

The Na⁺/H⁺ Exchanger Present in Trout Erythrocyte Membranes is Not Responsible for the Amiloride-insensitive Na⁺/Li⁺ Exchange Activity

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Abstract. The protein responsible for the Na⁺/Li⁺ exchange activity across the erythrocyte membrane has not been cloned or isolated. It has been suggested that a Na⁺/H⁺ exchanger could be responsible for the Na⁺/Li⁺ exchange activity across the erythrocyte membrane. Previously, we reported that in the trout erythrocyte, the Li⁺/H⁺ exchange activity (mediated by the Na⁺/H⁺ exchanger βNHE) and the Na⁺/Li⁺ exchange activity respond differently to cAMP, DMA (dimethyl-amiloride) and O₂. We concluded that the DMA insensitive Na⁺/Li⁺ exchange activity originates from a different protein. To further examine these findings, we measured Li⁺ efflux in fibroblasts expressing the βNHE as the only Na⁺/H⁺ exchanger. Moreover, the internal pH of these cells was monitored with a fluorescent probe. Our findings indicate that acidification of fibroblasts expressing the Na⁺/H⁺ exchanger βNHE, induces a Na⁺ stimulated Li⁺ efflux activity in trout erythrocytes. This exchange activity, however, is DMA sensitive and therefore differs from the DMA insensitive Na⁺/Li⁺ exchange activity. In these fibroblasts no significant DMA insensitive Na⁺/Li⁺ exchange activity was found. These results support the hypothesis that the trout erythrocyte Na⁺/Li⁺ exchange activity is not mediated by the Na⁺/H⁺ exchanger (βNHE) present in these membranes.

Key words: sodium-lithium countertransport — NHE — βNHE — Na⁺/H⁺ exchanger — trout

Introduction

Increased Na⁺/Li⁺ countertransport across the human erythrocyte membrane has been suggested to be a marker

for essential hypertension (Canessa et al., 1980; Rutherford, Thomas & Wilkinson, 1990; Delva et al., 1996), diabetic nephropathy (Krolewski et al., 1988; Mangili et al., 1988; Rutherford et al., 1992a; Rutherford, Thomas & Wilkinson, 1992c; Fujita et al., 1994) and hyperlipidaemia (Rutherford et al., 1992b). Possible links between the erythrocyte Na⁺/Li⁺ exchange and the Na⁺/H⁺ exchanger have been suggested (Rutherford et al., 1992a; Canessa, 1994). However, a genetic link between the Na⁺/H⁺ exchanger NHE-1 and an increased Na⁺/Li⁺ countertransport has been excluded (Lifton et al., 1991).

The hypothesis that the Na⁺/Li⁺ exchange activity across the erythrocyte membrane could be a mode of operation of the Na⁺/H⁺ exchanger is very tempting, because both processes are altered in hypertension and are influenced by insulin levels (Canessa, 1994). Busch et al. (1995) have clearly demonstrated that the NHE-1, the Na⁺/H⁺ exchanger thought to be present in the human erythrocyte, is able of exchanging Na⁺ for Li⁺, when expressed in *Xenopus* oocytes. So it is conceivable that the NHE-1 is responsible for the Na⁺/Li⁺ exchange activity as present in the human erythrocyte.

However, there are still several discrepancies in the behavior of the Na⁺/H⁺ exchange activity versus the Na⁺/Li⁺ exchange activity across the red cell membrane. One of the most important differences is their inhibition pattern: the Na⁺/H⁺ exchanger can be inhibited by amiloride and amiloride derivatives like 5-N,N-dimethyl-amiloride (DMA) and 5-N-ethyl-N-isopropyl-amiloride (EIPA), whereas the Na⁺/Li⁺ exchange activity is amiloride insensitive. This leaves the possibility that the erythrocyte Na⁺/Li⁺ exchange activity is due to the activity of a separate protein.

For the trout erythrocyte we recently reported that the Li⁺/H⁺ exchange activity can be stimulated by iso-

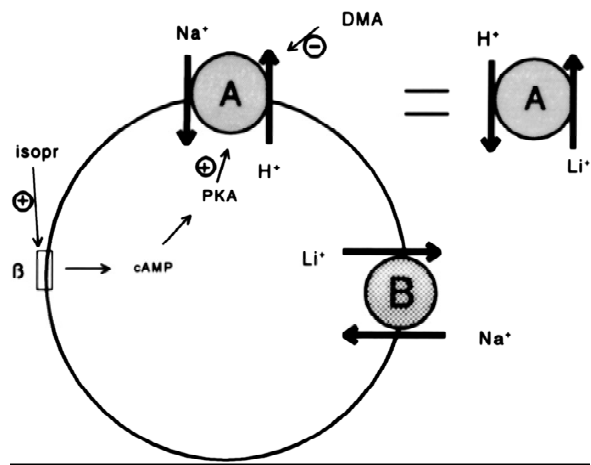


Fig. 1. Model of the trout erythrocyte Na^+/H^+ and Na^+/Li^+ exchange activities. (A) is the Na^+/H^+ exchanger β NHE, which, as indicated by the equal sign can also exchange Li^+ for H^+ . (B) is the protein responsible for the DMA insensitive Na^+/Li^+ exchange activity. isopr = isoproterenol, β = β adrenergic receptor, PKA = protein kinase A, DMA = 5-N,N-dimethyl-amiloride.

proterenol and inhibited by either DMA or O_2 (Borggreven et al., 1995) and therefore can be attributed to the trout erythrocyte Na^+/H^+ exchanger, β NHE (Motais, Garcia-Romeu & Borgese, 1987; Motais et al., 1990; Borgese et al., 1992; Guizouarn et al., 1993). The Na^+/Li^+ exchange activity in these membranes, however, was not influenced by either of these components (Borggreven et al., 1995). We therefore postulated the existence of two different Li^+ exchangers in this membrane.

This is summarized in a model (see Fig. 1). The Na^+/H^+ exchanger of the trout erythrocyte can exchange Li^+ for H^+ . The Li^+/H^+ activity is normally silent (Motais et al., 1990; Borgese et al., 1992; Guizouarn et al., 1993) and can be activated via the β -adrenergic receptor through cAMP. This Li^+/H^+ activity is DMA and O_2 sensitive, as can be expected from an exchange activity of the β NHE (Mori et al., 1991; Borgese et al., 1992; Guizouarn et al., 1993). The Na^+/Li^+ exchange activity, however, is active without stimulation and is not sensitive to isoproterenol, DMA or O_2 . So this model suggests that we are dealing with two different proteins, with different activation and inhibition pathways.

To examine whether the DMA-insensitive erythrocyte Na^+/Li^+ exchange is indeed not a mode of operation of the Na^+/H^+ exchanger present in these cells, we compared the exchange activities of human and trout erythrocytes with those of fibroblast cell lines expressing the Na^+/H^+ exchangers NHE-1 and β NHE (L'Allemain, Paris & Pouyssegur, 1984; Pouyssegur et al., 1984; Borgese et al., 1992).

In the fibroblasts no significant DMA-insensitive Na^+/Li^+ exchange activity was found indicating that the DMA-insensitive Na^+/Li^+ exchange activity present in

the trout erythrocyte can not be attributed to the Na^+/H^+ exchanger (β NHE) present in these membranes.

Materials and Methods

Li^+ EFFLUX MEASUREMENTS IN TROUT ERYTHROCYTES

The erythrocytes were washed and loaded with Li^+ as described earlier (Borggreven et al., 1995). The same methods as used for trout erythrocytes were used for human erythrocytes. The efflux temperature for the human erythrocytes, however, was 37°C instead of 22°C .

Li^+ EFFLUX MEASUREMENTS IN THE HAMSTER FIBROBLAST CELL LINES

In three different fibroblast cell lines the Li^+ efflux was measured. The first cell line 'the wild type' (CCL39) expressed the NHE-1 (present in the human erythrocyte) (L'Allemain et al., 1984; Pouyssegur et al., 1984). The second cell line (PS120) was mutated and did not contain the Na^+/H^+ exchanger anymore (L'Allemain et al., 1984). This PS120 cell line, lacking any Na^+/H^+ exchange activity, was used to overexpress the β NHE (Borgese et al., 1992). This cell line overexpressing the β NHE was called PS120- β NHE. Cells were maintained in Dulbecco's modified Eagle's medium (42430-025 GIBCO-BRL) supplemented with 15% fetal calf serum, 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 1 mM sodium pyruvate and 44 mM NaHCO_3 . The fibroblast cell lines were grown to 90% confluency, washed once with Li^+ -loading buffer (in mM): 130 LiCl , 5 KCl , 2 CaCl_2 , 1 MgCl_2 , 20 Tris-HCl ; pH 7.4 and incubated with Li^+ -loading buffer for 2 hr at 37°C . Cells were washed 8 times with NMG medium of 4°C (in mM): 130 N-methylglucamine, HCl, 5 KCl , 2 CaCl_2 , 1 MgCl_2 , 20 Tris-HCl ; pH 7.4). Then, the efflux was started by adding 6 ml of NMG-medium of 37°C . Immediately and after each min, a sample of 1 ml was taken and replaced by the same amount of fresh medium. After 5 min, 4 ml was replaced by either NMG medium or Na^+ medium (in mM): 130 NaCl , 5 KCl , 2 CaCl_2 , 1 MgCl_2 , 20 Tris-HCl ; pH 7.4. Thereafter, the previous sampling method was continued and either NMG medium or a mixture of 80% Na -medium and 20% NMG-medium was added. DMA was added to efflux media to a concentration of 0.1 mM. Data presented are corrected for dilution due to the sampling method. For example at $t = 2$ the concentration was: $0.2 [\text{Li}^+ t = 0] + 0.2 [\text{Li}^+ t = 1] + [\text{Li}^+ t = 2]$.

DETERMINATION OF Li^+

Li^+ concentrations were measured by atomic absorption spectrometry (Perkin Elmer, Model 4100). Li^+ standard solutions contained 0 to 80 μM LiCl .

pH MEASUREMENTS

Cells were grown on microscope glass cover slips, to a 90% confluency. They were treated in the same way as described above. Cells were incubated in Li^+ -loading buffer for 2 hr. The last 30 min BCECF (2',7'-bis(carboxyethyl)-5-(and 6)-carboxylfluorescein) was added to the medium. Thereafter the cells were washed in the Li^+ -loading medium without BCECF and placed in a perfusion cuvet containing Li^+ -loading buffer. Then the cells were perfused for 5 min with NMG medium

followed by a perfusion of 5 min with Na^+ medium. The fluorescence emission ratio at 525 nm was monitored as a measure of the average cytosolic H^+ concentration after excitation at 440 and 490 nm. For these experiments the spectrofluorophotometer Shimadzu RF-5000 was used. After each experiment the cells were calibrated at pH 6.0, 7.0 and 8.0 by perfusion with buffers containing $10 \mu\text{M}$ nigericin, a H^+ and K^+ ionophore.

ABBREVIATIONS

BCECF:	2',7'-bis(carboxyethyl-5(and 6)-carboxylfluorescein
CCL-39:	wild-type fibroblast cell line
DMA:	5-(N,N-dimethyl)-amiloride
EIPA:	5-N-ethyl-N-isopropyl-amiloride
NHE:	Na^+/H^+ exchanger
NHE1:	Na^+/H^+ exchanger type 1
β NHE:	trout erythrocyte Na^+/H^+ exchanger
NMG:	N-methylglucamine
PS120:	CCL-39 cell line lacking the NHE1
PS120- β NHE:	PS120 cell line expressing the β NHE

Results

To confirm our previous findings indicating that the trout erythrocyte DMA-insensitive Na^+/Li^+ exchange activity is not a mode of operation of the Na^+/H^+ exchanger present in these cells, we first measured the Na^+/Li^+ exchange activity in both trout and human erythrocytes, in the presence or absence of DMA. The human as well as the trout erythrocytes showed a Na^+ -stimulated Li^+ efflux (Fig. 2). The Na^+ -stimulated Li^+ efflux in human erythrocytes (Fig. 2a) is not or only hardly sensitive to DMA. The Na^+ -stimulated Li^+ exchange activity in the trout erythrocyte has previously been shown (Borggreven et al., 1995) to be completely DMA insensitive (Fig. 2b). Comparison of Fig. 2A and B reveals that the trout erythrocyte Na^+/Li^+ exchange activity is (even at 22°C) about ten times the human Na^+/Li^+ exchange activity (measured at 37°C).

To investigate whether the Na^+/H^+ exchangers present in these erythrocytes could be responsible for this DMA-insensitive Na^+/Li^+ exchange activity, hamster fibroblast cell lines were tested. The PS120 cell line lacking any Na^+/H^+ exchanger showed a very low Li^+ efflux rate (Fig. 3a). The Li^+ efflux rate was not significantly stimulated by Na^+ nor was it inhibited by DMA. The PS120- β NHE cell line, expressing the trout erythrocyte Na^+/H^+ exchanger showed a basal Li^+ efflux in the absence of Na^+ that could be inhibited by DMA (Fig. 3b). The rate of this basal efflux, which is an exchange of Li^+_{in} for H^+_{ex} (as confirmed in Fig. 4) started to decrease in the first 5 min (the curve bends). When Na^+ was added the Li^+ efflux rate significantly increased by about 50%. This Na^+ stimulated Li^+ efflux was completely inhibited by DMA. The CCL-39 cell line (Fig. 3c), expressing the NHE-1, generated the same efflux pattern as the PS120-

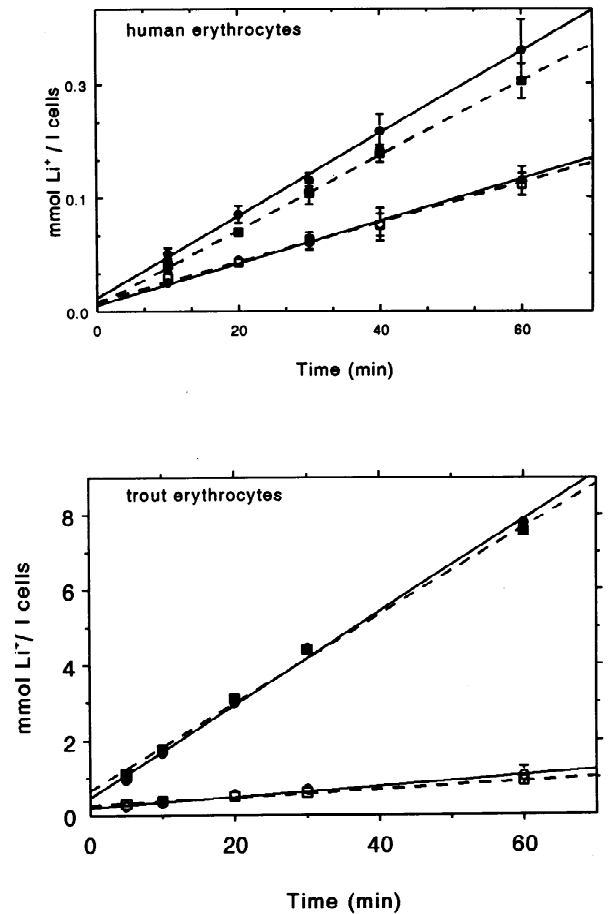


Fig. 2. The Na^+ -stimulated Li^+ efflux of human erythrocytes or trout erythrocytes. Li^+ -loaded erythrocytes were incubated in either a Na^+ -containing medium (closed symbols) or a N-methylglucamine-containing medium (open symbols). A continuous line represents the efflux in media without DMA, a broken line represents the efflux in medium containing 0.1 mM DMA. The data represent the mean and SE of 3 experiments. The data for trout erythrocytes were taken from Borggreven et al. (1995).

β NHE cell line: a basal Li^+ efflux that can be inhibited by DMA and a Na^+ -stimulated Li^+ efflux that is also completely DMA sensitive. To conclude: both Na^+/H^+ exchanger isoforms are capable of exchanging Na^+ for Li^+ , but only in a DMA-sensitive way. On the other hand, the erythrocyte Na^+/Li^+ exchange activity is DMA insensitive. These data confirm our previous findings that the erythrocyte DMA insensitive Na^+/Li^+ exchange activity is not mediated by the Na^+/H^+ exchanger present in these membranes.

Whereas the DMA insensitive Na^+/Li^+ exchange activity across the erythrocyte membrane is linear in time (Fig. 2), the DMA-sensitive Na^+/Li^+ exchange activity of fibroblasts is not (Fig. 3b and c). In fibroblasts, expressing either the NHE1 or the β NHE, the Li^+ efflux rate in the absence of Na^+ decreased with time, although the

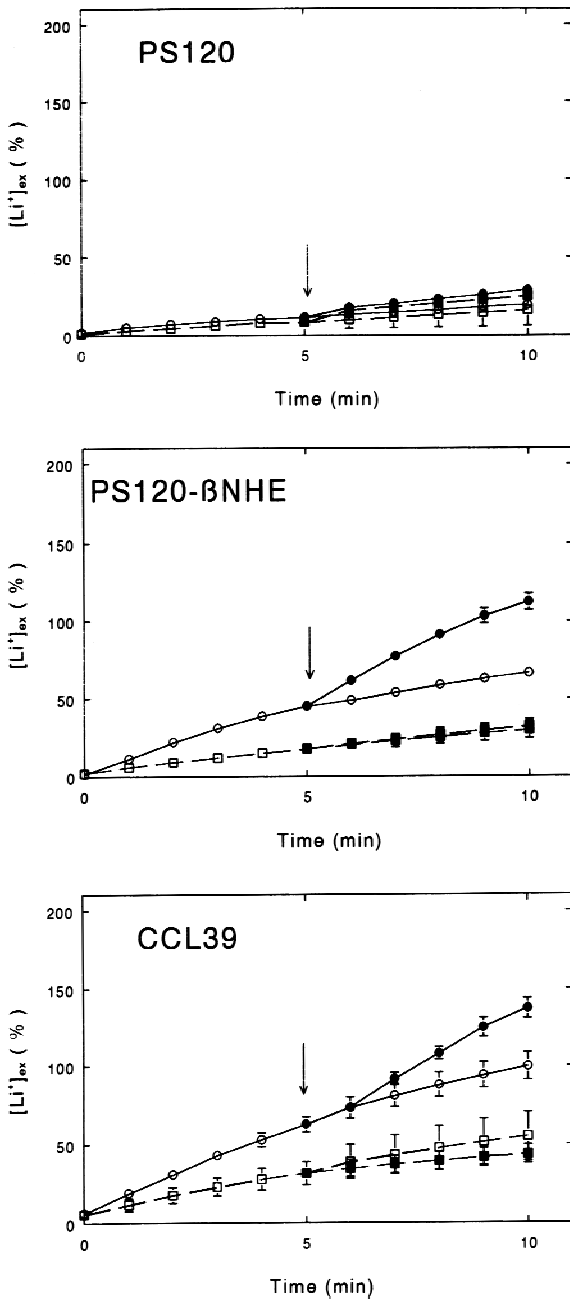


Fig. 3. The Na^+ -stimulated Li^+ efflux of different hamster fibroblasts cell lines. PS120, which lacks any Na^+/H^+ exchanger; PS120- β NHE, which contains the trout erythrocyte Na^+/H^+ exchanger β NHE and CCL39 which contains the NHE1, the Na^+/H^+ exchanger reported to be present in the human erythrocyte. Li^+ -loaded cells were incubated in either a Na^+ -containing medium (closed symbols) or a N-methylglucamine containing medium (open symbols). A continuous line represents the efflux in media without DMA, while a broken line represents the efflux in medium containing DMA. The arrow indicates the addition of Na^+ (filled symbols). The graphs were normalized for the Li^+ -efflux at $t = 10$ in NMG medium of the wild type, CCL39 cell line. Data represent the mean of 2 experiments. Each experiment was carried out in triplo. Error bars indicate SE.

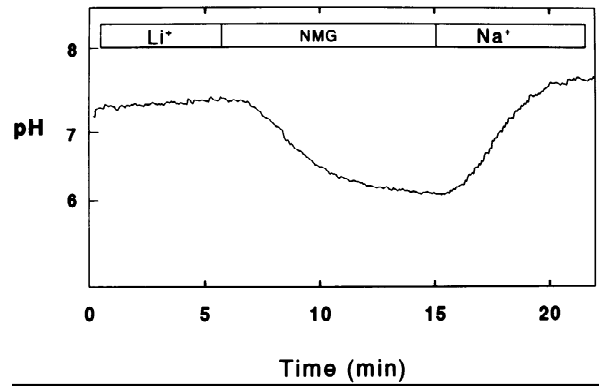


Fig. 4. The intracellular pH during an experiment as shown in Fig. 3b. The PS120- β NHE cells were loaded with Li^+ for 3 hr. During the last 30 min BCECF was added. The cells were washed with Li^+ -loading medium and placed in a perfusion cuvet. During the first time period the Li^+ -loaded cells were in the Li^+ -loading buffer, then, the medium was changed to Na^+ -free NMG medium, followed by a change to Na^+ -containing medium.

fibroblasts still contained sufficient Li^+ (60% of $[\text{Li}^+]_{in}$ at $t = 0$). The Li^+ efflux rate in both human or trout erythrocytes, however, was constant in time. One explanation for the difference might be that the DMA-sensitive Na^+/Li^+ exchange activity is influenced by proton fluxes. We therefore investigated whether fibroblasts expressing the β NHE or NHE1, became acidified during the experiments. We thus loaded these fibroblasts with BCECF in order to follow the intracellular pH during the Li^+ efflux experiments.

Figure 4 shows the intracellular pH of the PS120- β NHE cell line during an efflux experiment as indicated in Fig. 3b. When the cells were still in the Li^+ -loading buffer, the pH was neutral. When perfused with Na^+ -free medium, the pH dropped to 6.0. The rate of this drop in pH decreased in time, parallel with the decrease in time of the Li^+ efflux rate (Fig. 3b). The decrease in intracellular pH, probably, inhibited the exchange of Li^+_{in} for H^+_{ex} . Upon addition of Na^+ the pH raised to 7.0 again, resulting in a parallel increase in the Li^+ efflux rate (Fig. 3b). The recovery of the cells to neutral pH after efflux in Na^+ -free medium could be mimicked by the addition of Li^+ containing medium instead of Na^+ -containing medium (Fig. 5).

Discussion

Recently, we demonstrated that in Li^+ -loaded trout erythrocytes, a Li^+ for H^+ exchange can occur, after stimulation with isoproterenol. This Li^+ for H^+ exchange could be attributed to the trout erythrocyte Na^+/H^+ exchanger β NHE, because this exchanger was normally silent, could be stimulated by isoproterenol and then inhibited

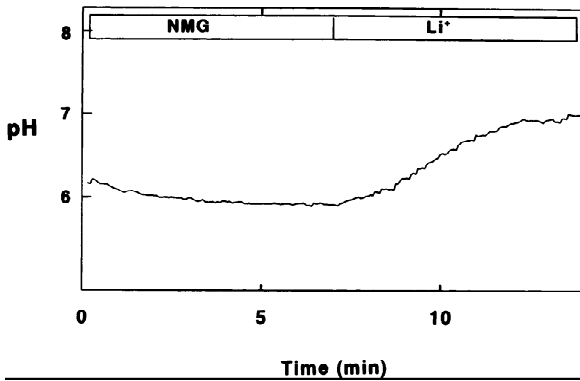


Fig. 5. The pH neutralizing effect of Na^+ can be mimicked by Li^+ . The intracellular pH during an experiment similar as described in Fig. 4, instead of addition of Na^+ , however, Li^+ was added. Li^+ and BCECF loaded PS120- β NHE cells were incubated in Na^+ -free NMG medium, followed by a change to Na^+ -free Li^+ -loaded buffer.

by DMA (Fig. 1) or O_2 (Borggreven et al., 1995). In addition, we found a Na^+/Li^+ exchanger activity present in these membranes, which is active in the absence of any stimulation and is not influenced by any of the components influencing the Na^+/H^+ exchanger. So we concluded that the Na^+/H^+ exchanger could not be responsible for the DMA-insensitive Na^+/Li^+ exchange activity as found in the trout erythrocyte membrane (Borggreven et al., 1995).

However, as shown in Fig. 1, the Na^+/H^+ exchanger should be capable of exchanging Na^+ for Li^+ . The summation of a Na^+ for H^+ exchange with a H^+ for Li^+ exchange, results in a Na^+ for Li^+ exchange. It is also very likely that the exchanger which can transport either of the ions can directly exchange Na^+ for Li^+ . Busch et al. (1995) already showed that the NHE-1, the Na^+/H^+ exchanger present in the human erythrocyte, can exchange Na^+ for Li^+ when expressed in *Xenopus* oocytes. In the present study, we confirm these findings for both the NHE-1 and the β NHE, the trout erythrocyte Na^+/H^+ exchanger. However, we show that the Na^+ stimulated Li^+ exchange, due to the NHE-1 or β NHE activity, is completely DMA sensitive. These results suggest as the model in Fig. 1 indicates, that the Na^+/H^+ exchanger is capable of exchanging Na^+ for Li^+ , but in a DMA-sensitive way. The Na^+/Li^+ exchange activity as found in both the human and trout erythrocyte, however is DMA insensitive. These results suggest that the Na^+/Li^+ exchange across the erythrocyte membrane is mediated by a different protein.

Another very strong argument, which points in the direction of two different proteins, is the fact that in trout erythrocytes the β NHE is quiescent, and becomes only active after stimulation with β -adrenergic agonists (Motaïs et al., 1990; Borgese et al., 1992; Guizouarn et al., 1993). The Na^+/Li^+ exchange activity, however, is always present in the trout erythrocyte. This DMA-

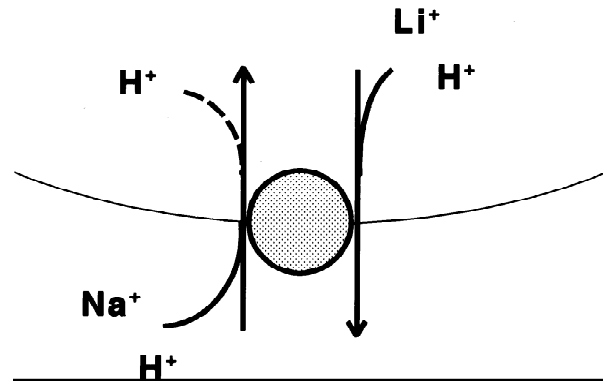


Fig. 6. A model of the exchange activities of the β NHE, the trout erythrocyte Na^+/H^+ exchanger. The Na^+/H^+ exchanger can exchange H^+ , Li^+ and Na^+ ions. The Li^+ concentration inside is high as compared to the external medium, while the cytosolic Na^+ concentration is low due to the Na^+/K^+ -ATPase activity. The dotted line represents the inhibition of Li^+ efflux in the presence of intracellular protons.

insensitive Na^+/Li^+ exchange activity remains the same, whether the β NHE is active or not (Borggreven et al., 1995).

To further investigate the kinetics of this DMA-sensitive Na^+/H^+ exchanger mediated Na^+/Li^+ exchange activity, we compared the proton fluxes (Figs. 4 and 5) with the Li^+ fluxes of the Li^+ -loaded fibroblasts expressing the trout erythrocyte Na^+/H^+ isotype (Fig. 3). We come to the following model (Fig. 6). If Li^+ loaded fibroblasts expressing the β NHE are placed in Na^+ - and Li^+ -free medium, the Li^+ gradient forces the Na^+/H^+ exchanger to exchange Li^+_{in} for H^+_{ex} . But due to this transport, the cellular activity and the fact that the pH regulation is hampered (because of the absence of extracellular Na^+ and HCO_3^-), the intracellular pH decreases. This pH gradient or the lower intracellular pH itself, inhibits the Li^+ efflux rate. When Na^+ is added, both Li^+ and H^+ can be transported against Na^+ . We postulate that at neutral intracellular pH, the rate-limiting step of Li^+ efflux is the binding of the Li^+ ion, but as soon as the intracellular pH decreases, the transport of the H^+ ion becomes rate limiting. This can be due to competition of Li^+ and H^+ for the intracellular binding site. But H^+ ions can not be transported, due to the absence of Na^+ . When Na^+ is added, β NHE is maximally activated due to the intracellular acidification. The Na^+/H^+ exchanger will exchange Na^+ for H^+ and the pH recovers. It appears as if there is a Na^+ stimulated Li^+ efflux, while in fact only the inhibition of Li^+ efflux due to the outward directed proton gradient is overruled.

The question, however, remains why there is no DMA-sensitive Na^+ stimulated Li^+ efflux in trout or human erythrocytes, while this activity is present in fibroblasts expressing the trout or human erythrocyte Na^+/H^+ exchanger. In the trout erythrocyte the Na^+/H^+ exchanger is silent and not sensitive to intracellular acidi-

fication (Motais et al., 1990; Borgese et al., 1992; Guizouarn et al., 1993; Borgese et al., 1994), which explains the absence of the DMA sensitive Na^+/Li^+ exchange activity. However, this does not explain the absence of a DMA-sensitive Na^+/Li^+ exchange activity in human erythrocytes. According to Fig. 6, the Na^+/H^+ exchanger mediated DMA-sensitive Na^+/Li^+ exchange is only apparent, when cells become acidified. This means that if there is no acidification during Li^+ efflux in Na^+ -free medium, the DMA-sensitive Na^+ -stimulated Li^+ efflux would not occur. This might be the case for the human erythrocytes.

To conclude: although the Na^+/H^+ exchanger present in both human and trout erythrocyte membranes is capable of exchanging Na^+ for Li^+ , the DMA-insensitive Na^+/Li^+ exchange activity as found in the erythrocyte can not be a mode of operation of the Na^+/H^+ exchangers NHE-1 (reported to be present in the human erythrocyte (Corry et al., 1993)) or β NHE (present in the trout erythrocyte). The possibility that, particularly in human, another NHE isotype is responsible for this exchange activity remains. In human erythrocytes, the evidence for the presence of NHE-1 is only based on immunohistochemistry (Corry et al., 1993), whereas the trout β NHE has been cloned from erythropoietic tissue (Borgese et al., 1992). We, therefore, also tested fibroblasts expressing the NHE-3, the isotype known to be most DMA insensitive (Counillon et al., 1993; Tse et al., 1993). But at a concentration of 10^{-4} M DMA (the concentration we use in all assays), still about 50% of the Na^+ -stimulated Li^+ efflux was inhibited (*data not shown*). The function of a DMA-insensitive Na^+/Li^+ exchange activity as present in the erythrocyte membrane becomes very obscure, if it is not mediated by a Na^+/H^+ exchanger. The fact that both human and trout erythrocyte possess this transport activity, directs to a preserved function. But why would a cell transport Li^+ for Na^+ or Na^+ for Na^+ ? Further investigations have to point out if we are dealing with a new NHE isotype or a completely different protein.

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