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Characterization of the mitochondrial Na^+–H^+ exchange. The effect of amiloride analogues

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The kinetic properties and inhibitor sensitivity of the Na^+–H^+ exchange activity present in the inner membrane of rat heart and liver mitochondria were studied. (1) Na^+-induced H^+ efflux from mitochondria followed Michaelis-Menten kinetics. In heart mitochondria, the K_m for Na^+ was 24 ± 4 mM and the V_{max} was 4.5 ± 1.4 nmol H^+/mg protein per s ($n = 6$). Basically similar values were obtained in liver mitochondria ($K_m = 31 \pm 2$ mM, $V_{max} = 5.3 \pm 0.2$ nmol H^+/mg protein per s, $n = 4$). (2) Li^+ proved to be a substrate ($K_m = 5.9$ mM, $V_{max} = 2.3$ nmol H^+/mg protein per s) and a potent competitive inhibitor with respect to Na^+ ($K_i \approx 0.7$ mM). (3) External H^+ inhibited the mitochondrial Na^+–H^+ exchange competitively. (4) Two benzamil derivatives of amiloride, 5-(*N*-4-chlorobenzyl)-*N*-(2′,4′-dimethyl)benzamil and 3′,5′-bis(trifluoromethyl)benzamil were effective inhibitors of the mitochondrial Na^+–H^+ exchange (50% inhibition was attained by approx. 60 μM in the presence of 15 mM Na^+). (5) Three 5-amino analogues of amiloride, which are very strong Na^+–H^+ exchange blockers on the plasma membrane, exerted only weak inhibitory activity on the mitochondrial Na^+–H^+ exchange. (6) The results indicate that the mitochondrial and the plasma membrane antiporters represent distinct molecular entities.

Introduction

The mitochondrial inner membrane possesses an Na^+–H^+ exchange system (see for a review Ref. 1). The existence of this transport route was first proposed by Mitchell and Moyle [2], who observed that Na^+ increased the decay of a previously established pH gradient between the two sides of the mitochondrial membrane. This phenomenon was observed in submitochondrial particles as well [3–5]. The passive swelling of rat liver [5], heart [6] and brain [7] mitochondria in isoosmotic sodium acetate confirmed the operation of an Na^+–H^+ exchange system. The equilibrium distribution of Na^+ and H^+ across the inner membrane was also in good agreement with the functioning of this transport route [8].

In spite of evidence proving the presence of the exchanger in the mitochondrial membrane, our understanding of the carrier molecule itself is

Abbreviations: BTFB, 3′,5′-bis(trifluoromethyl)benzamil hydrochloride sesquihydrate; CBDMB, 5-(*N*-4-chlorobenzyl)-*N*(2′,4′-dimethyl)benzamil; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; HMA, 5-(*N*,*N*-hexamethylene)amiloride; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; $[H^+]_o$, $[H^+]$ of the extramitochondrial compartment.

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rather poor. Few data are available about its kinetic properties, and no specific inhibitor has been described yet.

In recent years, much attention has been focused on the operation and physiological role of the amiloride-inhibitable Na^+–H^+ exchanger present in the plasma membrane of a wide variety of animal cells (see, for reviews Refs. 9–11). The characterization of the plasma membrane antiporter was largely promoted by the synthesis of more potent and specific amiloride derivatives, some of which inhibited Na^+–H^+ exchange [12–16], while others Na^+–Ca^{2+} exchange [17–20] rather selectively. The use of some of these drugs enabled us to compare the mitochondrial and the plasma membrane Na^+–H^+ exchange systems in respect of their kinetic parameters and inhibitor sensitivity. The results indicate that the plasma membrane and the mitochondrial Na^+–H^+ antiporters are distinct molecular entities.

Materials and Methods

Materials. Amiloride and its analogues were synthesized at the Merck, Sharp & Dohme Research Laboratories (West Point, PA, U.S.A.) as described previously [21,22] and were dissolved in dimethyl sulfoxide before use. Rotenone was purchased from K and K Laboratories (Plainview, NY, U.S.A.) carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and valinomycin from Calbiochem, antimycin A, mersalyl and monensin from Serva, oligomycin from Reanal (Hungary), [^{3}H]-acetate and [^{14}C]methylamine from Centre d'Etudes Nucleaires de Saclay (Gif-sur-Yvette, France).

Preparation of mitochondria. Rat liver and rat heart mitochondria were isolated by the methods described in Refs. 23 and 24, respectively. The mitochondrial protein content was assayed by the biuret method, using bovine serum albumin as standard.

Transport measurements. For the measurement of Na^+- and Li^+-induced H^+ efflux, mitochondria (1.9–2.3 mg protein/ml) were incubated in a medium with low buffer capacity composed of 100 mM KCl and 80 mM sucrose. In order to block the operation of other H^+-translocating pathways, i.e., the H^+ pumps of the respiratory chain, the F_0–F_1 ATPase and the H^+–P_i symporter, the basic medium contained rotenone (1 μM), antimycin A (4 μg/ml), oligomycin (2 μg/ml) and mersalyl (100 μM). H^+ movements were followed in an open reaction vessel at 22–24°C by a Radiometer pH electrode (type G 202 C) connected to a Radiometer pH meter (type 26) and a potentiometric recorder (Radelkis, OH-814/1). The 90% response time of the whole system after addition of 12.5 nmol H^+/mg protein at pH 6.9 was 2.1 s. Thus, the electrode response was far from being rate-limiting at the highest efflux rate measured. After 6 min preincubation of mitochondria, Na^+ or Li^+ were rapidly introduced into the medium. At this time, the extramitochondrial pH was 6.9. Amiloride analogues were added 1.5 min before Na^+, and the pH was titrated back with small amounts of HCl. (Neither dimethyl sulfoxide (DMSO) – the solvent of the analogues - nor the alkalinisation-back titration procedure influenced the Na^+-induced H^+ release.) The calibration of the electrode response was performed after each experiment as described previously [8]. Briefly, after the backflux of extruded H^+, known amounts of HCl were added and the recorder deflection was replotted according to a linear scale of ΔH^+. The initial rate of H^+ efflux was calculated from the first 3 s period after Na^+ or Li^+ addition. The electrode artifact due to added cations was determined in the presence of CCCP and valinomycin (see Fig. 1 and Ref. 8).

Ca^{2+} transport was monitored by a Ca^{2+}-selective electrode. The set up and the calibration of the electrode is described in Ref. 23.

The medium for these measurements contained 125 mM KCl, 20 mM Tris-HCl, 35 μM (endogenous) Ca^{2+}, 0.01% bovine serum albumin (pH 7.4).

Measurement of the intramitochondrial pH. The intramitochondrial pH was calculated on the basis of distribution of [^{3}H]acetate and [^{14}C]methylamine as described by Nicholls [25]. Liver mitochondria (2.5 mg/ml) were incubated in the basic medium supplemented with 10 mM Tris-HCl, 0.25 μCi/ml [^{14}C]methylamine and 1.5 μCi/ml [^{3}H]acetate, the final pH being 7.0. After different times of incubation in the presence or absence of Na^+ (detailed under Fig. 2), samples were withdrawn and spun down in a bench centrifuge (Janetzki TH-12) at $10000 \times g$ for 120 s. The

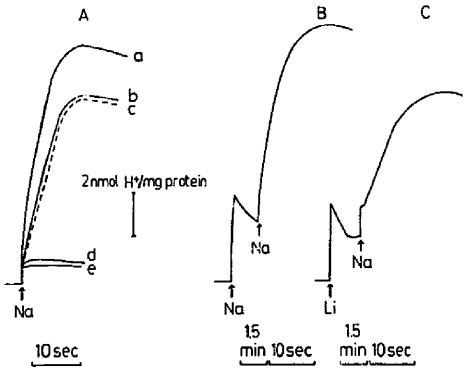

Fig. 1. Na^+-induced H^+ efflux from liver mitochondria. Trace of the pH electrode. Liver mitochondria (2.1 mg/ml) were incubated in the basic medium at pH 6.9. (A) After 6 min incubation, 15 mM NaCl was added in a, and 10 mM NaCl in b–e. Other additions were made 1.5 min before NaCl: a and b, none; c, 2 μM CCCP; d, 2 μM CCCP plus 1 μM valinomycin; e, 0.0625% Triton-X 100. (B) at the first arrow, 3.7 mM NaCl was added after 4.5 min incubation, and at the second arrow, 15 mM NaCl. (C) At the first arrow, 2.1 mM LiCl was added after 4.5 min incubation, and at the second arrow, 15 mM NaCl.

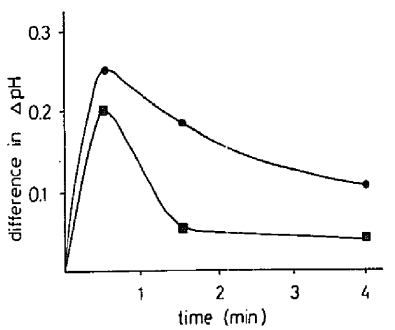

Fig. 2. Alkalinization of the intramitochondrial compartment following the addition of NaCl. After preincubation of mitochondria (2.5 mg/ml) for 1 min in the basic medium supplemented with 10 mM Tris-HCl, [^{14}C]methylamine (0.25 μCi/ml) and [^{3}H]acetate (1.5 μCi/ml), 10 mM (■) or 40 mM (●) NaCl was added and the incubation was continued for the period indicated in the figure. Control samples were incubated for the same period without NaCl. Thereafter, mitochondria were separated from the medium and treated as described under Materials and Methods. The ordinate indicates the difference of the transmembrane pH of control compared to NaCl-treated mitochondria. Experimental points represent the mean of two parallel determinations, the difference of which was less than 5%.

supernatant was discarded and the pellets were dissolved in 100 μl formic acid. The radioactivity was counted in a Beckman LS-250 scintillation spectrometer.

The mitochondrial Mg content was determined by atomic absorption spectrometry as described previously [26].

Results

Kinetic properties and substrate specificity of the mitochondrial Na^+–H^+ exchange system

In order to investigate the Na^+–H^+ exchange in intact mitochondria, Na^+-induced H^+ efflux was followed using a pH electrode. Fig. 1A shows that the addition of Na^+ to mitochondria incubated in the basic medium caused the rapid appearance of H^+ in the extramitochondrial compartment. The following findings indicate that the source of these H^+ ions was the matrix space. (1) Concomitant with the appearance of H^+ in the extramitochondrial space was the alkalinization of the intramitochondrial compartment, detected by the distribution of [^{14}C]methylamine and [^{3}H]acetate (Fig. 2). The time-course of the intramitochondrial pH change corresponded well to that observed outside the mitochondria. (2) After creating an artificial short-circuit for H^+ by the simultanous addition of the protonophore, CCCP and the K^+ ionophore, valinomycin, no H^+ efflux could be observed upon addition of Na^+ (Fig. 1A, d and see also Ref. 8). It has to be noted that in the presence of CCCP or valinomycin alone, the initial rate and extent of H^+ efflux decreased by less than 10% (Fig. 1A, c). (3) The solubilization of mitochondria with Triton X-100 completely abolished the Na^+-induced acidification of the medium (Fig. 1A, e).

Na^+-induced H^+ release, in principle, could occur through the K^+–H^+ exchanger of the inner membrane [27]. However the K^+–H^+ exchanger was shown to be active only in Mg-depleted mitochondria (< 2 nmol/mg [27]), while our preparation contained more than 20 nmol Mg/mg protein. Furthermore, 10 min incubation of mitochondria with 50 nmol/mg *N,N'*-dicyclohexylcarbodiimide (DCCD), a condition known to inhibit K^+–H^+ exchange [28], failed to affect the Na^+-induced H^+ ejection (data not shown).

Fig. 3 shows that the Na^+-induced H^+ efflux conformed to Michaelis-Menten kinetics. In the case of heart and liver mitochondria, the value of K_m for Na^+ at pH 6.9 was found to be 24 ± 4 mM ($n = 6$) and 31 ± 2 mM ($n = 4$), while the maximal velocity was 4.5 ± 1.4 and 5.3 ± 0.2 nmol H^+/mg protein per s, respectively. The kinetic parameters obtained in heart and liver are rather similar, suggesting that the same Na^+–H^+ transporter is present in both types of mitochondria.

Li^+ also proved to be a substrate for the exchanger (see Fig. 1C). The affinity for Li^+ was considerably higher (K_m 5.9 ± 0.2 mM, $n = 3$), whereas the rate of maximal H^+ efflux (2.3 ± 0.4 nmol H^+/mg protein per s) was lower as compared to Na^+. Preincubation of mitochondria with Li^+ inhibited the Na^+-induced H^+ efflux (Figs. 1C, 3 and 4A). The possibility that this inhibitory effect was only due to the formation of a ΔpH across the membrane as a consequence of Li^+–H^+ exchange can be eliminated on the basis of experiments shown in Fig. 1B and C. After addition of a small amount of Na^+ – just enough to produce H^+ efflux of a similar magnitude as that caused by 2.1 mM Li^+ – the further H^+ efflux evoked by a second Na^+ pulse was only negligibly inhibited.

Kinetic analysis (Lineweaver-Burk and Dixon plots) reported on Figs. 3 and 4A showed Li^+ to

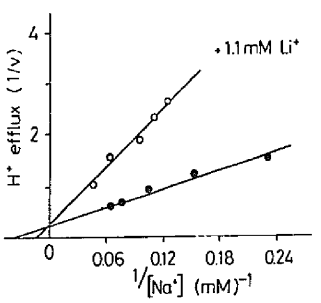

Fig. 3. The kinetics of the Na^+-induced H^+ efflux from heart mitochondria and its inhibition by Li^+. Double-reciprocal plots. The initial rate of Na^+-induced H^+ efflux (v, nmol H^+/mg per s) was measured in the absence (●) or presence (○) of 1.1 mM LiCl, as described in Materials and Methods. Mitochondria (1.9 nmol/mg) were incubated in the basic medium for 6 min before addition of NaCl. LiCl was added 1.5 min before NaCl.

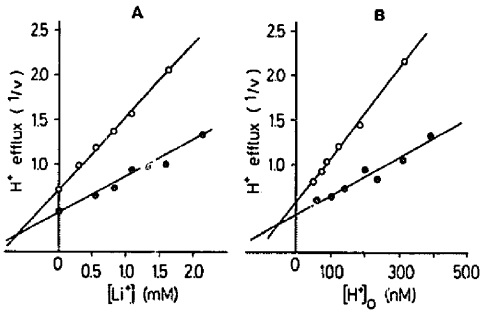

Fig. 4. Inhibition of Na^+-induced H^+ efflux from liver mitochondria by Li^+ (A) and by $[H^+]_0$ (B), plotted according to Dixon. Liver mitochondria (2.1 mg/ml) were incubated in the basic medium. In (A), after 4.5 min, the indicated concentrations of LiCl were added. In (B), after 4.5 min, small amounts of KOH or HCl were added to set the extramitochondrial pH to the desired value. At 6 min, 9.6 mM (○) or 15 mM (●) NaCl was added. (v, nmol H^+/mg per s).

be a competitive inhibitor with respect to Na^+ with a K_i of about 0.7 mM.

NH_4^+, which could exchange for H^+ through the antiporter of the renal microvillus membrane [29,30], did not cause any H^+ efflux from mitochondria and had no effect on the Na^+-induced H^+ extrusion, indicating that NH_4^+ did not interact with the carrier (not shown).

In the following experiments, we examined the effect of $[H^+]_0$ on the transport process. Increasing the H^+ concentration of the medium inhibited the subsequent Na^+-induced ejection of H^+. When $1/v$ is plotted against $[H^+]_0$ according to Dixon, a straight line is obtained (Fig. 4B), implying that in the investigated pH range (6.3–7.4), protons interact with the carrier at a single site. In accordance with this, the calculated Hill coefficient was 1.07 ± 0.11 ($n = 4$). The type of inhibition exerted by H^+ was apparently competitive with respect to Na^+ (Fig. 4B). The value of K_i ranged between 30 and 60 nM in four different mitochondrial preparations, indicating that the antiporter has a high affinity for external H^+.

The effect of amiloride analogues

Amiloride itself at 110 μM did not influence the mitochondrial Na^+–H^+ exchange (Fig. 5A, C). It was shown earlier that the 5-amino substituted analogues of amiloride possess much stronger

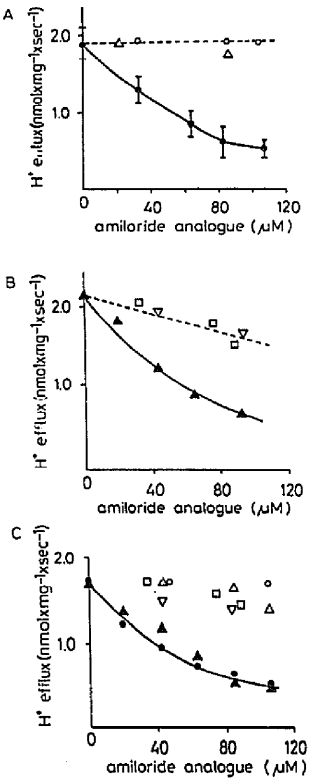

Fig. 5. The effect of amiloride and some of its derivatives on the Na^+-induced H^+ efflux from mitochondria. Heart (1.9 mg/ml, (A) and (B)) or liver (2.1 mg/ml (C)) mitochondria were incubated in the basic medium. The indicated concentrations of amiloride (○), EIPA (△), MIA (▽), HMA (□), CBDMB (●) or BTFB (▲) were added after 4.5 min, and the pH was immediately readjusted to 6.9. At 6 min, 15 mM NaCl was added. Initial rates (nmol/mg per s) were measured as described under Materials and Methods. In the case of CBDMB, data are expressed as means ± S.E., for four separate preparations.

inhibitory activity on the Na^+–H^+ exchange of the plasma membrane than the parent compound [12–16]. Therefore, we examined whether the most potent 5-amino derivatives, i.e., 5-(*N*,*N*-hexamethylene)amiloride (HMA), 5-(*N*-methyl-*N*-isobutyl)amiloride (MIA) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (their K_i values on the plasma membrane are between 0.1 and 0.2 μM [14–16]) were able to affect the mitochondrial Na^+–H^+ exchange. Fig. 5 shows that these compounds proved to be very weak inhibitors even at a concentration of 400–600-times higher than the value of their K_i on the plasma membrane. On the other hand, some amiloride analogues bearing substituents on the terminal guanidino nitrogen had marked inhibitory effects on the mitochondrial Na^+–H^+ exchange. These analogues are very poor inhibitors of the plasma membrane Na^+–H^+ antiporter ($K_i > 200$ μM) [14], while they block Na^+–Ca^{2+} exchange more efficiently [17–20]. The most potent compounds tested were two benzamil derivatives, 5-(*N*-4-chlorobenzyl)-*N*-(2′,4′-dimethyl)benzamil (CBDMB) and 3′,5′-bis(trifluoromethyl)benzamil hydrochloride sesquihydrate (BTFB). 50% inhibition was attained in the presence of 15 mM Na^+ at about 60 μM with both CBDMB and BTFB. The inhibitory potencies of these drugs were almost identical in heart and liver.

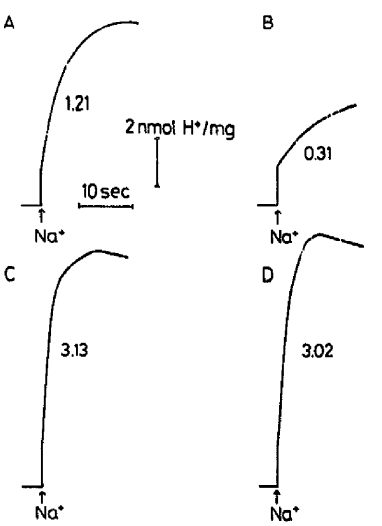

Fig. 6. The effect of CBDMB on the Na^+-induced H^+ efflux from mitochondria in the absence (A, B) and presence (C, D) of monensin. Liver mitochondria (2.1 mg/ml) were incubated as in Fig. 4.A, except that the medium contained 100 mM choline chloride instead of KCl, as monensin transports also K^+. Na^+ indicates 10.5 mM NaCl addition after 6 min incubation. Further additions: (A) none; (B) 85 μM CBDMB at 5 min; (C) 2 μg monensin/mg protein at 4.5 min; (D) 2 μg monensin/mg protein at 4.5 min plus 85 μM CBDMB at 5 min. The numbers beside the traces indicate initial rates as expressed in nmol H^+/mg per s.

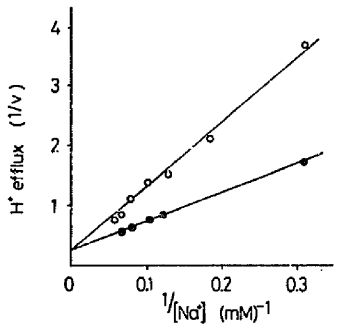

Fig. 7. Kinetic analysis of the inhibition of the Na^+-induced H^+ efflux from heart mitochondria by CBDMB. Double-reciprocal plots. Additions: none (●); 64 μM CBDMB (○) after 4.5 min incubation of mitochondria. Other experimental conditions are the same as in Fig. 5. v is expressed in nmol H^+/mg protein per s.

It was necessary to examine whether the observed inhibition was due to a direct interaction between the analogues and the Na^+–H^+ carrier. The following findings indicate that the general permeability properties of the mitochondrial membrane were not changed by potent amiloride analogues. (1) In the presence of the artificial Na^+–H^+ exchanger, monensin, the rate and the extent of the Na^+-induced H^+ efflux was almost identical, independently of whether mitochondria were preincubated with or without CBDMB (Fig. 6C and D). In the absence of monensin, the same amount of CBDMB inhibited the transport by 75% (Fig. 6A and B). Similar results were obtained with BTFB. (2) The Ca^{2+}-uptake capacity of mitochondria was not affected by CBDMB up to 80 μM (data not shown). In addition, CBDMB inhibited the Na^+-induced Ca^{2+} release from ruthenium red-treated mitochondria, a finding consistent with earlier reports about the effect of other amiloride analogues [31,32]. (3) Fig. 7 shows the kinetics of the inhibition of the mitochondrial Na^+–H^+ exchange by CBDMB. The competitive type of inhibition with respect to Na^+ also indicates direct interaction between CBDMB and the antiporter.

As amiloride analogues are weak bases, they may alter the matrix pH after permeating the mitochondrial membrane. The contribution of this possibility to the inhibitory actions of the drugs was excluded, since after the stabilization of the intramitochondrial pH by 0.2 mM potassium propionate [33], the effectivity of amiloride analogues was retained (data not shown).

Discussion

The present study provides the characterization of the Na^+–H^+ exchange activity present in the inner membrane of liver and heart mitochondria. This transport route satisfies the criteria of a carrier-mediated process: (1) it is saturable with its substrates (Na^+, Li^+) showing Michaelis-Menten type kinetics. (2) Li^+ and H^+ compete with Na^+ for binding (K_i 0.7 mM and approx. 50 nM.). (3) Some amiloride analogues proved to be rather potent inhibitors of this pathway.

The K_m value (31 mM) for Na^+ found by us in liver mitochondria is in good agreement with the K_m (27 mM) obtained by following the Na^+-induced changes of quinacrine fluorescence in respiring liver submitochondrial particles [3]. The close similarity of the K_m values observed in the inside-out and the right-side-out systems suggests that the Na^+-binding and transport properties of the exchanger might be symmetrical. In heart mitochondria, Crompton and Heid [8] have found a K_m of 5 mM and a V_{max} of 2 nmol H^+/mg protein per s, which are lower values than those obtained by us ($K_m = 24$ mM, $V_{max} = 4.5$ nmol H^+/mg protein per s). The different composition of our incubation medium (i.e., the absence of EGTA, the lower K^+ concentration, the presence of sucrose, rotenone and oligomycin) might account for this difference. Also, in accordance with the work of Rosen and Futai [3], Li^+ had a higher affinity for the carrier; however, in contrast with them, under our circumstances, the maximal transport rate was higher in the case of Na^+ than Li^+. Our observation corresponds well to the fact that mitochondria swell more rapidly in sodium than in lithium acetate [6].

Comparing the properties of the mitochondrial and the plasma membrane exchangers, some similarities and some basic differences can be established. (1) The K_m values for Na^+ are rather similar in the two systems (see Ref. 10 for a recent review on the plasma membrane antiporter). (2) Both carriers bind Li^+ with a higher affinity than Na^+, but transport it at a slower rate [10,34]. (3)

H^+ competes with Na^+ on both exchangers [30,35]. (4) In contrast with the antiporter present in the plasma membrane, the mitochondrial exchanger does not carry NH_4 ions. (5) The inhibitor sensitivity of the mitochondrial and the plasma membrane transporter is bascially different.

This is the first report in which some amiloride analogues were shown to inhibit the mitochondrial Na^+–H^+ antiporter. Derivatives bearing 5-amino substituents which are very strong Na^+–H^+ exchange blockers on the plasma membrane [12–16] were found to be only slightly effective in mitochondria. The most potent compound HMA (K_i on plasma membrane 0.16 μM [14]) caused less than 30% inhibition at 88 μM in mitochondria. On the other hand, CBDMB and BTFB, two derivatives substituted on the guanidino moiety, applied in the same concentration, reduced the transport activity by 70%. These latter compounds are very weak inhibitors of the plasma membrane Na^+–H^+ exchanger (K_i > 500 [20] and > 200 μM (Cragoe, E.J., Jr., unpublished observation)). These findings are not sufficient to declare a defined structure-potency relationship with respect to the mitochondrial antiporter, but the basic differences in the inhibitory activities of the various analogues on the mitochondrial and the plasma membrane exchangers suggest that these transporters represent distinct molecular entities.

It has been shown recently that amiloride analogues can be accumulated by the cells: at an extracellular concentration of 2 μM, the cytoplasmic drug level may reach several hundred μM [36]. This concentration is certainly enough to affect the mitochondrial Na^+–H^+ (and Na^+–Ca^{2+} [31,32]) transport processes, which might have relevance to the cellular effects of these drugs. On the other hand, the use of some of these derivatives may help to elucidate the physiological role of the mitochondrial Na^+–H^+ exchanger.

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