.

Other Determinations. Mitochondrial cations were determined by atomic absorption spectroscopy of acid extracts. Estimates of Na^+ uptake were corrected for extramitochondrial Na^+ using [^{3}H]water and [^{14}C]sucrose distribution as previously described (Jung et al., 1988). The buffering power of heart mitochondria was measured as described by Mitchell and Moyle (1969a). Amiloride analogues were prepared by the method of Cragoe et al. (1967).

Results

pH Gradients in Nonrespiring Heart Mitochondria. Heart mitochondria loaded with BCECF and suspended at 12 °C in a KCl medium show fluorescence indicative of a positive ΔpH ($pH_i > pH_o$) in the absence of respiration. This ΔpH varies with pH_o (Figure 1A), showing a maximum of about 0.8 pH unit when the external pH is 6.5 and approaching zero at a pH_o of 8. When respiration is initiated by addition of succinate, ΔpH increases somewhat at all values of pH_o

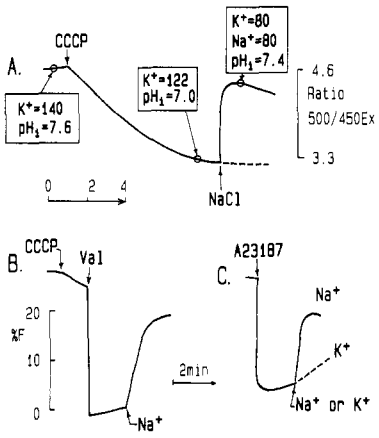

FIGURE 2: Time course of uncoupler-dependent and Na^+-dependent changes in pH_i for mitochondria suspended in choline chloride, pH_o 7.6 (conditions of Figure 1B). (A) Record of the change in fluorescence (excitation at 500 nm; emission at 530 nm) in response to the addition of CCCP (1.5 μM) followed by NaCl (50 mM). At the indicated points, the mitochondria were separated by rapid centrifugation, and mitochondrial cation content (in nanomoles per milligram of protein) was determined. Matrix Mg^{2+} showed no change in this protocol. Matrix pH (pH_i) was calculated from the ratio of fluorescence with excitation at 500 nm to that at 450 nm [see Jung et al. (1988)]. The latter value did not change significantly over the time shown. (B) Response of mitochondria treated with both CCCP (1.5 μM) and valinomycin (Val; 1.0 μM) under the conditions of (A). Virtually no K^+ is retained by the mitochondria under these conditions. In the absence of CCCP, addition of valinomycin produces a slower acid drift and a response to Na^+ that is nearly indistinguishable from the record shown. Note that (B) and (C) are recorded on a less expanded scale than (A) with the indicated range of ratios in (A) equivalent to 16% F. (C) Response of mitochondria to the addition of A23187 (1 μM) followed by either NaCl or KCl (50 mM).

(Figure 1A). Addition of an uncoupler (CCCP, 1.5 μM) to the nonrespiring mitochondria under these conditions results in a shift of pH_i to very near that of pH_o at neutral pH_o and to a somewhat more acid value at higher pH_o (Figure 1A).

BCECF-loaded mitochondria suspended in a K^+-free choline chloride medium at 12 °C also maintain a positive ΔpH over the pH_o range from 6.5 to about 7.6 (Figure 1B). When these mitochondria are treated with an uncoupler, pH_i drifts acid and approaches a new steady state after about 5 min (Figure 2A). The acid shift in pH_i following addition of an uncoupler appears to result from the inward conduction of H^+ (a reaction that should be very rapid) and a compensating loss of internal cations, a process that appears to be rate limiting. Some net anion loss may also occur during this period. The magnitude of the acid shift under these conditions varies somewhat with the mitochondria preparation and appears related to the ability of the uncoupled mitochondria to retain K^+ [see Carafoli et al. (1969) and Jung and Brierley (1984)]. In the experiment shown in Figure 2A, a ΔpH (interior acid) of 0.6 unit is established following the addition of CCCP at pH_o 7.6, and the loss of matrix K^+ amounts to 18 nmol/mg of protein.

An extensive and very rapid shift in pH_i is seen when valinomycin is also added to uncoupled mitochondria suspended in the K^+-free choline chloride medium (Figure 2B). Under these conditions, virtually all of the endogenous K^+ is lost, and the matrix pH is lowered to 6.6 or less at all values of pH_o tested (Figure 1B). In these mitochondria, the protonmotive force is zero, and at 12 °C, $\Delta\psi = 57\ \Delta pH$ (Mitchell & Moyle, 1969a). At elevated pH_o, these mitochondria show values of ΔpH in excess of −1.0 and Donnan potentials as high as −90 mV (Figure 1B).

Na^+-Dependent Extrusion of Matrix H^+. The addition of up to 75 mM Na^+ to nonrespiring heart mitochondria suspended in KCl or choline chloride produces little or no change

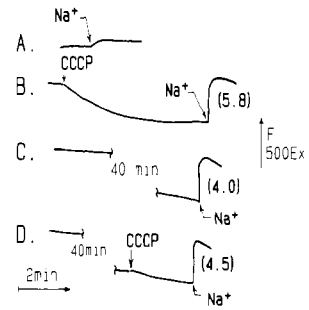

FIGURE 3: Na^+-dependent changes in pH_i do not require the presence of an uncoupler. Records of BCECF fluorescence of mitochondria suspended in the choline chloride medium described in Figure 1B (pH_o 7.6) at 25 °C. (A) Lack of response to NaCl (50 mM) in the absence of uncoupler. (B) Response of uncoupled mitochondria to 50 mM NaCl. The rate of Na^+-dependent change in pH_i (ΔpH per minute) is given in parentheses. (C) Response to Na^+ of BCECF-loaded mitochondria aged 40 min at 25 °C to dissipate ΔpH. (D) Response of aged mitochondria to Na^+ in the presence of uncoupler (CCCP, 2.0 μM).

in pH_i as detected by BCECF fluorescence over a wide range of pH_o values (Figure 3A, for example). These nonrespiring heart mitochondria maintain an alkaline pH_i (Figure 1), and it is apparent that, under these conditions, there is little tendency for Na^+ to be exchanged inward for matrix H^+. This is consistent with the concept that mitochondrial monovalent cation/H^+ antiport reactions normally serve to extrude excess Na^+ or K^+ from the alkaline matrix (Mitchell, 1970).

However, addition of Na^+ to uncoupled mitochondria suspended in choline chloride (Figure 2A) results in a rapid alkaline shift in pH_i. This Na^+-dependent extrusion of matrix H^+ is accompanied by the uptake of Na^+ (Figure 2A). A less extensive but more rapid extrusion of H^+ is seen with Li^+ addition, but there is no response to added K^+, Mg^{2+}, or TEA^+ under these conditions (not shown). The response to Na^+ is more extensive in mitochondria suspended in choline chloride and treated with valinomycin as well as uncoupler (Figure 2B). The magnitude of the Na^+-dependent pH change under these conditions varies with pH_o and can exceed a ΔpH of 1.0 at higher external pH values (Figure 1B).

The Na^+-dependent alkaline shift in pH_i is also readily apparent in mitochondria suspended in KCl (not shown). In this medium, the Na^+-dependent change in pH_i has the greatest extent (0.3 to 0.4 pH unit) and highest rate (2.2 ΔpH units min^{-1}) when the pH_o is about 7.2.

Role of the Uncoupler in Na^+-Dependent Changes in pH_i. The changes in pH_i and matrix Na^+ seen when Na^+ is added to uncoupled mitochondria (Figure 2) are consistent with the endogenous Na^+/H^+ antiport acting to conduct Na^+ down its concentration gradient into the matrix in exchange for internal H^+ (also moving in the direction of its concentration gradient). However, because an uncoupler is added in these protocols to acidify the matrix, the observed Na^+ for H^+ antiport could also be ascribed to an inward antiport of Na^+ combined with an outward conductance of H^+ by the uncoupler. The following experiments indicate that there is little or no contribution of Na^+ uniport to the observed Na^+/H^+ exchange and that the change in pH_i is due to the activity of the mitochondrial Na^+/H^+ antiport:

(a) The Na^+-dependent efflux of matrix H^+ does not depend on the concentration of uncoupler added. Varying the concentration of CCCP in the protocol of Figure 2A from 1.6 μM to 1.6 nM slowed the acidification of the matrix but did not alter the rate of Na^+-dependent change in pH_i. In addition, when the existing ΔpH is allowed to decay spontaneously by allowing the mitochondria to stand several minutes in the

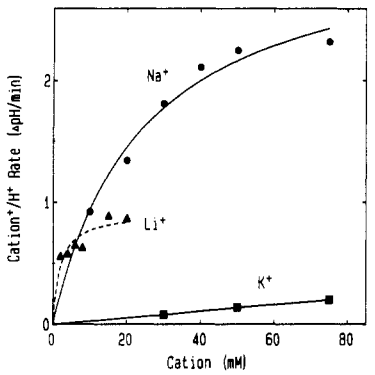

FIGURE 4: Rate of monovalent cation-dependent change in pH_i as a function of cation concentration. Rates were estimated at 12 °C using the protocol shown in Figure 2A and varying the amount of Na^+, Li^+, or K^+ added. Each addition was balanced with sufficient choline chloride to keep the $[Cl^-]$ constant at 50 mM with the exception of the 75 mM additions. The Na^+ points shown are the means of four to seven separate estimates with different preparations of mitochondria.

choline chloride medium at 25 °C, the response to Na^+ in the absence of uncoupler is nearly the same as that in the presence of 2 μM CCCP (Figure 3).

(b) The matrix of nonrespiring mitochondria can also be acidified by the addition of valinomycin to induce the loss of matrix K^+ in the absence of uncoupler. In the choline chloride medium, a portion of the K^+ is replaced by H^+ with a marked decrease in pH_i. When these mitochondria are then challenged with Na^+, an extensive alkaline shift in pH_i is observed, and the rate of H^+ extrusion is the same as that in the presence of uncoupler (see the legend for Figure 2B).

(c) When the matrix of nonrespiring heart mitochondria is acidified by the addition of A23187, an ionophore that exchanges matrix Mg^{2+} and Ca^{2+} for $2H^+$ (Reed & Lardy, 1972), a substantial acid ΔpH is established (Figure 2C). Addition of NaCl (50 mM) under these conditions produces a large (0.8 pH unit) and rapid alkaline shift in pH_i consistent with Na^+/H^+ antiport.

Since no uncoupler is added in the above protocols, it is clear that the pathways for inward Na^+ conduction and H^+ efflux must both be present in the mitochondrial membrane. The rapid acidification of the matrix following the addition of valinomycin indicates that pathways for H^+ influx in response to the outward uniport of K^+ are present in the membrane of fresh, unmodified mitochondria. The lack of response to added Na^+ (Figure 3A) argues that there is no effective inward uniport of Na^+ under these conditions. In contrast, Na^+/H^+ antiport is readily apparent in such mitochondria, as shown by the rapid swelling in sodium acetate [see Brierley (1983)].

The A23187 protocols (Figure 2C) also indicate that there is little or no contribution of the K^+/H^+ antiport to the observed changes in pH_i in these experiments. Depletion of matrix Mg^{2+} by treatment with A23187 is known to induce K^+/H^+ antiport, and this exchanger also transports Na^+ (Garlid, 1988b; Brierley & Jung, 1988b). However, there is little response to added K^+ following A23187 addition (Figure 2C), and it appears that the unfavorable acid pH_i [see Martin et al. (1984)] keeps the activity of this component very low even in Mg^{2+}-depleted mitochondria.

Kinetics of Cation/H^+ Antiport in Uncoupled Mitochondria. The rate of the Na^+-dependent alkaline shift in pH_i in protocols such as those in Figure 2 is dependent on the concentration of Na^+ added and shows saturation kinetics in the choline chloride medium when the pH_o is 7.6 (Figure 4). The response varies somewhat from preparation to preparation, but the apparent K_m for seven different preparations assayed under these conditions averaged 32 ± 4 mM Na^+ (mean ± SEM) with a V_{max} of 3.6 ± 0.3 ΔpH·min^{-1}. The apparent K_m for Li^+ under these conditions is near 2 mM with a much lower V_{max} than that for Na^+ (Figure 4). The low rates of K^+-dependent change in pH_i do not show saturation kinetics under these conditions (Figure 4). These apparent K_m values agree well with those previously reported for Na^+/H^+ antiport in mitochondria and SMP when estimated by other methods under similar conditions (Rosen & Futai, 1980; Nath & Garlid, 1988; Kapus et al., 1988).

Estimates of the buffering power of beef heart mitochondria agree well with the values reported by Mitchell and Moyle (1969a) for liver mitochondria. A change in pH_i of 1 pH unit amounts to about 27 ng ion of H^+/mg of protein at pH 6.5 and 12 ng ion/mg at pH 7.5. In addition, these studies have established that the presence of BCECF has no significant effect on matrix buffering power. The values for buffering power of the mitochondria permit rates of Na^+-dependent change in pH_i, such as those shown in Figure 4, to be expressed as rates of Na^+-dependent H^+ extrusion. The pH_i of the mitochondria used in the kinetic analysis shown in Figure 4 was about 6.9 just prior to addition of Na^+, and the initial Na^+-dependent changes in pH_i used to estimate the rates shifted this to the region of 7.1. The buffering power for this pH range averages 21 ng ion of H^+·(mg of protein)$^{-1}$·(pH unit)$^{-1}$. When this value is used, the average V_{max} of 3.6 ΔpH·min^{-1} can be converted to a rate of H^+ extrusion of about 75 ng ion of H^+·min^{-1}·mg^{-1} at 12 °C.

The rate of Na^+-dependent H^+ extrusion using the protocol of Figure 2A varies with temperature with a Q_{10} of 2 and a ϵ_a just over 11 000 cal/mol. In a typical experiment using the protocol of Figure 2A, the rate of Na^+-dependent change in pH_i was 3.6 at 11 °C, 4.6 at 17 °C, 7.6 at 24 °C, and 10.9 at 30 °C. This would put the rate of H^+ extrusion at 160 ng ion of H^+·min^{-1}·mg^{-1} at 24 °C and 230 ng ion of H^+·min^{-1}·mg^{-1} at 30 °C, values quite comarable to the V_{max} of 170 and 400 ng ion of H^+·min^{-1}·mg^{-1} reported by Nath and Garlid (1988) using $^{22}Na^+$ uptake and osmotic swelling procedures and 110 ng ion of H^+·min^{-1}·mg^{-1} found by Crompton and Heid (1978).

Inhibition of Na^+/H^+ Antiport by Li^+. Nath and Garlid (1988) have reported that Li^+ is a competitive inhibitor of Na^+/H^+ antiport and shows an apparent K_i of 1.6 mM. This finding is confirmed by the studies shown in Figure 5. When Li^+ is added prior to Na^+ to BCECF-loaded, uncoupled mitochondria suspended in choline chloride at pH_o 7.5, the Na^+-dependent reaction is strongly inhibited with an I_{50} of 0.7 mM Li^+ (Figure 5A). The somewhat slower rate of Na^+/H^+ antiport seen in the KCl medium is also inhibited by Li^+ with a value for I_{50} that is quite comparable to that seen in choline chloride (1.2 mM, Figure 5A). These results indicate that K^+ not only does not inhibit Na^+/H^+ antiport but also does not interfere with the ability of Li^+ to inhibit the reaction. The inhibition of Na^+/H^+ antiport by Li^+ appears competitive, since there is a large effect of Li^+ on the K_m for Na^+ but little effect on the V_{max} (Figure 5B). Because Li^+ does not inhibit the K^+/H^+ antiport (Garlid, 1988b), the sensitivity of the Na^+-dependent H^+ extrusion to this cation is strong evidence that the bulk of the Na^+/H^+ exchange seen in these studies takes place on the Na^+/H^+ antiport.

Effect of pH_o and pH_i on Na^+/H^+ Antiport. The simplest model for a membrane Na^+/H^+ antiport involves a single binding site (E^-) for cation or H^+ that can be oriented alternatively toward the cytosolic and the matrix aspect of the

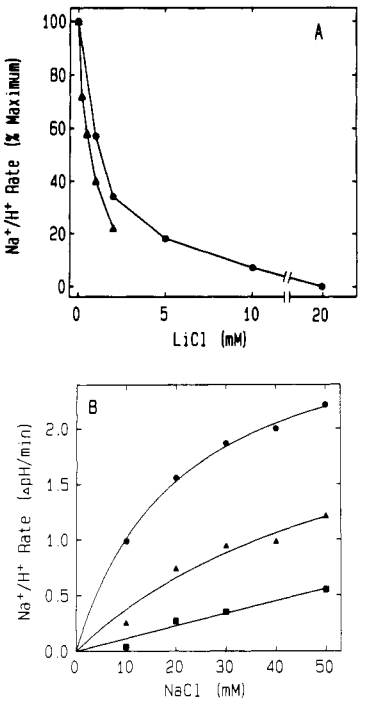

FIGURE 5: Inhibition of Na^+/H^+ antiport by Li^+. (A) Effect of increasing concentrations of LiCl on the Na^+-dependent change in pH_i in a choline chloride medium [protocol of Figure 2A (▲)] and in the same medium in which KCl is substituted for the choline salt (●) with increasing levels of LiCl. In each case, the LiCl was added after the CCCP-dependent acidification had reached a steady state, and the change in pH_i due to Li^+ addition was allowed to stabilize prior to addition of NaCl (50 mM). (B) Effect of Li^+ on a plot of velocity of Na^+/H^+ antiport vs Na^+ concentration. The conditions were identical with those of Figure 2A with none (●), 0.7 mM (▲), or 2.0 mM (■) LiCl added after CCCP as described for (A). Double-reciprocal plots (not shown) indicate that the inhibition is competitive (V_m near 3.2 $\Delta pH \cdot min^{-1}$ with varied K_m).

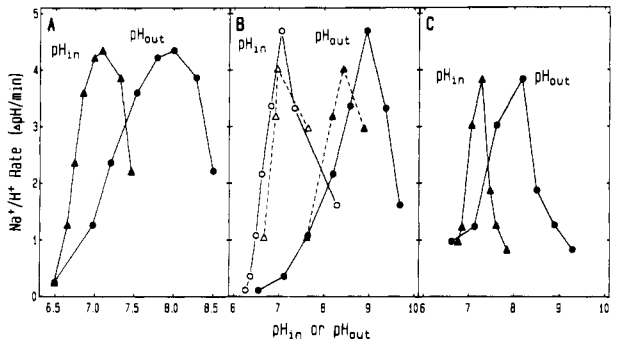

FIGURE 6: Dependence of the rate of Na^+/H^+ antiport on pH_o and pH_i. (A) Protocol of Figure 2A with pH_o adjusted as shown (●). The rate of Na^+-dependent alkaline shift in pH_i following the addition of NaCl (50 mM) was evaluated and plotted vs pH_{out} or pH_{in}, the internal pH just prior to Na^+ addition (▲). (B) Protocol of Figure 2B; mitochondria suspended in choline chloride and treated with CCCP and valinomycin. The Na^+/H^+ antiport rate for two different preparations as a function of pH_{out} (●, ▲) and pH_{in} (○, △) is shown. (C) Protocol of Figure 2C; mitochondria suspended in choline chloride and treated with A23187.

membrane [see Brierley et al. (1978) for example]. In such a model, the inward movement of Na^+ can be viewed as occurring in two stages:

$$[Na^+]_o + EH \leftrightarrow E^-Na^+ + [H^+]_o \quad \text{(i)}$$

$$E^-Na^+ + [H^+]_i \leftrightarrow EH + [Na^+]_i \quad \text{(ii)}$$

It is apparent that both pH_o and pH_i will contribute to the rate of such a reaction sequence with external H^+ competing with Na_o^+ for the binding site and internal H^+ necessary to remove

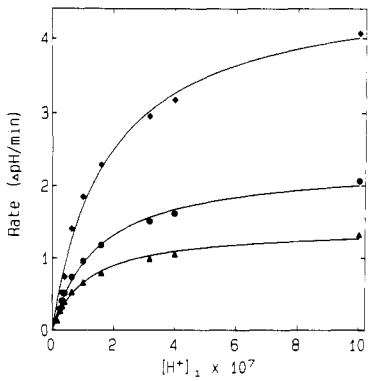

FIGURE 7: Dependence of the rate of Na^+-dependent H^+ extrusion on matrix $[H^+]$. Mitochondria were treated with CCCP and valinomycin in choline chloride at 12 °C (protocol of Figure 2B) with pH_o set at 7.5 (▲), 7.8 (●), or 8.3 (♦). Just prior to the addition of Na^+ (50 mM), the matrix $[H^+]$ was adjusted to the indicated value by the addition of KCl (0.5–8 mM). In the presence of valinomycin, the added K^+ rapidly enters the matrix and displaces H^+, giving a higher pH_i with no change in the highly buffered pH_o. Values for K_m (expressed as pH_i) of 6.9, 6.8, and 6.8, and of V_{max} of 1.4, 2.3, and 4.7 $\Delta pH \cdot min^{-1}$, were obtained from least-squares plots of $1/v$ vs $1/[H^+]_i$ (not shown) for pH_o 7.5, 7.8, and 8.3, respectively.

Na^+ from the anionic site in the matrix compartment.

The observed rate of Na^+/H^+ antiport in uncoupled mitochondria suspended in the choline chloride medium (protocol of Figure 2A) increases from near zero to a maximum as the pH_o is increased from 6.5 to 8.0 (Figure 6A). The rate then declines as pH_o is increased above 8.0 (Figure 6A). This result appears to be in line with the predictions of the model just described. In this protocol, the pH_i attained by the uncoupled mitochondria prior to the addition of Na^+ also varies as the pH_o is varied, and a plot of the initial rate of Na^+-dependent H^+ extrusion vs pH_i shows a maximum near 7.1 (Figure 6A). The pH_o profile for the extensive Na^+-dependent alkaline shift of pH_i in mitochondria treated with CCCP and valinomycin in a choline chloride medium (protocol of Figure 2B) is somewhat variable but shows an optimum between pH_o 8.5 and 9.0, depending on the preparation (Figure 6B). The optimum pH_i for this reaction still falls very nearly at 7.1 (Figure 6B). A similar situation prevails when the pH dependency of Na^+/H^+ antiport in the presence of A23187 is examined (Figure 6C). In this case, the optimum rate is seen when pH_o is 8.2 and pH_i is 7.3. An optimum value of 7.2 for pH_i is also seen for Na^+-dependent extrusion of H_i^+ in KCl (not shown).

It should be noted that studies of the pH dependency of the rate of osmotic swelling of mitochondria in sodium acetate (Brierley et al. 1978; Nakashima & Garlid, 1982) or in NaCl following the induction of Cl^-/OH^- antiport with tripropyltin (Brierley et al. 1978) have shown an optimum pH_o near 7.3 and that both of these reactions are thought to be limited by Na^+/H^+ antiport. BCECF fluorescence shows that pH_i is very nearly equal to pH_o under both of these conditions (data not shown). These studies lead to the conclusion that the inward exchange of Na_o^+ for matrix H^+ is optimal when the pH_i is about 7.2 for all of the conditions studied.

The pH_i can be varied at constant pH_o in the choline chloride medium when valinomycin and uncoupler are present (protocol of Figure 2B) by adding small amounts of K^+ (0.5–8 mM; records not shown). Equilibration of the K^+ via the valinomycin uniport produces a rapid efflux of H^+ from the matrix and a more alkaline pH_i. The rate of Na^+-dependent H^+ efflux decreases as pH_i increases under these conditions, and the decrease is nearly linear with pH_i over the limited spans that are generated. A plot of the rate vs matrix H^+

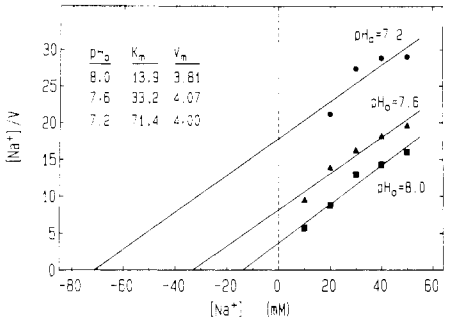

FIGURE 8: Hanes plot at three different values of pH_o showing dependence of the rate of Na^+/H^+ antiport on external Na^+ concentration and competitive inhibition by external $[H^+]$. The mitochondria were suspended in choline chloride at 12 °C with uncoupler added (protocol of Figure 2A).

concentration, $[H^+]_i$, at various values of pH_o shows a hyperbolic relationship (Figure 7). In these plots, the apparent V_{max} increases with increasing pH_o while the K_m for matrix H^+ remains essentially the same at 148 nM, which corresponds to a pH_i of 6.8 ± 0.1 (mean ± SD for seven separate determinations on four preparations of mitochondria). These results are compatible with the model above in which $[H^+]_i$ serves as a substrate for the membrane antiport.

Plots of the rate of Na^+/H^+ antiport vs Na^+ concentration are hyperbolic when pH_o is 7.2 or above (see Figures 4 and 5B) but become linear at lower values of pH_o. Hanes plots of such data at different values of pH_o (Figure 8) indicate that the V_{max} is essentially independent of pH_o and that the apparent K_m for Na^+ increases markedly as the external H^+ concentration increases. When these experiments are carried out under conditions that keep pH_i nearly constant and near its optimal value of 7.2, it is clear that the data are consistent with H_o^+ acting as a competitive inhibitor of Na^+ influx and H_i^+ extrusion [see also Kapus et al. (1988)]. Extrapolation of a plot of apparent K_m vs $[H^+]_o$ to zero external $[H^+]$ gives a K_m of 4.6 mM for Na^+ (average of three determinations). The K_i for external H^+ averages 3 nM (pH_o of 8.6 ± 0.2) in this treatment. Again, these results appear quite compatible with the above model.

Inhibition of Na^+/H^+ Antiport by Amiloride Analogues. The Na^+-dependent extrusion of matrix H^+ is only marginally sensitive to amiloride (0.5 mM inhibits by about 10% in the protocol of Figure 2A,B). However, the reaction is strongly inhibited by benzamil, with a 100 μM sample of this amiloride analogue inhibiting up to 90% (Figure 9). The antiport is also inhibited by 4′-fluorobenzamil in this concentration range and by amiloride analogues substituted on the 5-amino nitrogen atom. The most effective of these reagents tested, N^5,N^5-hexamethyleneamiloride, inhibits the mitochondrial Na^+/H^+ antiport by about 50% at 60 μM (conditions of Figure 2A). The N^5-ethyl-N^5-isopropylamiloride analogue and N^5,N^5-dimethylamiloride are somewhat less effective (100 μM inhibits 52% and 30%, respectively, under the conditions of Figure 2A).

The effectiveness of benzamil as an inhibitor of mitochondrial Na^+/H^+ antiport varies with measuring conditions. The I_{50} value for this reagent against Na^+-dependent H^+ extrusion in KCl is 150 μM at the optimum pH_o of 7.2. The corresponding I_{50} for benzamil when the reaction is run in choline chloride in the presence of CCCP at pH_o 8.0 (protocol of Figure 2A) is 100 μM, whereas that in this medium in the presence of both CCCP and valinomycin at pH_o 8.8 (protocol of Figures 2B and 9) is closer to 50 μM. These values indicate that benzamil is a more effective inhibitor of Na^+/H^+ antiport

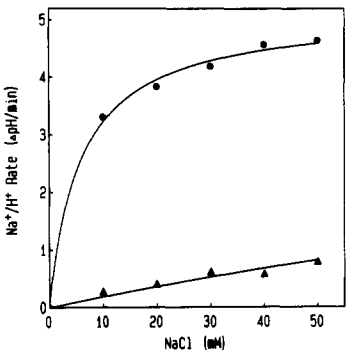

FIGURE 9: Inhibition of Na^+/H^+ antiport by benzamil. The rate of Na^+-dependent H_i^+ extrusion was measured in the choline chloride medium using mitochondria treated with CCCP and valinomycin at pH_o 8.8 (protocol of Figure 2B). Nothing (●) or 100 μM benzamil (▲) was added prior to the Na^+. The addition of benzamil increased the pH_i from 6.9 to about 7.2. Amiloride analogues have been reported to quench the fluorescence of BCECF (Paradiso et al., 1986). However, benzamil (100 μM) decreases the BCECF fluorescence ratio at any given pH value by only about 0.1 ratio unit and does not alter the extent of the ΔpH_i observed on addition of Na^+.

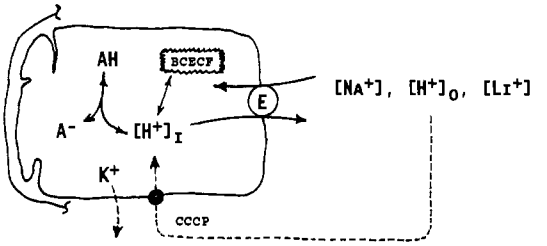

FIGURE 10: Conditions for the assay of Na^+/H^+ antiport using the fluorescence of matrix BCECF. The normally alkaline matrix compartment is first acidified by addition of uncoupler (CCCP) which conducts H^+ inward. The entering H^+ displaces a portion of the interior K^+ from matrix anionic binding sites (A). Addition of Na^+ then results in an uptake of Na^+ and a decrease in matrix $[H^+]$ due to the activity of the Na^+/H^+ antiport (E). The reaction shows a hyperbolic dependence on $[H^+]_i$ and on external $[Na^+]$. Both Li^+ and external H^+ act as competitive inhibitors of Na^+ uptake. The presence of an active cation uniport (as when valinomycin and external K^+ are present) will also produce a cation/H^+ exchange due to the ability of entering K^+ to displace H^+ from the anionic sites and the high H^+ conductance in the presence of CCCP.

when pH_o is elevated and ΔpH is large.

Amiloride has been shown to dissipate pH gradients by virtue of its properties as a weak base (Dubinsky & Frizzell, 1983), and benzamil produces a small alkaline shift in pH_i when added to mitochondria in the protocol of Figure 9. However, the inhibition of Na^+/H^+ antiport produced by the amiloride analogues is considerably greater than that due to increasing pH_i into a less than optimal range. In a typical experiment using the protocol of Figure 9, for example, 50 μM N^5,N^5-hexamethyleneamiloride increased pH_i from 7.0 to 7.4 and inhibited the Na^+-dependent change in pH_i by 84%. Increasing pH_i to 7.4 by addition of ammonium chloride produced only about 20% inhibition under the same conditions. Such results indicate that the amiloride analogues inhibit by interaction with the Na^+/H^+ antiport and not simply by altering the pH gradient.

Discussion

These studies have established that the fluorescence of BCECF can be used to follow Na^+-dependent changes in pH_i that are consistent with the activity of the endogenous Na^+/H^+ antiport of the mitochondrion. This measuring system (see Figure 10) requires that the ΔpH (interior alkaline) maintained by intact mitochondria be dissipated and at least a

portion of the original complement of matrix cations be replaced by H^+. This provides a pool of internal H^+ to support the inward exchange of Na^+ and puts pH_i in a region appropriate for measurement with the fluorescent probe. This condition can be achieved by allowing ΔpH to decay spontaneously (Figure 3). However, this spontaneous process takes some time at 25 °C and can lead to other changes in permeability and loss of internal anions. The alkaline interior can be acidified more conveniently by the addition of an uncoupler, as shown in Figure 10, or by addition of A23187 to exchange H^+ for internal divalent cations. In the presence of 1.5 μM CCCP, the protonmotive force is essentially zero, proton conductivity is high, and $\Delta\psi$ (the Donnan potential) is approximately equal to 57 ΔpH at 12 °C (see Figure 1B).

When such a mitochondrial population is challenged with external Na^+, there is an increase in matrix Na^+ and an alkaline shift in pH_i (Figure 2A) consistent with the extrusion of matrix H^+. The magnitude of the Na^+-dependent change in pH_i is determined by the existing ΔpH when Na^+ is added. The rate of the Na^+-dependent change in pH_i is determined by a temperature-sensitive membrane component that shows properties consistent with those of the Na^+/H^+ antiport as defined in other experimental systems [see Brierley and Jung (1988b) or Garlid (1988b) for reviews]. The evidence that the exchange of Na^+ for H^+ does not depend on Na^+ uniport or the activity of the K^+/H^+ antiport has been discussed above. The present work establishes that Na^+/H^+ antiport shows a hyperbolic dependence on both $[H^+]_i$ and external $[Na^+]$, is inhibited competitively by external $[H^+]$, and is sensitive to low concentrations of amiloride analogues. The competitive inhibition of Na^+ uptake by Li^+ reported by Nath and Garlid (1988) and Kapus et al. (1988) is also confirmed by using this measuring system (Figure 5).

The mitochondrial Na^+/H^+ antiport resembles the analogous exchange component found in the plasma membrane of mammalian cells [see Mahnensmith and Aronson (1985) for a review] in that H^+, Na^+, and Li^+ appear to compete for the external transport site and there is no reaction with K^+. However, internal H^+ appears to interact with both transport and activator sites on the plasmalemmal antiport, and thus far, there is no indication of an activator site for H^+ on the mitochondrial Na^+/H^+ antiport. In addition, the plasmalemmal exchanger shows an apparent K_i for external H^+ at 35 nM or pH_o 7.5 (Aronson et al., 1983), whereas the corresponding value for the mitochondrial component is a full pH_o unit higher (pH_o of 8.6). This would indicate that the mitochondrial Na^+/H^+ antiport is capable of reacting effectively at a much more alkaline pH than the plasmalemmal component and would be consistent with the putative role of the antiport in extruding Na^+ from the alkaline matrix of respiring mitochondria. Because of the significant difference in effective operating pH of the two Na^+/H^+ antiports, it is possible that they involve different groups at the catalytic sites. Such differences could explain the failure of diethyl pyrocarbonate, an effective inhibitor of the plasmalemmal Na^+/H^+ antiport, to inhibit the corresponding mitochondrial antiporter (Davis et al., 1987b).

The mitochondrial Na^+/H^+ antiport also shows a response to the amiloride analogues that differs significantly from that of the Na^+/H^+ antiport in plasma membranes (Vigne et al., 1984; Simchowitz & Cragoe, 1986). Benzamil strongly inhibits the mitochondrial reaction (Figure 9) but is not an effective inhibitor of the plasma membrane antiport. The latter antiport has been shown to require an unsubstituted guanidino group for inhibition by amiloride analogues (Simchowitz & Cragoe, 1986). In addition, the 5-N-substituted analogues inhibit the plasma membrane activity at concentrations as low as 0.16 μM (Simchowitz & Cragoe, 1986) compared to the 50–100 μM found necessary in the present studies of mitochondria Na^+/H^+ antiport.

The pattern of inhibition of the mitochondrial Na^+/H^+ antiport by amiloride analogues corresponds more closely to that reported for Na^+/Ca^{2+} exchange in plasma membrane vesicles. This activity is sensitive both to benzamil and to amilorides with substitutions on the 5-amino nitrogen atom (Kaczerowski et al., 1985). The mitochondrial Na^+/Ca^{2+} antiport is also inhibited by benzamil (Jurkowitz et al., 1983), and a more recent survey has found that this activity is sensitive to N^5,N^5-hexamethyleneamiloride and other 5-N-substituted amilorides in the same range of concentrations necessary to inhibit Na^+/H^+ antiport activity.[2] It therefore seems possible that the mitochondrial Na^+/H^+ and Na^+/Ca^{2+} antiports may share certain features, or even a common subunit, and a more complete comparison of the effect of amiloride analogues on the two activities is currently in progress.

The Na^+/H^+ antiport of red cell membranes shows a functional asymmetry in that it is inhibited by external but not by internalized amiloride (Grinstein & Smith, 1987). In contrast, the enhanced effectiveness of benzamil as an inhibitor of mitochondrial Na^+/H^+ antiport at elevated pH_o suggests that these reagents may be effective only when they enter the matrix of the mitochondria. The pK_a of these amiloride analogues is near 8.5, and protonation of the guanidino moiety is essential for inhibition of the Na^+/Ca^{2+} exchange in plasma membrane vesicles (Kaczorowski et al., 1985). In the vesicles, the effectiveness of these reagents falls off as pH is increased in the region of the pK_a value. The ability of benzamil to inhibit mitochondrial Na^+/H^+ antiport at elevated values of pH_o when the matrix pH is near neutral (conditions of Figure 9) is consistent with an interior reactive site. Entry of amiloride derivatives into intact cells has been shown to occur exclusively via the neutral (uncharged) form of the amines (Simchowitz et al., 1987). It therefore seems quite likely that benzamil would enter the matrix as the uncharged species generated at elevated pH_o and be converted to the reactive protonated form in the more acid matrix. The alkaline shift in pH_i seen on addition of benzamil (Figure 9) indicates that at least a portion of the inhibitor enters the matrix under these conditions. It has also been established that these amiloride analogues inhibit Na^+/H^+ antiport in SMP which have the opposite membrane orientation from that of intact mitochondria (Brierley et al., 1988a).

A complication in the interpretation of the inhibition of Na^+-dependent H^+ extrusion by benzamil and the other amiloride analogues arises when the effect of these reagents on swelling reactions is examined. Benzamil has little, if any, effect on the passive swelling of mitochondria in 50 mM sodium acetate at pH_o 7.2 or in 50 mM NaCl in the presence of tripropyltin (records not shown). These reactions are generally accepted to be dependent on Na^+/H^+ antiport activity (Brierley et al., 1978). The basis for the insensitivity of these swelling reactions to amiloride analogues is not clear and is currently being investigated.

Benzamil and the other effective amiloride analogues also show significant activity against Na^+/H^+ antiport in KCl, and there is little ΔpH maintained by uncoupled mitochondria under these conditions (Figure 1A). It is therefore possible that benzamil in the protonated form can also react with the

[2] G. P. Brierley and D. W. Jung unpublished results.

antiporter on the external surface, but in this case, there would be no concentrating effect of ΔpH. It is clear that inhibition of mitochondrial Na^+/H^+ antiport by 100 μM benzamil can vary from near zero in the swelling reactions to as high as 90% for the Na^+-dependent change in pH_i (Figure 9). Kapus et al. (1988) have recently reported that two analogues of benzamil, N^5-(4-chlorobenzyl)-2′,4′-dimethylbenzamil and 3′,5′-bis(trifluoromethyl)benzamil, inhibit mitochondrial Na^+/H^+ antiport. In this study, the activity was followed with a glass electrode as a Na^+-dependent change in external $[H^+]$ at pH_o 6.9. Under these conditions, the inhibition was competitive with respect to Na^+ and showed an I_{50} value of 60 μM at 15 mM Na^+. Such results appear compatible with an external reactive site for amiloride analogues. Further study of the effects of these reagents on the various Na^+/H^+ antiport assay systems will be necessary to establish whether these variations can be ascribed to asymmetry of the mitochondrial Na^+/H^+ antiport.

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