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Transport Pathways for Therapeutic Concentrations of Lithium in Rat Liver

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Abstract. Although both amiloride- and phloretin-sensitive Na^+/Li^+ exchange activities have been reported in mammalian red blood cells, it is still unclear whether or not the two are mediated by the same pathway. Also, little is known about the relative contribution of these transport mechanisms to the entry of therapeutic concentrations of Li^+ (0.2–2 mM) into cells other than erythrocytes. Here, we describe characteristics of these transport systems in rat isolated hepatocytes in suspension. Uptake of Li^+ by hepatocytes, preloaded with Na^+ and incubated in the presence of ouabain and bumetanide, comprised three components. (*a*) An amiloride-sensitive component, with apparent K_m 1.2 mM Li^+, V_{max} 40 μmol · (kg dry wt · min)$^{-1}$, showed increased activity at low intracellular pH. The relationship of this component to the concentration of intracellular H^+ was curvilinear suggesting a modifier role of $[H^+]_i$. This system persisted in Na^+-depleted cells, although with apparent K_m 3.8 mM. (*b*) A phloretin-sensitive component, with K_m 1.2 mM, V_{max} 21 μmol · (kg · min)$^{-1}$, was unaffected by pH but was inactive in Na^+-depleted cells. Phloretin inhibited Li^+ uptake and Na^+ efflux in parallel. (*c*) A residual uptake increased linearly with the external Li^+ concentration and represented an increasing proportion of the total uptake. The results strongly suggest that the amiloride-sensitive and the phloretin-sensitive Li^+ uptake in rat liver are mediated by two separate pathways which can be distinguished by their sensitivity to inhibitors and intracellular $[H^+]$.

Key words: Lithium therapeutic concentrations — Transport kinetics — Hepatocytes — Amiloride — Phloretin — Li/Na exchange — Na/H exchange

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Introduction

Lithium salts are widely used in the treatment of mania and are effective in reducing the recurrence of manic-depressive episodes (Johnson & Johnson, 1978). While its therapeutic activity is well established, very little is known of the transport mechanisms by which therapeutic or mildly toxic concentrations of Li^+ (0.2–2.0 mM and 2–3 mM, respectively) enter cells of the major organs. A substantial body of work with human erythrocytes has indicated a number of possible pathways for membrane transport of Li^+, but the relative importance of each has not been established. The emphasis on red cells appears to result from interest in the proposal that an increased transmembrane exchange of intracellular Li^+ for medium Na^+ may provide a diagnostic test of a propensity to contract essential hypertension (Canessa, Adragna & Solomon, 1980; Van Norren et al., 1998). Also, because of their availability, red blood cells are a convenient model to study different transport systems although, as non-nucleated cells, they are not a suitable tool for delineation of the molecular biological properties of transporters and other proteins.

In addition to the work with red blood cells, Li^+ transport has also been studied in plasma-membrane vesicles of some other cells (Kinsella & Aronson, 1981; Kahn et al., 1989; Zerbini et al., 1997). Overall, there is evidence that Li^+ can partially replace Na^+ in a number of transport systems. These include some Na^+ channels (Keynes & Swan, 1959), the coupled active transport of Na^+ and K^+ (Beaugé, 1978; Pandey et al., 1978; Wood, Elphick & Grahame-Smith, 1989), cotransport of $Na^+/K^+/Cl^-$ (Pandey et al., 1978) and $Na^+/CO_3^{2-}/HCO_3^+$ (Soleimani et al., 1991), amiloride-sensitive Na^+/H^+ exchange (here referred to as "Na(Li)/H exchange" or NHE) (Kinsella & Aronson, 1980, 1981; Busch, Burckhardt & Siffert, 1995) and a phloretin-sensitive Na^+/Li^+ exchange (referred to as "Na/Li exchange") (Sarkadi et

al., 1978; Duhm & Becker, 1979; Zerbini et al., 1997). The last two of these have attracted most attention, but there is continuing discussion as to whether the phloretin-sensitive Na^+/Li^+ exchange is a functional mode of the amiloride-sensitive pathway or is an independent entity (e.g., Kahn, 1987; Kahn et al., 1989; Busch, Burckhardt & Siffert, 1995; Chi, Mo & de Freitas, 1996; Van Norren et al., 1998).

Rat liver is a convenient model for studying transport activity by nucleated cells of a solid organ (Boyer, Graf & Meier, 1992). A well-established model for the study of ion movements in vitro, with either liver slices or freshly isolated hepatocytes, uses the net, metabolism-dependent uptake of K^+ and extrusion of Na^+ and Cl^- that occur during recovery from a period of cold preincubation (Elshove & van Rossum, 1963; McLean, 1963; Macknight, Pilgrim & Robinson, 1974; Mazet, Claret & Claret, 1974). To our knowledge, Li^+ transport (at its therapeutic concentrations) by these cells and under these conditions has not been studied previously. The work described here, concentrated on the two Na^+-Li^+ exchange mechanisms and it was felt desirable to simplify the experimental system by limiting the number of pathways that might contribute to Li^+ transport. Thus, ouabain and bumetanide were used throughout to inhibit Na^+/K^+-activated adenosine triphosphatase and $Na^+/K^+/Cl^-$ cotransport, respectively. Also, $Na^+/CO_3^{2-}/HCO_3^-$ cotransport was avoided by using HCO_3^--free medium. Further, the two Na^+/Li^+ exchange mechanisms are both considered to be of the exchange-diffusion type so that the movement of Li^+ should be modified by the transmembrane gradient of Na^+. Experiments were therefore carried out with cells that were either loaded with, or depleted of Na^+.

The results show that in isolated rat hepatocytes the uptake of Li^+ at therapeutically relevant concentrations, took place by two saturable systems that can be distinguished by their sensitivity to pH and inhibitors. Li^+ also entered cells by a residual leak, which was a linear function of the medium Li^+ concentration. Further experiments describe characteristics of Li^+ uptake which suggest the presence of independent amiloride- and phloretin-sensitive systems. Preliminary accounts of some of the results have been published in abstract form (Shahabi & van Rossum, 1998, 1999).

Materials and Methods

Reagents

The acetoxymethyl ester of 2′,7′-bis (2-carboxyethyl)-5(and 6)-carboxyfluorescein (BCECF-AM) was purchased from Molecular Probes (Eugene, OR) and LiCl from J.T. Baker Chemical, Phillipsburg, NJ. Ouabain, amiloride, bumetanide, phloretin, collagenase (type IV), nigericin and all other reagents were purchased from Sigma Chemical (St. Louis, MO).

Tissue Preparation

Male albino rats of a Sprague-Dawley strain (Zivic-Miller, Allison Park, PA) were fed *ad libitum* and used at 150–250 g body wt. The experimental protocol was approved by Temple University Animal Care and Use Committee, according to which the animals were anesthetized with pentobarbital (35 mg/kg, i.p.) prior to any further operation.

Hepatocytes were isolated according to Seglen (1976) as modified by Starke & Farber (1985). Briefly, the liver of an anesthetized rat was first perfused with a Ca^{2+}-free medium containing (mM): 137 NaCl, 4.7 KCl, 2.0 Na^+ phosphate, 0.6 $MgSO_4$, 1.0 ethyleneglycol tetraacetic acid (EGTA) and 10 tris-hydroxymethylaminoacetic acid (Tris) · HCl, pH 7.4. This was followed by perfusion with collagenase (130 U/ml, Type IV) in Ca^{2+} medium (mM): 126 NaCl, 6.7 KCl, 4.8 $CaCl_2$, 40 Tris · HCl, pH 7.6. The capsule was stripped away and the cells separated by gentle agitation in a medium containing either Na^+ or choline as the major cation (*see below*). Cells were recovered by settling under gravity and washed with the same medium. Cell viability, as determined by exclusion of trypan blue, was 85–90% immediately after isolation and fell by less than 10% during subsequent experimental incubation.

Li^+ Uptake by Isolated Hepatocytes

The basic experimental medium used for hepatocytes was an isoosmotic, Tris- and phosphate-buffered Ringer medium (Na^+-medium) containing (mM): 140 NaCl, 5 KCl, 2.0 Na^+ phosphate, 1.0 $MgSO_4$, 1.3 $CaCl_2$, 10 Tris · HCl (pH 7.4). Ouabain (1 mM) (Farber et al., 1989) and bumetanide (100 μM) were added to all incubation media to inhibit the ATPase-dependent Na^+ and K^+ exchange and Na^+-K^+-Cl^- cotransport, respectively (Canessa et al., 1982; Dunham & Senyk, 1977; Ehrlich & Diamond, 1979). Lithium, amiloride and phloretin were added as indicated in Results. A Na^+-free medium was prepared by substituting 140 mM choline for Na^+ and 2 mM K^+ phosphate for the Na^+ salt (choline-medium); varying the substitution with choline produced media of intermediate Li^+ or Na^+ concentrations.

Experiments with Na^+-Preloaded Cells

Hepatocytes were washed with Na^+-medium and then preloaded with Na^+ by incubation at 1°C for 30 min in the same medium at pH values stated in Results. Intracellular pH was modified in two ways for different series of experiments (as noted in Results): (i) all cells were loaded with Na^+ at pH 7.4 followed by equilibration in media at pH 7.4 or 6.5 for 10 min at 37°C in oxygenated choline-medium; (ii) batches of cells were loaded with Na^+ at 1°C at different pH. External Na^+ was then removed by washing the cells three times with ice-cold, choline-medium. Lithium uptake was initiated by the addition of LiCl to the cell suspensions to give the desired final concentrations (*see* Results). Samples of the suspensions were taken at intervals, transferred to tared microcentrifuge tubes and immediately centrifuged at 12,000 × *g* for 5 sec. The supernatants were removed by aspiration and the cell pellets dried at 110°C overnight.

Experiments with Na^+-Depleted Cells

Hepatocytes were prepared and washed three times with ice-cold choline-medium to remove residual extracellular Na^+ and then depleted of

intracellular Na^+ by incubation in the same medium for 30 min at 1°C. The suspensions were then centrifuged and the cell pellets were kept on ice until used (1–10 min). To study the fluxes of Li^+ at low concentrations of Na^+, cells were first depleted of Na^+, as above, and then equilibrated at 1°C in media in which different concentrations of Na^+ and Li^+ replaced equimolar quantities of choline. For the experimental incubation, the cell pellets were resuspended in oxygenated choline-medium, containing different concentrations of Na^+ and Li^+ together with ouabain, bumetanide (*as above*) and amiloride and/or phloretin at concentrations required by the experiments. Incubation was at 37°C in a shaking water bath. Samples were taken at intervals, centrifuged and dried, as above.

Intracellular pH Measurements

Cytosolic pH was measured by dual-wavelength excitation fluorescence of BCECF (Thomas et al., 1979; Tsien, 1989). Isolated hepatocytes were loaded with BCECF-AM (5–10 μM) for 30 min in oxygenated choline or Na^+-medium at 1°C. Cells were then washed briefly with ice-cold medium and incubated in oxygenated choline-medium of different pH for 10 min at 37°C, in a shaking water bath. Cell samples (0.5 ml) were transferred to a cuvette in the fluorimeter (Perkin-Elmer, model 203). Fluorescence emission at 530 nm was monitored upon excitation at 500 nm (pH-dependent wave length) with intermittent measurements at 440 nm (pH-independent wave length). Intracellular pH was determined by comparing the ratio of fluorescence emitted upon excitation at the two wavelengths (500/440) with a calibration curve. The calibration was derived from cells incubated in high-K^+ modifications of the Tris-Ringer (120 mM K^+ replacing Na^+) at five different pH values in the presence of nigericin (1 μM) (Thomas et al., 1979).

Analytical Methods

Cell pellets were weighed after drying overnight at 110°C to determine the dry weight (dw). Ions were extracted from the dried samples with 0.1 N HNO_3 (Little, 1964). Na^+ and Li^+ contents were measured by flame-photometry (Perkin-Elmer, model 2380, Atomic Absorption Spectrometer, used in the emission mode).

Expression of Results

The ionic contents of the cells are expressed as $\text{mmol} \cdot (\text{kg dw})^{-1}$. To determine the rate of net uptake of Li^+, the slope of the regression line of net Li^+ uptake *vs.* time was calculated with the least squares method and expressed as $\mu\text{mol}/(\text{kg dw} \cdot \text{min})^{-1}$. The kinetic constants, K_i, K_m and V_{max}, were determined either by fitting the Michaelis-Menten equation to the experimental points by an iterative procedure, or by least-squares, linear-regression analysis of the Lineweaver-Burk plot. Each experiment was repeated at least five times with cells from different rats. Values in tables and figures are expressed as mean ± SEM (number of observations). Tests for statistical significance were done by "Student's" *t* test with $P \leq 0.05$ considered to be significant.

Results

Experiments were performed both with cells loaded with, or depleted of Na^+ in order to determine the extent to which Li^+ movements were dependent on exchange for Na^+.

Li^+ Uptake by Na^+-Preloaded Hepatocytes

Experiments were done with freshly isolated hepatocytes in suspension. To simplify interpretation of the results, ouabain and bumetanide were used to inhibit Na^+ and Li^+ movements driven directly or indirectly by Na^+-K^+ coupled active transport or $Na^+/K^+/Cl^-$ cotransport. The cells were preloaded with Na^+ by pre-incubation in Na^+-medium at pH 7.4 (unless otherwise stated) for 30 min at 1°C; net fluxes of Na^+ and Li^+ at 37°C were then initiated by transferring cells to Na^+-free, choline-medium containing varying concentrations of Li^+ (*see* Materials and Methods). Preliminary experiments suggested that Li^+ uptake at a medium concentration of 0.2 mM was usually linear for the first 10 min. Samples were routinely taken for analysis at 3, 6 and 10 min and the earliest linear part of the curve was used to represent the initial flux rate.

Dependence on Li^+ Concentration

The dependence of the initial rate of Li^+ uptake on medium concentration of this cation suggested the presence of a high-affinity, saturating mechanism superimposed on a second uptake that showed no indication of saturating at the Li^+ concentrations studied (Fig. 1, inset). Two agents known to inhibit uptake of Li^+ by red cells (e.g., Pandey et al., 1978), amiloride (100 μM) and phloretin (100 μM), each reduced uptake into the hepatocytes. When the two inhibitors were added simultaneously, Li^+ uptake was reduced to a linear portion which ran parallel to the total uptake at 2 to 3 mM medium Li^+ (Fig. 1, inset). Subtraction of the linear uptake from the total uptake and from that in the presence of each of the two inhibitors revealed components each of which was fitted to the Michaelis-Menten equation (Fig. 1, main figure). The values of the apparent K_m of the amiloride- and phloretin-sensitive fractions of uptake are both close to 1 mM (Table 1); nevertheless the V_{max} of the former is twice as great as the phloretin-sensitive fraction. Further, the effects of the two inhibitors are closely additive. The concentration of 100 μM used for each inhibitor gave the maximum effect. This is seen for phloretin in Fig. 2. With respect to amiloride, 100 μM is some 30 times its IC_{50} of 3 μM against both the common isoform, NHE-1, of Na^+/H^+ exchanger and NHE-2 (Noël & Pouysségur, 1995); further, in a direct comparison we found that 1.0 mM and 100 μM amiloride reduced Li^+ uptake by 42 and 37%, respectively. Since both inhibitors were used at, or close to, their maximally effective concentrations, the additivity of their effects strongly suggests the presence of two separate transport systems.

The residual, linear portion of the uptake curve represented a substantial and increasing proportion of the total uptake as the Li^+ concentration in the medium in-

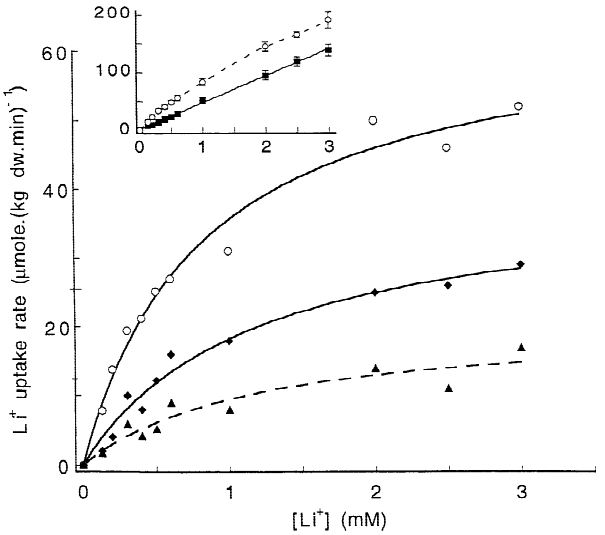

Fig. 1. Effects of amiloride and phloretin on the saturable components of Li^+ uptake by isolated hepatocytes preloaded with Na^+. After isolation, the cells were loaded with Na^+ by preincubation for 30 min at 1°C, then washed twice in Na^+-free, choline-medium and incubated in this medium for 10 min at 37°C with the concentrations of Li^+ indicated on the abscissa. Ouabain (1 mM) and bumetanide (100 μM) were present in all media at 37°C. The figure shows the net uptake in the absence (○) or presence of 100 μM amiloride (▲) or 100 μM phloretin (◆), after subtraction of the uptake resistant to both these agents. Lines were drawn by fitting the Michaelis-Menten equation to the points. Inset. Total uptake of Li^+ (○) and uptake persisting in the presence of amiloride *plus* phloretin (■). Points are mean ± SEM (n = 7). Units on the main Fig. also apply to the Inset.

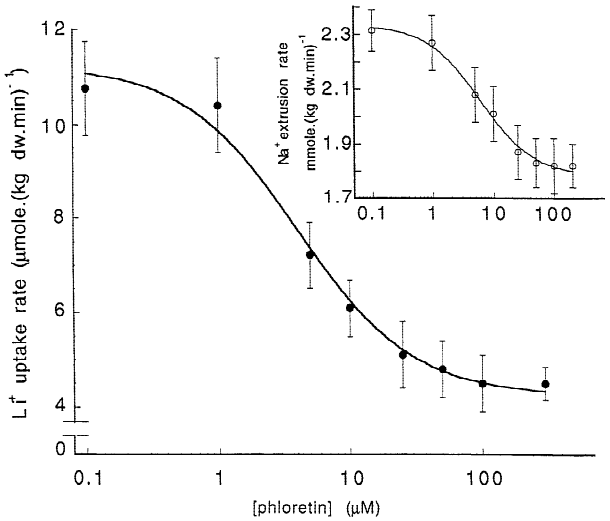

Fig. 2. Concentration-dependence of the effect of phloretin on rate of Li^+ uptake; (inset) rate of Na^+ loss. Hepatocytes were preloaded with Na^+ at 1°C and then incubated for 10 min at 37°C in the presence of 0.2 mM Li^+ and the concentrations of phloretin shown on the abscissa. Ouabain (1 mM), amiloride (100 μM) and bumetanide (100 μM) were present throughout. Values are mean ± SEM (n = 13). Units on the abscissa of the main Fig. also apply to the Inset.

Table 1. Apparent kinetic constants of saturable components of Li^+ uptake by isolated rat hepatocytes

Component	Apparent K_m[a] (mM)	V_{max}[a] mmoles · (kg dry wt · min)$^{-1}$
Total	0.83 ± 0.11	65.0 ± 3.6
Amiloride-sensitive	1.17 ± 0.21	39.5 ± 3.1
Phloretin-sensitive	1.19 ± 0.48	20.7 ± 3.7

[a] Values are from the experiments of Fig. 1. They were determined by fitting the Michaelis-Menten equation to the experimental points.

creased. Thus, at 0.2 mM Li^+ the residual uptake represented 24% of the total, rising to 63% at 0.6 mM and 73% at 3 mM (Fig. 1, inset).

Inhibition of Li^+ Uptake and Na^+ Extrusion by Phloretin

To study the characteristics of the phloretin-sensitive system in isolation, the initial rate of net uptake of 0.2 mM Li^+ was studied in the presence of ouabain, bumetanide and amiloride. The last of these was used in preference to its more specific analogues e.g., 5-(N,N-dimethyl) amiloride (Kleyman & Cragoe, 1988) to inhibit more generally the contribution of Na(Li)/H exchange as well as fluxes through certain Na^+ channels (Garty & Benos, 1988). The maximally inhibiting concentration of 100 μM phloretin reduced the rate of Li^+ uptake by more than 50%, from 11.0 ± 0.9 to 4.5 ± 0.4 μmole · (kg dw · min)$^{-1}$ ($P < 0.0002$, n = 13) (Fig. 2). Half-maximal inhibition was given by 4.04 ± 0.78 μM phloretin.

The effects of phloretin concentration on the net efflux of Na^+ showed similarities to its effects on Li^+ uptake. The concentration for half-maximal inhibition was 6.01 ± 0.88 μM phloretin and the maximum effect was at approximately 100 μM (Fig. 2, inset). However, the efflux of Na^+ was maximally reduced from 2.33 ± 0.07 to 1.82 ± 0.08 mmol · (kg dw · min)$^{-1}$ ($P < 0.001$), i.e., a decrease of 510 μmol · (kg dw · min)$^{-1}$ which greatly exceeded the reduction of 6.5 μmol in the uptake of Li^+.

Effect of Cell pH on Li^+ Uptake Mechanisms

Evidence from red blood cells (e.g., Ehrlich & Diamond, 1979) and our finding of a partial inhibition of Li^+ uptake by amiloride (Fig. 1) indicated a role for a Na^+/H^+ exchanger in the Li^+ and Na^+ transport. We therefore compared the uptake of 0.2 mM Li^+ by isolated hepatocytes preloaded with Na^+ and equilibrated at pH 6.5 and 7.4 (procedure i, *see* Materials and Methods). The intracellular pH, measured with BCECF (*see* Materials and

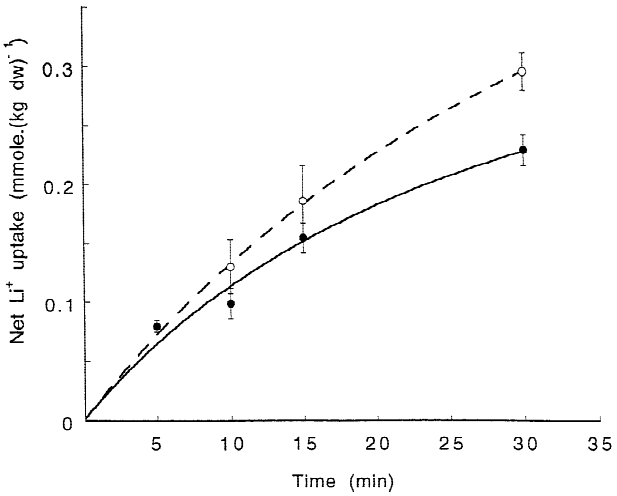

Fig. 3. Effect of pH on the time course of net uptake of Li^+ by hepatocytes. The cells were preloaded with Na^+ at 1°C, washed free of external Na^+ and equilibrated for 10 min at 37°C in choline-medium at pH 7.4 (●) or 6.5 (○). Uptake was then initiated by addition of 0.2 mM Li^+. Ouabain (1 mM) and bumetanide (100 μM) were present throughout. Intracellular pH was determined to be 7.2 (medium pH 7.4) and 6.6 (medium pH 6.5) by dual-wavelength excitation fluorescence of BCECF. Points are mean ± SEM (n = 12).

Methods), was estimated to be 6.63 ± 0.05 and 7.20 ± 0.03, respectively, after the period of equilibration. At this point, Li^+ was added and its uptake at 37°C determined at intervals. After 30 min, uptake was 25% greater in the more acidic medium (Fig. 3). In these experiments, the incubation media during uptake of Li^+ had the same pH values as those in which the cells were equilibrated (i.e., pH 6.5 or 7.4), suggesting that the stimulation of Li^+ uptake was mainly due to the intracellular acidification rather than to the extracellular/intracellular pH gradient.

At both pH 7.4 and pH 6.5 the influx of Li^+ was partially inhibited by 100 μM amiloride, indicating involvement of NHE in the transport (Fig. 4*a* and *b*). A further reduction was seen in the additional presence of 100 μM phloretin. Whereas inhibition by amiloride was significantly greater at pH 6.5 than at pH 7.4, the extra inhibition caused by the further addition of phloretin differed little between the two values of medium pH. Also, the residual, presumably diffusive uptake of Li^+ in the simultaneous presence of both inhibitors was virtually unaffected by the change of pH.

Further information on the pH-dependence of Li^+ uptake was obtained by measuring the uptake rate at 0.8 mM Li^+ over the range, pH 5.5–7.4. In these experiments, the cells were preloaded with Na^+ and H^+ according to procedure ii (*see* Materials and Methods). Lithium uptake by the cells was measured in choline-medium at 37°C (pH 7.4) for up to 10 min with and without amiloride (1 mM) or phloretin (100 μM). The intracellular pH for each batch of cells was determined (*see* Materials and

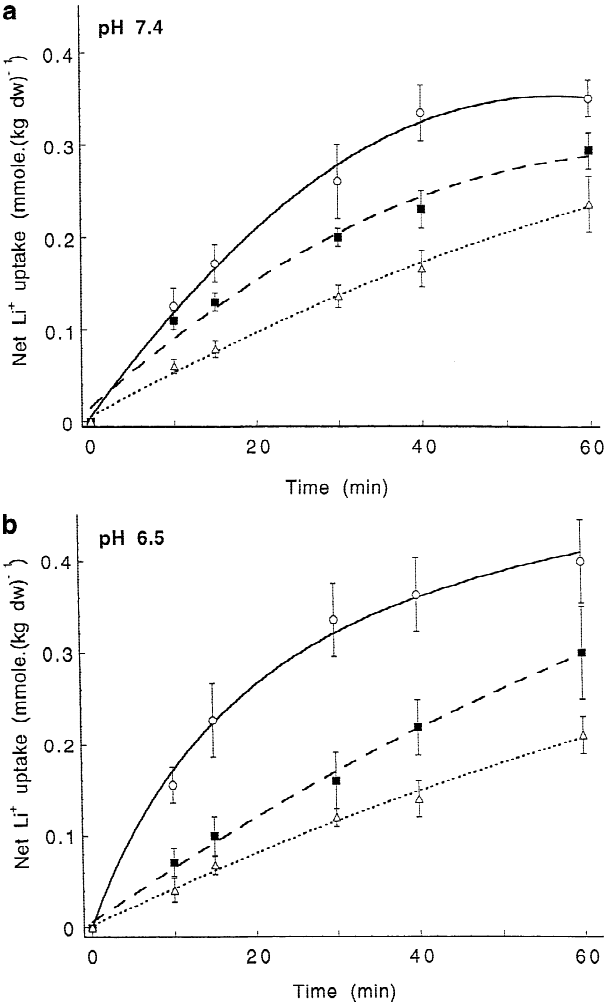

Fig. 4. Effects of amiloride and phloretin on the time course of Li^+ uptake by hepatocytes at (*a*) medium pH 7.4 and (*b*) medium pH 6.5. The cells were preloaded with Na^+ at 1°C and then divided into three batches for equilibration for 10 min at 37°C in the media of different pH in the absence (○) or presence of 0.1 mM amiloride alone (■) or together with 0.1 mM phloretin (△). Ouabain and bumetanide were present in all media. Lithium uptake was initiated by addition of 0.2 mM at time 0. Values are mean ± SEM (n = 9).

Methods) after the preloading phase. Total Li^+ uptake was dependent on intracellular pH and was particularly stimulated at pH less than 6.5 (Fig. 5). Whereas the pH-sensitive uptake was completely inhibited by amiloride, the phloretin-sensitive uptake was little affected by pH, i.e., the difference between the control uptake and that in the presence of phloretin remained approximately constant over the whole range tested (Fig. 5). These results confirm and extend the results from the time-course experiments (Figs. 3 and 4). Furthermore, the relation of the amiloride-sensitive Li^+ uptake to the intracellular concentration of H^+ was curvilinear in a manner characteristic of greater than first-order kinetics (Fig. 5, inset). A similar phenomenon, relating Na^+ uptake to $[H^+]_i$ was

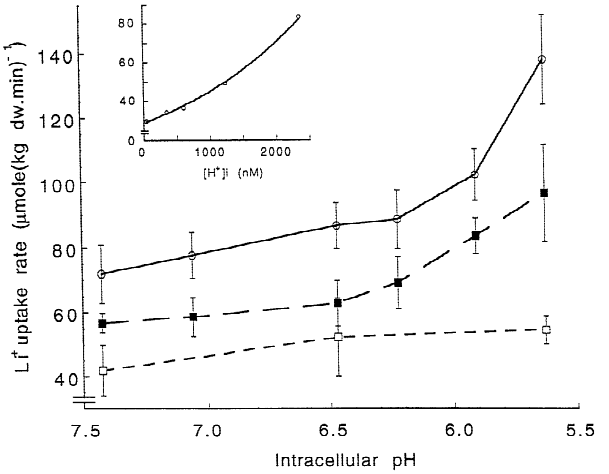

Fig. 5. Effects of pH, amiloride and phloretin on the initial rate of Li^+ uptake in Na^+-preloaded cells. The cells were preloaded with Na^+ and H^+ at 1°C for 30 min (method ii for pH adjustment, *see* Materials and Methods) and Li^+ uptake (0.8 mM) was measured for up to 9 min at 37°C in choline medium (pH 7.4) in the absence (○) or presence of 1 mM amiloride alone (□) or 100 μM phloretin alone (■). Ouabain and bumetanide were present in all media. Values are mean ± SEM ($n = 5$). Inset. Relation of the rate of amiloride-sensitive Li^+ uptake to the intracellular concentration of protons, derived from the main figure. Uptake values are the differences between the total uptake and the uptake continuing in the presence of amiloride; values on the amiloride line required for the subtraction at pH 7.1, 6.3 and 5.9 were obtained by interpolation. The plot shows the values between pH 7.1 and 5.5 only. The uptake curve was fitted to the experimental points by a second-order polynomial function. Units on the ordinate of the main figure apply also to the Inset.

also observed in renal microvillus membrane vesicles (Aronson, Nee & Suhm, 1982) where it was suggested to be due to an allosteric activation of the amiloride-sensitive NHE by protons.

Li^+ Uptake by Na^+-Depleted Hepatocytes

It has been proposed that both the amiloride- and phloretin-sensitive mechanisms for Li^+ uptake into red cells involve exchange of entering Li^+ for an efflux of Na^+ or, in the former case, possibly amiloride-sensitive efflux of H^+. The experiments described so far were all done in the presence of a large Na^+ gradient, intracellular greater than extracellular, which would be expected to facilitate Li^+ entry. We therefore examined the effects of reducing intracellular Na^+ on Li^+ uptake patterns. Hepatocytes were isolated and washed in the Na^+-free, choline medium. At this point, cells retained approximately 14.9 ± 1.3 mmole $Na \cdot (kg\ dw)^{-1}$; during the subsequent experimental incubation the content decreased further to 6.8 ± 0.7 $mmol \cdot (kg\ dw)^{-1}$.

Effect of Internal [Na^+] on Li^+ Influx

The effect of the intracellular Na^+ concentration on Li^+ influx was determined with hepatocytes that were depleted of Na^+, as described in the preceding paragraph. The intracellular Na^+ concentration was then adjusted by incubation for 30 min at 1°C in media in which 0, 70 and 140 mM Na^+ replaced the equivalent amount of choline. The cells were washed in Na^+-free, choline medium, leaving the intracellular Na^+ contents shown in Table 2 ('Initial' content). The cells were then warmed to 37°C in the choline-medium and uptake of Li^+ was initiated by addition of Li^+ over a range of medium concentrations (0.2–2.0 mM). The uptake was measured for up to 10 min. During this period, the Na^+ contents declined further (Table 2). Reducing the initial intracellular Na^+ content from 121 to 73 $mmole \cdot (kg\ dw)^{-1}$ had little effect on the initial rate of Li^+ uptake but the further reduction to 15 $mmol \cdot kg^{-1}$ caused an overall inhibition of 20–25% at all external concentrations of Li^+. This result suggests that the efflux of Na^+ exerts a trans-stimulation on Li^+ uptake, making the binding sites more readily available to the external Li^+.

Effects of pH and Inhibitors on Li^+ Influx

To investigate further the carrier systems involved in Li^+ uptake by the Na^+-depleted cells, the effects of pH and the two inhibitors, amiloride and phloretin, were studied at a medium concentration of 0.2 mM Li^+. The time course of Li^+ entry indicated an approach towards a steady-state content of Li^+ after 50 min (Fig. 6). In contrast to the case of Na^+-loaded cells (Fig. 3), the rate of Li^+ entry did not differ significantly between pH 6.5 and 7.4. The uptake at either pH was reduced by 50% in the presence of 100 μM amiloride (Fig. 6) but, in a further contrast to the Na^+-loaded cells, the addition of phloretin together with amiloride had no further effect on the uptake. We conclude that facilitated uptake of Li^+ by the Na^+-depleted cells involved only the amiloride-sensitive Na^+/H^+ exchange system, the phloretin-sensitive mechanism being abolished when the cells were depleted of Na^+. Also consistent with this conclusion is the similarity of the 25% reduction of Li^+ uptake caused by Na^+-depletion to the fraction of total uptake inhibited by phloretin in Na^+-loaded cells (*cf.* Table 2 and Fig. 1).

Because only the NHE-dependent, Na(Li)/H mechanism for facilitated Li^+ uptake appeared to be functional, the Na^+-depleted cells offered a means to study the kinetics of influx by this mechanism. A double-reciprocal plot of the concentration-dependence of Li^+ uptake (Fig. 7, inset) leads to an estimated apparent K_m of 3.78 mM Li^+.

Inhibitory Interactions Between Li^+ and Na^+

Two series of experiments were done to obtain further information on the mutual interaction between Na^+ and Li^+ in the Na^+-depleted cells, i.e., when uptake was due

Table 2. Effect of intracellular Na^+ and external Li^+ on the uptake of Li^+ by hepatocytes in suspension

Intracellular $[Na^+]^a$ mmol · (kg dw)$^{-1}$		Medium $[Li^+]$ (mM)				
		0.2	0.5	1.0	2.0	*n*
Initial	Final	Initial rate of Li^+ uptake μmol · (kg dw · min)$^{-1}$ [b]				
14.9 ± 1.3	6.8 ± 0.7	15 ± 1	31 ± 5	87 ± 10	149 ± 14	17
72.9 ± 3.5	49.5 ± 6.9	20 ± 1[c]	40 ± 4	98 ± 6[c]	189 ± 14[c]	6
120.5 ± 6.1	75.6 ± 7.4	22 ± 1[c]	42 ± 1[c]	113 ± 12[c]	220 ± 12[c]	17

[a] Intracellular Na^+ was modified by leaching the cells during 30 min preincubation at 1°C in medium containing 0, 70 or 140 mM Na^+ (with choline to maintain osmolarity), followed by washing twice in Na^+-free, choline-medium. They were then reincubated in choline-medium containing different concentrations of Li^+, for 10 min at 37°C. Ouabain and bumetanide were present throughout. Na^+ content of the cells was determined before (initial) and after (final) the 10 min incubation with Li^+.

[b] Values are the mean ± SEM of the rate of Li^+ uptake during the first 10 min after its addition to the incubation medium.

[c] $P \leq 0.01$ when compared to value at lowest initial Na^+ content.

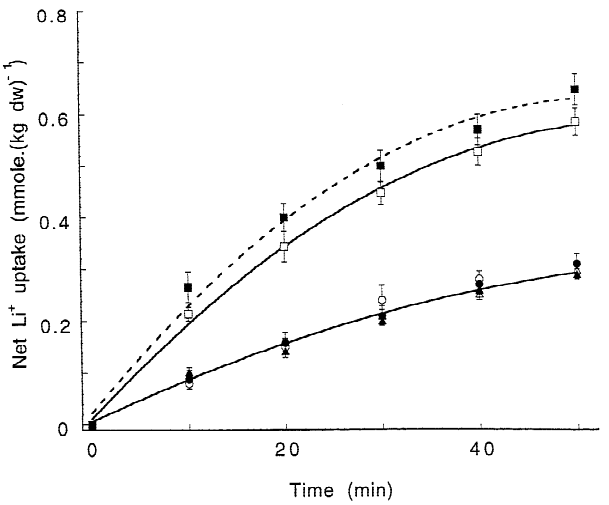

Fig. 6. Effects of pH, amiloride and phloretin on the time-course of Li^+ uptake by Na^+-depleted hepatocytes. Cells were depleted of Na^+ by isolation and pre-incubation (30 min at 1°C) in Na^+-free, choline medium. Batches were then equilibrated with choline media at pH 7.4 (closed symbols) or 6.5 (open symbols) for 10 min at 37°C, in the presence of ouabain and bumetanide (squares). Amiloride (100 μM) (circles) and phloretin (100 μM) (triangles) were added as indicated in methods. All results with amiloride and amiloride *plus* phloretin at either pH followed a closely similar course; the line shown has been fitted to the points with amiloride at pH 6.5. Values are mean ± SEM ($n = 5$).

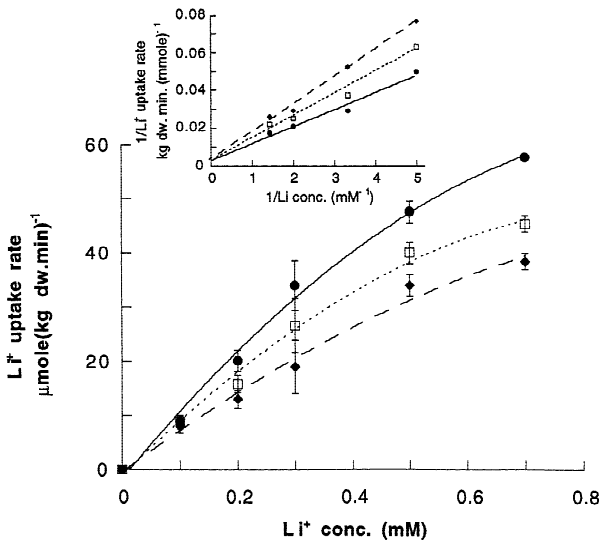

Fig. 7. Effect of external Na^+ on the uptake of Li^+ by Na^+-depleted hepatocytes. Cells were depleted of Na^+ as described in this figure and Li^+ uptake was measured at 37°C for up to 10 min in choline medium (●) or in choline medium in which 20 (□) or 40 mM (♦) choline was substituted for an equal amount of Na^+. Ouabain and bumetanide were present throughout. Inset shows the double-reciprocal plot of the results. Values are mean ± SEM ($n = 5$).

to Na(Li)/H mediated by the NHE exchange system. First, replacement of 20 and 40 mM medium choline by 20 and 40 mM Na^+, respectively, significantly inhibited entry of Li^+ into the Na^+-depleted cells (Fig. 7). The inhibition by Na^+ appeared to be competitive with an apparent K_i of 55.5 mM Na^+ ($n = 6$), as determined from the slope and intersection of linear-regression analysis of the Lineweaver-Burk plot (Fig. 7, inset). The competitive nature of this inhibition suggests that, when inwardly transported by the NHE mechanism, Li^+ interacts with the Na^+-binding site of the transporter and does so with greater affinity than Na^+.

By contrast, the expected trans-stimulation of Na^+ efflux by external Li^+ was not observed. Instead, low concentrations of external Li^+ inhibited Na^+ efflux from the depleted cells (Fig. 8). The inhibition was half maximal at 0.1 mM Li^+ and maximal at 0.3 mM. The rate of Na^+ efflux was reduced from 0.75 ± 0.06 mmol · (kg dw · min)$^{-1}$ in the absence of Li^+ to 0.45 ± 0.04 at 0.3 mM Li^+. The absence of a trans-stimulation by external Li^+ on the efflux of Na^+ in this case, presumably resulted from the internal Na^+ concentration in the Na^+-depleted cells being insufficient to compete successfully with the

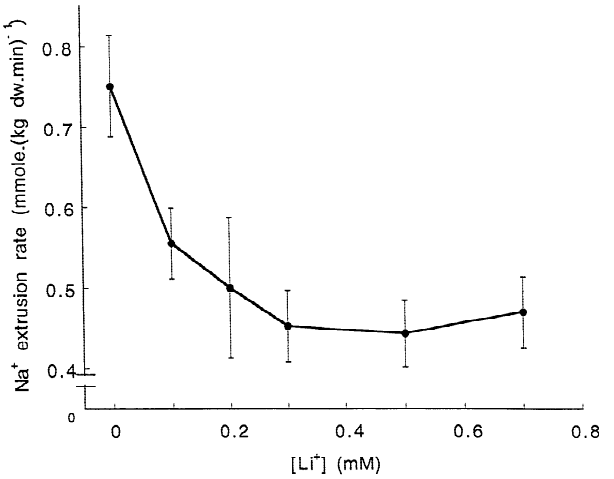

Fig. 8. Inhibition of Na^+ extrusion from hepatocytes by extracellular Li^+. Cells were depleted of Na^+ and then incubated for 10 min at 37°C in the presence of different concentrations of Li^+. Ouabain and bumetanide were present throughout. Samples were taken for analysis of Na^+ content before and after the warm incubation. Values are the mean ± SEM ($n = 5$).

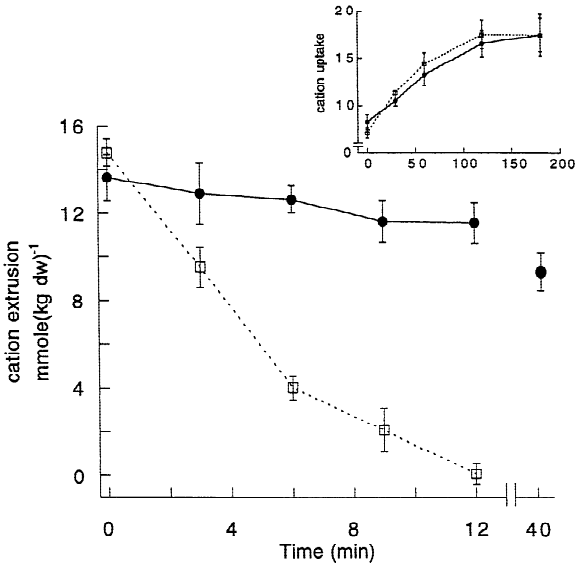

Fig. 9. Time course of Na^+ and Li^+ entry into and extrusion from isolated hepatocytes. Isolated cells in suspension were loaded with Na^+ (□) and Li^+ (●) by preincubation at 1°C for up to 180 min (inset). The extrusion of both cations was measured in Na^+- and Li^+-free, choline-medium containing ouabain (1 mM) and bumetanide (100 μM), at 37°C ($n = 4$).

incoming Li^+ for binding sites on the carriers. This is again consistent with a much greater affinity of Li^+ than of Na^+ for the carrier concerned. A similar conclusion has been drawn for other experimental systems (Aronson, 1985; Pandey et al., 1978).

Na and Li^+ Efflux from Isolated Hepatocytes

Despite the apparently greater affinity of Li^+ for the transport systems, its translocation across some plasma membranes has been reported to be much slower than that of Na^+ e.g., in frog muscle fibers (Keynes & Swan, 1959) and red blood cells (Maizels, 1954). It was therefore of interest to compare the efflux patterns of intracellular Li^+ and Na^+. For this purpose, hepatocytes were preloaded with Na^+ and Li^+ (20 mM each) by incubation at 1°C for up to 180 min. Both cations followed the same time course of entry into the cells at 1°C (Fig. 9, inset). In contrast, when the same cells were subsequently incubated at 37°C in Na^+- and Li^+-free medium, containing ouabain and bumetanide, efflux of Na^+ was much more rapid than that of Li^+. Almost all of the Na^+ gained during the cold incubation was lost in less than 15 min, whereas a significant amount of Li^+ (approximately 60%) still remained in the cells, even after 40 min incubation at 37°C suggesting that overall permeability of the plasma membrane to Li^+ was much less than to Na^+.

Discussion

The work described shows, for the first time, that therapeutic concentrations of Li^+ enter the intact cells of a solid organ in vitro by at least two carrier-dependent mechanisms. Kinetic studies of Li^+ entry into the hepatocytes indicated that a high-affinity, saturable uptake was superimposed on an inhibitor-resistant component. The saturable uptake was itself divisible into two systems, distinguishable by their sensitivity to the inhibitors, amiloride and phloretin, and to pH. A number of characteristics of these two systems have been defined. In addition, an apparently nonsaturable system is shown to contribute substantially to the total uptake.

Previous work on Li^+ transport has been based on the early studies of human erythrocytes which, in addition to a 'leak' pathway (Duhm & Becker, 1977, 1979), gave evidence for saturable exchange of Li^+ for Na^+ by two mechanisms: (i) an amiloride-sensitive Na^+/H^+ exchange system in which Li^+ competitively replaces either Na^+ or H^+ (Haas, Schooler & Tosteson, 1975), here referred to as the Na(Li)/H exchange; (ii) a phloretin-sensitive Na^+/Li^+ exchange. Argument as to the separate identity of these two is unresolved, for it has been suggested that both are aspects of Na(Li)/H activity (e.g., Aronson, 1983; Funder et al., 1984; Chi, Mo & de Freitas, 1996). The lack of agreement may be at least partly due to the difference in relative activities of the two mechanisms between species and organs (Kahn, 1987; Kahn et al., 1989; Van Norren et al., 1998). As discussed below, our results appear to favor the existence of separate mechanisms in rat hepatocytes.

Inhibitor-resistant, 'Nonsaturable' Influx

A residual uptake of Li^+ persisting in the combined presence of amiloride and phloretin was linearly related to medium Li^+ over the concentration range studied. It was also resistant to ouabain and bumetanide (present in all experiments) and to the absence of HCO_3^-, which is to say that it was apparently not due to any of the carrier-dependent transport systems that have been considered for Li^+ uptake (*see* Introduction). It was thus either due to a so far unrecognized carrier-facilitated mechanism of high K_m or, more probably, to simple diffusion. Sodium channels could provide a route for the leak, but they would have to be different from the channels blocked by amiloride (Koch & Leffert, 1979; Garty & Benos, 1988). As the saturable components of Li^+ uptake approached their maximal activity at the upper end of the therapeutic concentration range, the residual inward leak came to represent an ever larger fraction of the total entry. Within the therapeutic concentration range the saturable mechanisms contributed at least as much to the total uptake as did the leak, but at toxic concentrations of Li^+ the latter predominated. In many of our experiments studying the saturable uptake, the diffusive component was minimized by use of the low external concentrations of 0.2–0.8 mM Li^+.

Saturable Li^+ Uptake

In preliminary experiments on the possible mechanisms of the saturable portion of Li^+ uptake, we studied the effects of inhibitors and ions on the total uptake of Li^+. At maximally inhibiting concentrations, amiloride and phloretin each inhibited only a part of the whole and their separate effects were additive. This suggested the existence of two independent pathways. A contribution from the Na(Li)/H exchange was indicated by the stimulation of total Li^+ uptake in an acidic medium. The occurrence of exchange of Na^+ for Li^+ by exchange diffusion was shown by the reduction of total Li^+ uptake both when the cells were depleted of Na^+ and when excess external Na^+ was present. In the latter case a hundredfold excess of Na^+ over Li^+ (20 mM Na^+ *vs.* 0.2 mM Li^+) reduced uptake by some 20%. The inhibition by Na^+ was competitive, suggesting interaction with the same exterior-facing binding site(s) on a carrier, having a substantially greater affinity for Li^+ than for Na^+. Nevertheless, the inhibition of Na^+ efflux by extracellular Li^+ and the slower efflux of Li^+ than of Na^+ suggest that the rate of release of Li^+ from the internal carrier site might limit Na^+ efflux. However, it is likely that Na^+ efflux is also occurring by other pathways including channels which do not mediate Li^+ fluxes.

For the further characterization of the components of saturable uptake, experiments were done separately in the presence of amiloride or phloretin.

Na(Li)/H Exchange

Inhibition by amiloride is a strong indication that Li^+ is transported on one or more of the five mammalian isoforms of the Na^+/H^+ exchanger protein (NHE). At the concentration used here, amiloride is a reasonably specific inhibitor particularly of the widely distributed, 'housekeeping' NHE-1 isoform (Aronson, 1985; Ramamoorthy et al., 1991; Noël & Pouysségur, 1995). The apparent K_m of the amiloride-sensitive portion of Li^+ uptake was 1.2 mM, a value very similar to that found for erythrocytes (Duhm & Becker, 1977). Further evidence for a role of Na(Li)/H exchange in Li^+ uptake comes from the effects of pH. A notable characteristic of NHE is that increasing concentrations of protons appear to augment Na^+ transport not only by virtue of their role as transported substrate, but also by a modulating, possibly allosteric, effect (Aronson, Nee & Suhm, 1982). In our experiments with Na^+-loaded cells, the total uptake of Li^+ was increased approximately 50% by reducing the cytoplasmic pH to 5.5. This resulted solely from an increase in the amiloride-sensitive uptake, the effect of phloretin being unchanged. In these Na^+-loaded cells, the apparent intracellular concentration of Na^+ (65 mM) was very much higher than that of protons (<1 μM) making it probable that the stimulated influx of Li^+ exchanged predominantly for internal Na^+ rather than protons. Other results also suggest that the stimulation of Li^+ by protons was due to the intracellular concentration of H^+ rather than to its gradient across the plasma membrane (Fig. 3). Further analysis (Fig. 5, inset) showed that the amiloride-sensitive stimulation of Li^+ uptake by intracellular $[H^+]$ had kinetics higher than first order, possibly indicative of interaction of H^+ with a modifier binding-site. These results are closely analogous to the findings of Aronson et al. (1982) with Na^+/H^+ exchange and are the first report of such an effect on Li^+ uptake. The importance of Na^+ exchange in the amiloride-sensitive uptake of Li^+ is also seen in that the apparent K_m for Li^+ was increased to 3.8 mM in cells depleted of Na^+ while the effect of acidifying the cytoplasm was much reduced. However, the V_{max} of 37 $\mu mol \cdot (kg\ dw \cdot min)^{-1}$ for the Li^+ uptake under these conditions was close to that found with Na^+-loaded cells.

Na/Li Exchange

In human red cells, the existence of Na^+/Li^+ exchange separately from the Na(Li)/H exchange, has been upheld largely on the basis of its inhibition by phloretin, resistance to amiloride, and independence of pH. Our experi-

ments give similar evidence for entry of low concentrations of Li^+ into rat hepatocytes. First, the V_{max} of the phloretin-sensitive uptake was half that of the amiloride-sensitive system although the apparent K_m values of the two systems were similar. Second, unlike the pH-sensitivity of the proposed Na(Li)/H-dependent uptake, the phloretin-inhibited Li^+ transport by Na^+-loaded cells was little affected by changing the internal pH. Further, the phloretin-sensitive system, unlike Na(Li)/H exchange, was inactive in Na^+-depleted hepatocytes (Fig. 6). That the phloretin-sensitive uptake of Li^+ takes place in exchange for intracellular Na^+ is also strongly suggested by the simultaneous inhibition of both Li^+ influx and Na^+ efflux by phloretin, with similar values of IC_{50} (Fig. 2). However, an unexpected aspect of this last experiment is the quantitatively much greater loss of Na^+ which is coupled to the gain of Li^+ compared to the reported 1:1 stoichiometry of the system in red cells (Sarkadi et al., 1978). Possible explanations include a rapid recycling to the medium, *via* a leak pathway, of Li^+ initially transported into the cells, or an action of phloretin as a Na^+ channel blocker, reducing the efflux of this ion. We have as yet no evidence to support either suggestion.

In conclusion, the experiments have for the first time studied the entry of Li^+ at therapeutically relevant concentrations into intact cells prepared from a major mammalian organ. Evidence is given that, at these concentrations, Li^+ enters liver cells by at least three transport components, some of the characteristics of which we have elucidated. Two of these components, an amiloride-sensitive, pH- and Na^+-dependent transport and a phloretin-sensitive, Na^+-dependent but pH-independent mechanism, predominate at concentrations of Li^+ below 1 mM. Our evidence supports the contention that these systems are independent of each other. A third component, not affected by inhibitors and linearly related to the medium concentration of Li^+ contributes increasingly to the uptake at concentrations in or near the pharmacologically toxic range. These findings with nucleated cells are likely to be of importance for eventual investigation of the molecular biological properties of the Li^+ transporting proteins.

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