SODIUM/PROTON ANTIPORTER OF RAT LIVER MITOCHONDRIA

Barry P. ROSEN and Masamitsu FUTAI+

Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201, USA and +*Department of Microbiology, Faculty of Pharmaceutical Sciences Okayama University, Tsushima-Naka, Okayama 700, Japan*

Received 3 June 1980

1. Introduction

Antiport systems which catalyze exchange of protons and monovalent cations have proven to be ubiquitous in bacteria [1–7] and mitochondria [8–10]. Mitchell [11] first proposed that exchange of protons for monovalent cations was involved in regulation of ΔpH between the mitochondrial matrix and cytosol. Cations such as Na^+ increased the rate of decay of proton gradients established by electrogenic proton translocation through the electron transport chain, suggesting the existence of a Na^+/H^+ antiporter [8]. Similar conclusions were reached for both rat liver and bovine heart mitochondria by observing the passive swelling of mitochondria in the presence of various cations [10,12,13]. Using that technique, it was established that bovine heart mitochondria exchange protons for a variety of monovalent cations, including Na^+ and Li^+ [10]. However, that method is not suitable for competition experiments, so it was not possible to determine whether separate antiporters exist for each cation, whether there are families of antiporters, as has been found in bacteria [2], or whether a single system was responsible for the observations. Uptake of Na^+ and K^+ into submitochondrial particles was measured directly from rat liver using ion-specific electrodes [9,10]. Again, neither the number nor specificities of monovalent cation antiporters was determined.

The energy-dependent uptake of the lipophilic weak base quinacrine was used as a measure of the ΔpH in submitochondrial particles of rat liver [14]. Formation of a pH gradient results in the quenching of the fluorescence of quinacrine, which provides a convenient assay for that component of the protonmotive force. Since exchange of protons for cations decreases the steady-state ΔpH, examination of the effects of cations on the quenching of quinacrine fluorescence has allowed for elucidation of the properties of the antiporters of *Escherichia coli* [2,15–18], *Bacillus alcalophilus* [6], and the thermophilic bacillus PS3 [7]. Here we have used this technique with submitochondrial particles from rat liver, demonstrating that there is a single Na^+/H^+ antiporter with a selectivity of $Li^+ > Na^+$. The antiporter catalyzed electroneutral cation/proton exchange.

2. Materials and methods

Rat liver submitochondrial particles were prepared as in [19]. The submitochondrial particles were suspended to 5 mg membrane protein/ml in a solution containing 10 mM Tris–HCl, 140 mM choline chloride, 0.5 mM dithiothreitol, and 10% (v/v) glycerol (pH 7.5), diluted with an equal volume of glycerol and stored at −20°C until use.

The ΔpH was estimated from the energy-dependent quenching of quinacrine fluorescence, as in [2]. The assay medium consisted of 10 mM Tris–HCl, 140 mM choline chloride and 1 μM quinacrine (pH 8.0) in 2 ml final vol. In the experiments described in table 1, choline chloride was replaced by mixtures of various potassium salts, as described in the table. Submitochondrial particles and the choline salt of ATP were added at 0.03 mg membrane protein/ml and 1 mM, respectively. Choline ATP was prepared from disodium ATP using an ion exchange resin. Quenching was initiated by addition of $MgSO_4$ to 5 mM, which activated the H^+-translocating ATPase (F_0F_1). Relative fluorescence was measured with a Hitachi Spectrofluorometer, with excitation at 420 nm and emission at 500 nm. Protein was measured by the method in [20].

Table 1
Effect of permeant anions of Li^+/H^+ and Na^+/H^+ exchange

Substrate	Buffer[a]	Initial rate of cation/H^+ exchange (% fluorescence increase/min)	% Control
LiCl (2.5 mM)	Choline-Cl	429	100
	KCl	511	119
	KNO_3	410	96
	KSCN	350	82
NaCl (50 mM)	Choline-Cl	120	100
	KCl	45	38 (100)
	KNO_3	45	38 (100)
	KSCN	54	45 (120)

[a] The buffers consisted of 10 mM Tris–HCl (pH 8.0) containing 1 μM quinacrine–HCl, and the appropriate salt mixtures as follows: 140 mM choline chloride; 140 mM KCl; 100 mM KNO_3 plus 40 mM KCl; or 50 mM KSCN plus 90 mM KCl

3. Results

The energy-dependent quenching of the fluorescence of quinacrine by submitochondrial particles [14] and everted membrane vesicles from bacteria [2] has been related to the formation of a ΔpH, acid interior. The final level of fluorescence quenching reflects a steady-state between active proton uptake and secondary proton extrusion. Addition of substrates of cation/proton antiporters would be expected to perturb that steady state by increasing the rate of proton efflux in exchange for cation. As shown in fig.1, addition of ATP to submitochondrial particles has no effect on the quenching of quinacrine fluorescence until $MgSO_4$ is added, at which time rapid loss of fluorescence is observed. Subsequent addition of the F_0F_1 inhibitor *N,N'*-dicyclohexylcarbodiimide reverses the fluorescence quenching. These results shows that Mg^{2+}-dependent hydrolysis of ATP by the *N,N'*-dicyclohexylcarbodiimide-sensitive F_0F_1 produces a proton gradient, acid interior.

After a steady-state proton gradient is attained, addition of either NaCl or LiCl produced reversal of fluorescence quenching, suggesting that the steady-state proton gradient was decreased by addition of those salts (fig.1). Since the assays were performed in 140 mM choline chloride, the effect is most likely caused by the cations Na^+ or Li^+. In support of this, neither KCl nor RbCl reversed the quenching at 50 mM. The steady-state proton gradient was the same in the presence of LiCl whether LiCl was added prior to formation of the proton gradient (fig.1B) or subsequent to active proton uptake (fig.1A). The same was true for the effect of NaCl (fig.1C,D). If LiCl were added before NaCl, the latter had no effect on the proton gradient (fig.1A,B). Similarly, prior to addition of NaCl prevented reversal of fluorescence by LiCl (fig.1C,D). These results demonstrate that the effects of Li^+ and Na^+ are not additive but, rather, are competitive. ATP hydrolysis by the F_0F_1 was unaffected by 100 mM NaCl or 20 mM LiCl (not shown). In addition, essentially the same results were obtained using 5 mM Tris–succinate as the source of energy to establish a proton gradient (not shown). The most reasonable explanation for these results is that there is a cation/proton antiporter which accepts either Na^+ or Li^+ as substrates. The lack of effect of K^+ or Rb^+ suggest that the antiporter is specific for Na^+ and Li^+. Since the reactions occur in 140 mM choline chloride, the cation choline$^+$ is probably not a substrate.

When the initial rate of fluorescence increase was measured as a function of NaCl or LiCl concentration, hyperbolic curves similar to saturation curves were observed (fig.2). Such treatments have been used to estimate the affinities of bacterial antiporters for substrates [2,6,15–18]. With submitochondrial particles, the antiporters exhibited much higher affinity for Li^+ than for Na^+, which app. K_m 1.0 and 26.5 mM, respectively. The app. V_{max} was 3-fold greater with Li^+ than Na^+. The app. V_{max} value was variable from one assay to another, but the 3-fold

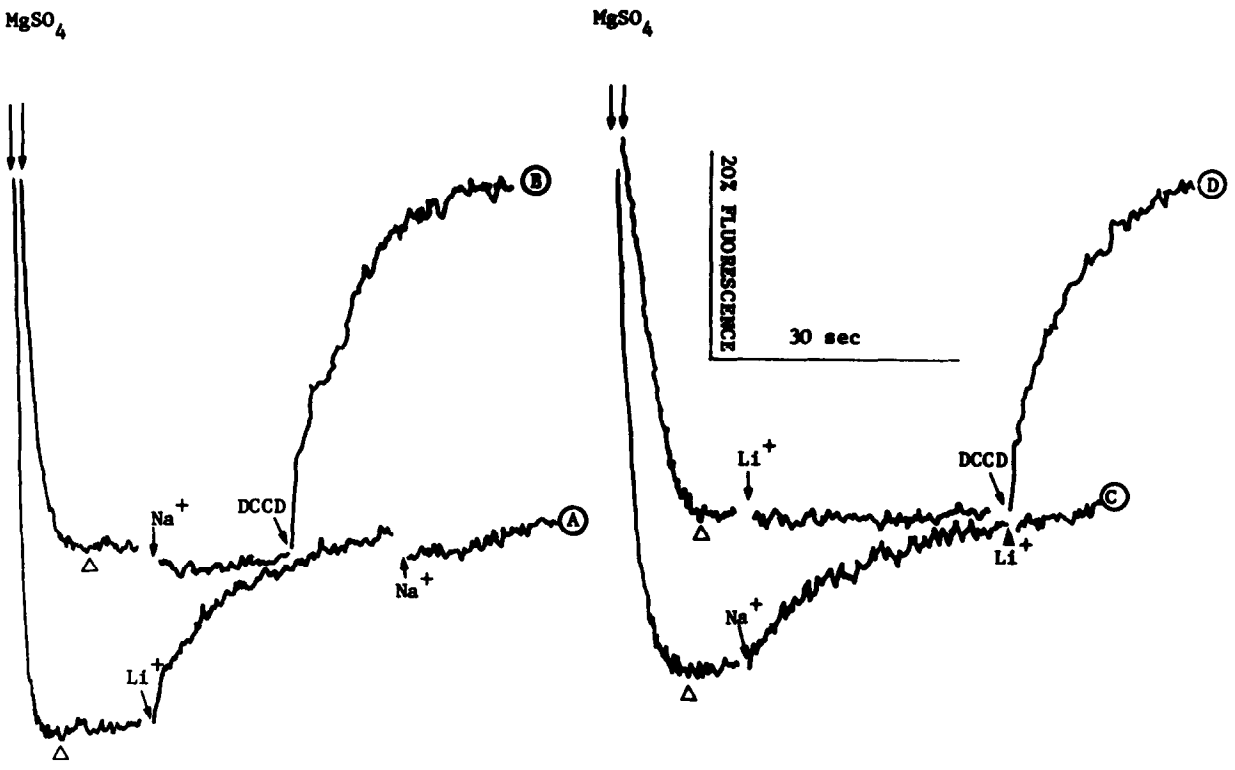

Fig.1. Li^+/H^+ and Na^+/H^+ antiporter activity in submitochondrial particles. Activity was estimated from the reversal of the quenching of quinacrine fluorescence, as in section 2. At the time indicated concentrated solutions were added to give the following final concentrations: 5 mM $MgSO_4$; 2.5 mM LiCl; 50 mM NaCl; 0.1 mM *N,N'*-dicyclohexylcarbodiimide. (B) LiCl was added to 2.5 mM prior to addition of $MgSO_4$. (D) NaCl was added to 50 mM prior to addition of $MgSO_4$. At the positions indicated by (△), the chart speed was increased 8-fold to that indicated in the figure. Each assay contained 0.06 mg submitochondrial particle protein.

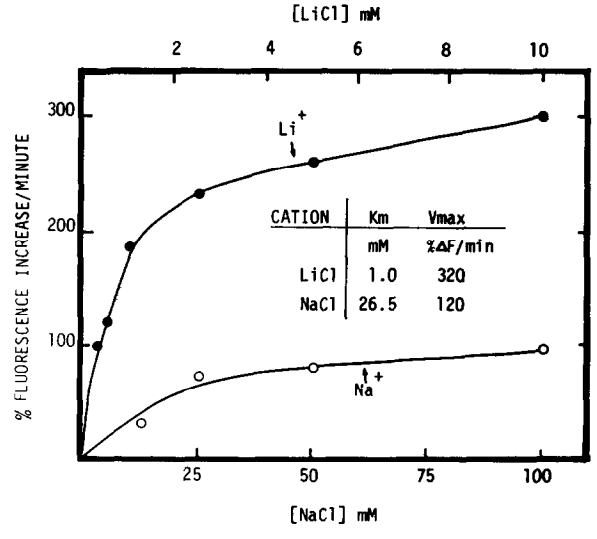

Fig.2. Concentration dependence of Na^+/H^+ and Li^+/H^+ exchange. Activity was assayed as in section 2 using 0.06 mg submitochondrial particle protein/assay. The initial rate in units of percentage increase in fluorescence/min was calculated from the initial increase in fluorescence following addition of varying amounts of NaCl or LiCl. The app. K_m and V_{max} values were calculated from reciprocal plots of the data.

difference between the values for Li^+ and Na^+ was found consistently. The cause of the variability is not known, but the same phenomenon was observed for the Na^+/H^+ antiporter of *E. coli* [15].

The effect of the permeant anions nitrate and thiocyanate on Na^+/H^+ and Li^+/H^+ exchange was examined (table 1). Both anions produced a large increase in the quenching of quinacrine fluorescence (data not shown), as has been observed using everted bacterial membrane vesicles [15]. This is interpreted

as an increase in the steady-state ΔpH. In both submitochondrial particles and everted bacterial membrane vesicles, the $\Delta\psi$ component of the protonmotive force is positive interior. Thus permeant anions would be accumulated in response to the $\Delta\psi$. $\Delta\psi$ would decrease until a steady-state accumulation of anion were attained. At high concentration of anion. This produces a depolarization which, although transient, should last the length of the assay. Since proton uptake via the F_0F_1 is restricted through a mass action effect by $\Delta\psi$, depolarization stimulates proton uptake; thus, ΔpH increases. When the conditions are such that the amount of fluorescence quenching is the same in the presence and absence of permeant anion, it is assumed that ΔpH is constant but that $\Delta\psi$ is near zero in the presence of anion. Thus, the effect of cation on ΔpH can be examined in the presence and absence of $\Delta\psi$. With everted membrane vesicles from bacteria ΔpH was adjusted to a constant value with NH_4Cl or K^+ in the presence of nigericin [15]. With submitochondrial particles fluorescence quenching was much greater in the presence of permeant anion, but the level of fluorescence spontaneously increased after a few minutes. In the experiments summarized in table 1, NaCl or LiCl were added to each assay when the same amount of fluorescence quenching was attained. Different baseline values were observed in the presence of chloride, nitrate or thiocyanate, but when those values were subtracted from the initial rate of fluorescence increase produced by addition of NaCl or LiCl, no difference in the rate of cation/proton exchange was observed (table 1). Substitution of potassium salts for choline salts was without effect on Li^+/H^+ exchange, although a 50–60% decrease in Na^+/H^+ exchange was noted. It is not clear whether this reflects a low affinity of the antiporter for K^+.

4. Discussion

Mitchell [11] first suggested that cation/proton antiport systems may be involved in mitochondrial energy metabolism. Na^+/H^+ and K^+/H^+ antiporters in rat liver mitochondria were subsequently demonstrated [8,12]. These systems have been studied in rat liver mitochondria and bovine heart mitochondria and submitochondrial particles in several laboratories [8–13]. A number of techniques have been used, including measurements of passive swelling of mitochondria [8–10] and of cation uptake using ion-specific electrodes [9,13]. We have studied analogous systems in *E. coli* by measurement of the effect of cations on the energy-linked quenching of quinacrine fluorescence [2,15–18]. Quenching of quinacrine fluorescence has been used in submitochondrial particles [14] and everted bacterial membrane vesicles [2,6,7] to estimate the ΔpH component of the protonmotive force. Since perturbation of the steady state ΔpH by cations has been correlated with the activity of bacterial cation/proton antiporters [2,15–18], it seemed logical to extend the studies of such antiporters in mitochondria with the tools successfully used in bacterial systems. The methods used here have several advantages over those used in previous studies of mitochondrial antiporters:

(i) The assay is simple and extremely sensitive.
(ii) It allows measurement of antiporter activity under conditions of constant ΔpH, which, as discussed below, is important for determination of electroneutrality.

Studies based on passive swelling of mitochondria have suggested that cation/proton exchange occurs with a number of monovalent cations, including Na^+ and Li^+ [10]. The data do not distinguish between a common system for all monovalent cations or separate systems for each. These results, however, show that Na^+ and Li^+ are fully competitive with each other, and that there is only single system kinetically for the two cations. Moreover, the antiporter has considerably greater affinity for Li^+ than for Na^+; likewise, the maximal velocity is greater with Li^+ than Na^+. Considering the importance of lithium in clinical medicine, this observation may not be insignificant.

In most discussions of the Na^+/H^+ antiporter it has been assumed without direct evidence that the porter catalyzes a neutral one-for-one exchange. This assumption is based in part on analogy with nigericin, which does catalyze electroneutral exchange. However, our results with the similar Na^+/H^+ antiporter of *E. coli* suggest that the porter catalyzed electrogenic exchange where $H^+:Na^+ > 1$ [15]. The results with rat liver and bovine heart mitochondria in [8–13] are consistent with either stoichiometry. Permeant anions stimulated Na^+ uptake by submitochondrial particles [9,13] but that does not necessarily indicate a stimulation of cation/proton exchange, especially in light of the observation that the same anions stimulated respira-

tion [13]. The stimulation of uptake may have been due to an increase in ΔpH as a result of the stimulation of H^+ uptake catalyzed by the electron transport chain. By measuring cation/proton exchange in the presence and absence of $\Delta\psi$ at constant ΔpH (table 1), we demonstrated that membrane depolarization, which under these conditions should have reduced the magnitude of the protonmotive force, did not decrease Na^+/H^+ or Li^+/H^+ exchange. The mitochondrial Na^+/H^+ antiporter must, therefore, catalyze electroneutral exchange.

The Na^+/H^+ antiporters of bacteria such as *E. coli* [15] and *Halobacterium halobium* [14] are electrogenic. The function of those systems appears to be conversion of the electrochemical proton gradient into an electrochemical sodium gradient, which is utilized for Na^+/solute symports [4,21]. The K^+/H^+ antiporter of *E. coli*, on the other hand, catalyzes electroneutral exchange of protons for most monovalent cations, including Na^+ and Li^+ [17]. We have postulated that the function of this system is regulation of intracellular pH by exchange of intracellular K^+ for external H^+ [17,18]. We were unable to observe K^+/H^+ exchange in submitochondrial particles, but this activity is negligible in rat liver mitochondria [13]. We would postulate that the Na^+/H^+ antiporter of mitochondria plays a role similar to the K^+/H^+ system of *E. coli*, namely preventing electrogenic proton pumping from creating a large pH differential between cytosol and matrix. The necessity for such exchange in regulation of ΔpH was first recognized in [11]. Perhaps this is a general phenomenon: electrogenic antiporters may function as transformers, switching one type of current into another, while the function of electroneutral antiporters may be related to regulatory phenomena.

Acknowledgements

We thank Miss Midori Hirano for technical assistance. This work was supported by grants to B. P. R. from the National Institute of General Medical Sciences of the National Institutes of Health (GM 21648) and the National Sciences Foundation (PCM 77-17652), to M. F. from the Ministry of Education, Science and Culture of Japan, and by a joint grant under the US-Japan Cooperative Science Progam sponsored by the National Science Foundation and the Japan Society for the Promotion of Science (R-BAM-0037).

References

[1] West, I. C. and Mitchell, P. (1974) Biochem. J. 144, 87–90.
[2] Brey, R. N., Beck, J. C. and Rosen, B. P. (1978) Biochem. Biophys. Res. Commun. 83, 1588–1594.
[3] Schuldiner, S. and Fishkes, H. (1978) Biochemistry 17, 706–711.
[4] Lanyi, J. K. and MacDonald, R. E. (1976) Biochemistry 15, 4608–4613.
[5] Niven, D. F. and MacLeod, R. A. (1978) J. Bacteriol. 134, 737–743.
[6] Krulwich, T. A., Mandel, K. G., Bornstein, R. F. and Guffanti, A. A. (1979) Biochem. Biophys. Res. Commun. 91, 58–62.
[7] Goto, K., Hirata, H. and Kagawa, Y. (1980) J. Bioenerg. Biomemb. in press.
[8] Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162.
[9] Cockrell, R. S. (1973) J. Biol. Chem. 248, 6828–6833.
[10] Brierly, G. P., Jurkowits, M. and Jung, D. W. (1978) Arch. Biochem. Biophys. 190, 181–192.
[11] Mitchell, P. (1961) Nature 191, 144–148.
[12] Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 9, 149–155.
[13] Douglas, M. G. and Cockrell, R. S. (1974) J. Biol. Chem. 249, 5464–5471.
[14] Lee, C. P. (1971) Biochemistry 10, 4375–5381.
[15] Beck, J. C. and Rosen, B. P. (1979) Arch. Biochem. Biophys. 194, 208–214.
[16] Brey, R. N. and Rosen, B. P. (1979) J. Biol. Chem. 254, 1957–1963.
[17] Brey, R. N., Rosen, B. P. and Sorensen, E. N. (1980) J. Biol. Chem. 255, 39–44.
[18] Plack, R. H. and Rosen, B. P. (1980) J. Biol. Chem. in press.
[19] Spitsberg, V. L., Morris, H. P. and Chan, S. H. P. (1979) Arch. Biochem. Biophys. 195, 136–144.
[20] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[21] Hasan, S. M. and Tsuchiya, T. (1977) Biochem. Biophys. Res. Commun. 76, 26–31.