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Lithium's Inhibition of Erythrocyte Cation Countertransport Involves a Slow Process in the Erythrocyte

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Summary. Chronic administration of lithium (Li^+) to human subjects results in reduction of Li^+/Na^+ countertransport in their erythrocytes (RBC). The time course of development of inhibition is much slower than one would expect for an immediate effect of Li^+ on the RBC membrane. Possible explanations include pharmacokinetic delays, a mediating humoral agent, and a slow process in the RBC. To discriminate among these possibilities, we incubated human RBC in sterile culture by the method of Freedman (Freedman, J.C. 1983. *J. Membrane Biol.* **75**:225–231), which permits much longer incubations than other methods.

As gauged by eight measures, the incubated RBC remain viable for two weeks. Small changes in intracellular concentrations with time during incubation are in the same direction as the changes associated with natural aging of RBC *in vivo*, except for a rise in ATP and related cation shifts during the first few days of incubation. Treatment of incubated RBC with 2 mM Li^+ inhibits countertransport by 48% without affecting Li^+ leak efflux. The inhibition develops slowly: it is half-maximal after 1–2 days and maximal by 4–7 days. Differences between *in vivo* results and our incubated cells in the time course of inhibition are as expected from the pharmacokinetic delays operating *in vivo*. The inhibition is reversible on removing Li^+. Li^+ inhibits countertransport similarly slowly and to a similar degree from inside the RBC and from outside.

Hence the slow time course of inhibition *in vivo* is not due to a humoral factor or to the time required for intracellular Li^+ accumulation and is only partly due to pharmacokinetic delays. The delay must involve an unidentified slow process at the level of the RBC.

Key Words countertransport · erythrocyte · lithium · manic-depressive illness

The erythrocyte (RBC) extrudes lithium against an electrochemical gradient by a countertransport mechanism coupling downhill Na^+ entry to uphill Li^+ exit (Haas, Schooler & Tosteson, 1975; Duhm, Eisenreid, Becker & Griel, 1976; Pandey et al., 1978; Ehrlich & Diamond, 1979). This Li^+/Na^+ countertransport is found to be inhibited by about 50% in RBC of humans whose plasma contains Li^+ at the therapeutic concentration of ~1 mM used to treat manic-depressive illness (Meltzer et al., 1977; Rybakowski, Frazer & Mendels, 1978). The inhibition involves a decrease in the apparent affinity of the countertransport mechanism for Li^+ (threefold increase in the apparent K_m), without a change in the maximum countertransport rate V_{max} (Ehrlich, Diamond & Gosenfeld, 1981). Li^+ exerts a similar inhibition in human muscle (Clausen, Ehrlich, Diamond & Gosenfeld, 1983).

The slowness with which this inhibition develops poses a puzzling problem in membrane biology. In patients commencing Li^+ therapy by taking oral Li_2CO_3 doses several times daily, 4–7 days are required for maximal inhibition, and 3–4 days for half-maximal inhibition. If Li^+'s inhibitory effect involved a direct action on the membrane, one would expect the effect to develop much more rapidly.

The time course of countertransport inhibition is significant clinically as well as biologically. Li^+/Na^+ countertransport is clinically relevant because Li^+'s action might be intracellular and because countertransport is the process maintaining intracellular Li^+ below electrochemical equilibrium not only in RBC but also in nerve and muscle (Ehrlich & Diamond, 1980; Ehrlich, Clausen & Diamond 1980; Ehrlich & Russell, 1981; Ehrlich & Wright, 1982; Diamond et al., 1983). Nerve is the putative site of Li^+'s therapeutic effect while muscle is a site of toxic side-effects. For unknown reasons, Li^+ therapy takes about a week to control manic symptoms rather than acting quickly. Toxic side effects of Li^+ therapy, when they occur, are also slow to develop. Hence the slow development of countertransport inhibition could be relevant to the slow development of Li^+'s therapeutic effects.

At least three types of explanations could contribute to the slowness of countertransport inhibition:

1) It might be due to pharmacokinetic delays *in vivo* in reaching a steady-state plasma Li^+ level.

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That is, external Li^+ might act instantly on the membrane, but 2–7 days are required for plasma Li^+ concentration to attain its steady-state value after regular oral Li^+ dosing is begun (Bergner et al., 1973). While this time is roughly on the order of the time required for maximal countertransport inhibition, a quantitative comparison has not been made.[1]

2) Li^+ might not act through an instantaneous effect on the RBC membrane but through some slow process in the RBC, such as altering membrane transport of another solute whose intracellular concentration affects countertransport (e.g., choline: Jope, Jenden, Ehrlich & Diamond, 1978), or else through a chain of events beginning with modification of a protein.

3) Countertransport inhibition might be due to Li^+ releasing elsewhere in the body a humoral agent that then acts on the RBC, rather than Li^+ acting directly on the RBC.

To evaluate these possibilities, we wished to study whether, or how rapidly, Li^+ added to RBC *in vitro* inhibits countertransport. For example, if explanation No. 3 were the whole explanation for the delay observed with Li^+ taken *in vivo*, addition of Li^+ *in vitro* would cause no inhibition. If explanation No. 1 were the whole explanation, then Li^+ added *in vitro* would instantly produce maximal inhibition. *In vitro* $(Li^+)_o$ or $Li^+)_i$ can be set rapidly, permitting quantitative comparison of the time courses of countertransport inhibition, $(Li^+)_o$, and $(Li^+)_i$.

The practical problem in executing this experiment is that it would require a week if Li^+'s effect *in vitro* is anything like as slow as its effect *in vivo* (this proves to be true). Conventional methods available for measuring RBC fluxes *in vitro* are limited by RBC deterioration to experiments of about 1 day duration even with antibiotics are added to suppress bacterial growth. However, methods for longer incubations of sheep RBC have been reported (Kepner & Tosteson, 1972; Benderoff, Blostein & Johnstone, 1978). In the accompanying paper Freedman (1983) describes solutions and methods for maintaining human RBC in sterile culture at room temperature with good viability for a week or more. We have therefore used Freedman's techniques to measure countertransport with or without preincubation with external or internal Li^+ for up to 14 days.

[1] Following establishment of an external Li^+ level (abbreviated $(Li^+)_o$), the internal Li^+ concentration (abbreviated $Li^+)_i$) builds up with a half-time of a few hr. Hence, if Li^+ acted internally, the time required for equilibration of $(Li^+)_i$ with $(Li^+)_o$ during oral Li^+ dosing would make only a small contribution to the observed delay in countertransport inhibition.

Materials and Methods

Incubations

All manipulations were carried out in a sterile box with autoclaved or presterilized items. Solutions were sterilized by filtration through presterilized 0.45 μm filters.

Under sterile conditions we centrifuged blood from normal volunteer donors at $1500 \times g$ in the cold and aspirated the plasma and buffy coat. The RBC were washed once with 166 mM choline chloride and resuspended at 2% hematocrit in nutrient medium in 50-cc tubes. The tubes were rotated at 1 RPM at room temperature for the duration of the experiment, up to 1 or 2 weeks. As criteria of RBC viability we used assays for ATP, 2,3-DPG, GSH, Na^+, and K^+, maintenance of biconcave disc shape, absence of hemoglobin in the supernatant (a control for lysis), and absence of bacteria.

The nutrient medium had the following composition. Salts (mM): 116.4 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1.0 NaH_2PO_4, 15.0 $NaHCO_3$, 18.0 HEPES. Amino acids, each at 0.5 mM: cysteine, glycine, glutamic acid, glutamine. Other nutrients (mM): 5.6 glucose, 0.25 adenine. Vitamins (mg/liter): 0.244 D-biotin, 0.477 Ca^{++} D-pantothenate, 0.140 choline chloride, 0.441 folic acid, 0.122 nicotinamide, 0.204 pyridoxal HCl, 0.038 riboflavin, 0.337 thiamine HCl. Other ingredients: 5 g/liter bovine serum albumin, 100 units/ml streptomycin, 100 units/ml penicillin. The pH was 7.4, the osmolarity 295 mM. Experimental cells were maintained in medium containing in addition 2 mM Li^+, added as isotonic LiCl. In all experiments control cells were incubated in parallel in medium without Li^+, for comparison with experimental cells.

Analyses

Coefficients of variation (CV) for assays were determined from 10–18 replicate determinations on the same blood sample. ATP was measured by Sigma kit 366-VU (CV 5.2%), 2,3-DPG by Sigma kit 35-UV (CV 3.2%), GSH by the method of Beutler (1975) (CV 2.0%), Na^+ (CV 2.2%), and K^+ (CV 3.2%) by flame photometry, and Li^+ by flameless atomic absorption spectroscopy (Ehrlich & Diamond, 1978) (CV 2.0%). We routinely did Li^+ analyses in triplicate, other analyses in duplicate. Concentrations were expressed as millimoles per liter RBC, by measuring RBC hemoglobin as cyanomethemoglobin and converting to liter RBC.

Effect of RBC Age

Circulating RBC have a life-span of about 120 days. A blood sample contains RBC of all ages from 0 to 120. To determine how Li^+ transport and assayed concentrations vary with RBC age *in vivo* for comparison with *in vitro* results, we separated fresh RBC into age fractions by density-gradient centrifugation (*see* Vettore, De Matteis & Zampini, 1980, for details). Briefly, 0.5 ml whole RBC were layered over 10 cc of a mixture of 3.5 ml aqueous suspension of colloidal silica particles (Percoll, by Pharmacia), 20 ml of 60% meglamine diatrizoate (Renografin 60, by Squibb), and 4.5 ml distilled water. The mixture plus RBC was centrifuged 20 min at $35{,}000 \times g$ at 4 °C in 15-cc Corex tubes, causing the mixture to form a density gradient and causing the cells to separate into several bands of different densities and ages. The major bands near the top (young-cell

fraction, ca. 20% of total RBC) and near the bottom (old-cell fraction, ca. 20% of total RBC) were collected. Material from eight tubes was pooled and washed three times with 166 mM choline chloride to remove the density gradient material. The washed young and old fractions were then compared for ATP, Na^+, K^+, 2,3-DPG, and Li^+ transport.

Countertransport Determinations

RBC in nutrient medium were centrifuged at $1500 \times g$, the medium was removed by aspiration, and the RBC were washed once in 166 mM choline chloride. RBC were loaded with Li^+ and fluxes were measured as previously described (Ehrlich & Diamond, 1979). Briefly, RBC to be loaded were suspended in NaCl saline (140 mM NaCl, 4 mM KCl, 10 mM Hepes-Tris at pH 7.4, 10 mM glucose) in which 20 mM Na^+ had been replaced with Li^+. After incubation for 1 hr at 37 °C the RBC were washed three times in choline saline to remove external Li^+.[2] The resulting values of Li_i^+ ranged from 0.42 to 1.4 mM. To compare efflux data between different experiments, we assumed a linear efflux-*vs.*-Li_i^+ relation in this range (based on Fig. 2 of Ehrlich & Diamond, 1979) and normalized efflux to $(Li^+)_i = 0.75$ mM. Efflux was measured by adding Li^+-loaded RBC to test tubes containing either Na^+ or choline saline (both Li^+-free) prewarmed to 37 °C and at a final hematocrit of 7.5%.Aliquots of the suspension were withdrawn immediately and again 1 hr later for determining $(Li^+)_o$ and $(Li^+)_i$. Total Li^+ efflux was calculated from the increase in $(Li^+)_o$ over this time in Na^+ saline; leak efflux alone, from the increase in Na^+-free saline. Efflux by Li^+/Na^+ countertransport was taken as the total efflux minus the leak efflux.

Reversibility

To study the reversibility of Li^+ inhibition of countertransport, we incubated RBC with 2 mM Li^+ in the medium for 1 week, spun them down, washed them with sterile choline chloride, and resuspended them in nutrient medium without Li^+. A second set of RBC was similarly incubated with 2 mM Li^+ for 1 week, then resuspended in the same Li^+-containing medium, to determine unreversed inhibition of countertransport for comparison. A third, control set was incubated in Li^+-free medium for 1 week, then resuspended in fresh samples of the same medium. For all three sets, Li^+ transport was compared after one further week. In effect, this procedure compares transport in inhibited-and-reversed, inhibited, and never-inhibited RBC.

[2] It may seem confusing to talk of comparing RBC exposed or not exposed to Li^+, and of the time course of inhibition, when *all* RBC have to be exposed to Li^+ for 1 hr to load them with Li^+ for countertransport measurements. The explanation of this paradox is that we are concerned with changes in countertransport (measured after such a 1-hr loading) that result from chronic exposure to Li^+ over previous days or weeks. Figure 2 will demonstrate that chronic exposure produces only 8% inhibition of countertransport after 4 hr and half-maximal inhibition only after 1–2 days. Hence, as a result of the 1-hr loading period, all RBC that we studied presumably have countertransport reduced by about 2% compared to the value that would be observed if it were possible instantly to load RBC and to measure countertransport. This reduction is negligible and in any case applies to all samples.

Results

Viability of Cells

Before asking whether Li^+ affects countertransport during 2-week incubations *in vitro*, one must first compare viability in incubated RBC and fresh RBC, compare the aging process *in vitro* and *in vivo*, and examine the effect of Li^+ on viability

Table 1. Solute concentrations and countertransport rates in RBC

		ATP (mM)	Na^+ (mM)	K^+ (mM)	2,3-DPG (mM)	GSH (mM)	Li^+ counter transport efflux (mmol/liter RBC, hr)
Fresh Cells							
Pooled		1.19±0.08 (12)	6.2±0.4 (9)	88.0±6.5 (9)	4.76±0.32 (6)	2.21±0.23 (6)	0.156±0.025 (3)
Younger cells		1.54±0.07 (4)	5.1±0.5 (4)	87.8±8.7 (4)	5.51±0.49 (2)	Not measured	0.169±0.050 (3)
Older cells		1.12±0.07 (4)	6.5±0.7 (4)	72.5±8.7 (4)	4.31±0.29 (2)	Not measured	0.113±0.028 (3)
Incubated cells							
$t=0$		1.19±0.09 (10)	6.3±0.4 (8)	90.0±6.9 (8)	5.43 (1)	2.32±0.25 (5)	0.187±0.018 (10)
$t=1$ day	no Li^+	1.49±0.09 (9)	5.4±0.4 (7)	90.0±6.7 (7)	5.24 (1)	1.84±0.19 (4)	0.189±0.021 (10)
	Li^+	1.43±0.13 (9)	5.8±0.5 (7)	92.6±5.6 (7)	5.53 (1)	1.85±0.16 (4)	0.144±0.022 (10)
$t=1$ week	no Li^+	1.76±0.13 (8)	6.5±0.8 (7)	79.1±6.2 (7)	2.74 (1)	1.56±0.14 (5)	0.177±0.018 (9)
	Li^+	1.76±0.15 (8)	10.2±1.7 (7)	76.9±5.3 (7)	2.86 (1)	1.42±0.16 (5)	0.110±0.016 (9)
$t=2$ weeks	no Li^+	1.63±0.20 (5)	14.2±3.5 (7)	76.9±4.8 (7)	Not measured	1.03±0.16 (5)	0.125±0.031 (4)
	Li^+	1.64±0.22 (5)	9.8±1.4 (7)	70.4±4.8 (7)	Not measured	1.04±0.15 (5)	0.065±0.023 (4)

Fresh RBC were studied either as the entire RBC population from a blood sample ("pooled") or else as separated fractions of younger and older RBC. RBC incubated in nutrient medium were sampled immediately at the start of incubation ("$t=0$"), and after 1 day, 1 week, or 2 weeks in the presence or absence of 2 mM Li^+ in the medium. Each entry gives the mean value, standard error of the mean, and (in parentheses) number of preparations studied. Units of concentration are millimoles per liter RBC. Each preparation was from a different donor. *See* text for comparisons of same preparation at different times or under different conditions, by paired *t* tests.

of incubated RBC. Table 1 summarizes assays and Li^+ countertransport rates for all preparations. To minimize effects of interindividual variation, the following statistical comparisons are mostly based on paired two-tailed t tests that use each RBC preparation as its own control (e.g., young *vs.* old RBC, incubated *vs.* fresh RBC, or Li^+-treated *vs.* non-Li^+-treated RBC from the same preparation). In one case to be mentioned, we also report independent t tests comparing means for all preparations studied. The level of significance was taken as $P<0.05$.

Young vs. Old RBC. Fresh RBC were separated into young and old cells as described under Methods. Comparison of the means in Table 1 (row 2 *vs.* row 3) shows that ATP, K^+, 2,3-DPG, and countertransport decrease, and Na^+ increases, with age. Each of these changes agrees with previous observations by Bernstein (1959), except that Bernstein did not measure countertransport. Paired comparisons show that these changes are highly significant for ATP ($P<0.001$), K^+ ($P<0.01$), and Na^+ ($P<0.02$). As expected, the mean ATP, Na^+, 2,3-DPG, and countertransport values for young and old RBC straddle the corresponding mean values for pooled RBC not separated by age (row 1 of Table 1), and this is nearly true for the K^+ values.

Incubated vs. Fresh RBC. In incubated RBC, whether treated or not treated with Li^+, there is apparently a decrease in K^+, 2,3-DPG, GSH, and countertransport and an increase in Na^+ with time, and ATP decreases from 1 week to 2 weeks (lines 4–10 of Table 1). Paired t tests of RBC incubated 2 weeks compared with fresh RBC show that the decline in GSH is significant ($P<0.01$ for Li^+-treated RBC, $P<0.02$ for non-Li^+-treated RBC), as is the decline in countertransport for Li^+-treated RBC ($P<0.01$), while the changes in ATP, Na^+, and K^+ at 2 weeks do not quite reach the $P<0.05$ level. Independent t tests yield the same conclusion. The directions of the changes with time in incubated RBC are the same as the directions by which old fresh RBC differ from young fresh RBC, as discussed in the preceding paragraph. Thus, incubated RBC continue to age *in vitro* as do RBC *in vivo*. Even after 2 weeks of incubation, though, the incubated RBC are still viable by the criteria used (*see* second paragraph of Materials and Methods). Experiments were not extended beyond 2 weeks, because RBC changed in appearance from biconcave discs to irregular, deformable shapes.

As assessed by hemoglobin in the supernatant, the rate of lysis was approximately constant at $0.33\pm0.09\%$/day ($n=28$) up to at least 2 weeks. It is interesting that this rate is even lower than the rate of RBC destruction *in vivo* (ca. 0.8%/day). The explanation may be the spleen removes RBC *in vivo* at a stage before they would have lysed under *in-vitro* conditions.

In one respect the change with time *in vitro* is clearly opposite to the aging trend *in vivo*: ATP rises during the first day of incubation ($P<0.01$ for non-Li^+-treated RBC, $P<0.05$ for Li^+-treated RBC), and rises further until 1 week ($P<0.001$). Only after 1 week *in vitro* does ATP decline, as for aging *in vivo*, and the 2-week ATP levels are still above the fresh-RBC levels. A possible explanation for the initial rise in ATP is that the adenine level in our bathing medium may have been above that required to maintain RBC ATP initially in a steady state at 25 °C. Accompanying the early marked rise in ATP are a slight drop in Na^+ in the first day for both Li^+-treated and non-Li^+-treated RBC, a slight rise in K^+ in the first day for Li^+-treated RBC, and a slight but regularly observed rise in countertransport for non-Li^+-treated RBC (Fig. 1). These early changes in Na^+, K^+, and countertransport are also opposite to the aging trend *in vivo* and to the *in-vitro* changes after 1 day, but they do not reach the $P<0.05$ level. The various changes associated with this initial "rejuvenation" and subsequent aging of incubated RBC are qualitatively consistent, as raised ATP would tend to stimulate the Na^+/K^+ pump and hence to lower cell Na^+, raise cell K^+, and (by increasing the Na^+ gradient) stimulate countertransport. However, the quantitative relevance of this ATP effect to understanding the ionic changes is doubtful, as the ATP levels are well above K_m for ATP's effect on pumping.

Li^+-Treated vs. Non-Li^+-Treated Incubated RBC. Paired t tests show that Li^+ treatment does not affect ATP, Na^+, or K^+ in incubated RBC, whether after 1 day, 1 week, or 2 weeks of incubation. For GSH the difference between Li^+-treated and non-Li^+-treated RBC is significant at 1 week ($P<0.05$) but nonexistent at 1 day or two weeks. The rate of lysis as assessed by supernatant hemoglobin is identical for Li^+-treated ($0.33\pm0.19\%$/day, $n=14$) and non-Li^+-treated ($0.33\pm0.23\%$/day, $n=14$) RBC. Thus, Li^+ treatment does not affect viability of incubated RBC. However, Li^+ treatment causes a large decline in countertransport, as will now be discussed.

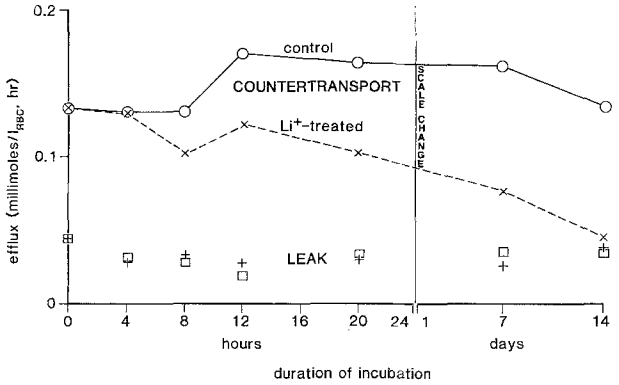

Fig. 1. Li^+ countertransport efflux and Li^+ leak efflux as a function of duration of incubation, with (×, +) or without (○, □) 2 mM Li^+ in the medium. Results are from a single batch of RBC. Note the change of abscissa scale after 24 hr. The leak efflux is constant with time and is not affected by incubation with Li^+. The countertransport efflux is inhibited within 8 hr by Li^+, and the inhibition increases with time. Countertransport efflux in the absence of Li^+ rises between 8 and 12 hr, then remains steady for a week before declining

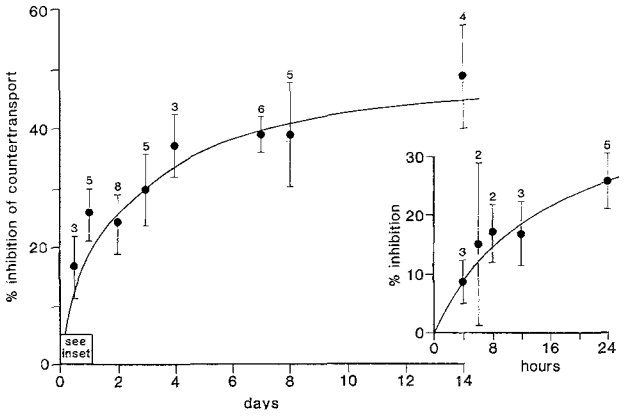

Fig. 2. Development of countertransport inhibition with duration of exposure to Li^+. The inset gives results for the first 24 hr on an expanded time scale. Inhibition of countertransport was measured in 12 experiments similar to that of Fig. 1. At each time the point plotted is the mean reduction in countertransport in Li^+-treated RBC compared to control RBC, the bars give ±1 SEM, and the number over the bar is the number of experiments on which the point is based. The curve through the points is drawn by eye and has no theoretical significance

Inhibition of Countertransport

Comparison of countertransport in paired RBC samples incubated with and without Li^+ shows that Li^+ treatment reduces countertransport by 22% after 1 day ($P<0.001$), 40% after 1 week, and 48% after 2 weeks (Table 1).

Figure 1 illustrates the time course of inhibition in an experiment on one batch of RBC from a single donor. Countertransport in control RBC incubated without Li^+ (○, Fig. 1) rises between 8 and 12 hr, then remains constant for 1 week (an indication of the good condition of the cells), and thereafter declines slightly by 2 weeks. This time course was observed in all 12 such experiments performed. As discussed in the preceding section, the initial rise in countertransport is associated with the initial "rejuvenating" rise in ATP and perhaps K^+ and drop in Na^+. The Li^+-treated RBC also exhibit this rise in countertransport between 8 and 12 hr (×, Fig. 1). However, inhibition of countertransport in the Li^+-treated RBC compared to the control RBC is already evident at 8 hr (23% inhibition), and increases to 53% at 1 week and 67% at 2 weeks in this experiment. The decline in countertransport in Li^+-treated RBC from 12 hr to 1 week is wholly due to this inhibition, while aging as in the control RBC also contributes to the decline after 1 week.

In contrast, the leak efflux for both Li^+-treated RBC (+, Fig. 1) and control RBC (□) does not change during 2 weeks of incubation. Paired t tests for all such experiments show that there is no significant difference in leak efflux between Li^+-treated and control RBC at any time.

Figure 2 summarizes the results of 12 experiments similar to that of Fig. 1. The obvious conclusion is that, *in vitro* as *in vivo*, inhibition is far from instantaneous. At the earliest time measured, 4 hr, there is a small but significant inhibition by 8% (inset of Fig. 2). Half-maximal inhibition of 24% is reached at 1–2 days, and the inhibition reached after 2 weeks is 48%. The average maximal value reached *in vitro* is essentially the same as the average maximal inhibition of 50% that we observed *in vivo* (Ehrlich, et al., 1981).

The time course of development of inhibition is compared in Fig. 3 for *in-vitro* and *in-vivo* conditions. In both cases it requires 4–7 days to achieve close to maximal inhibition. However, inhibition initially develops more rapidly *in vitro* than *in vivo*: an inhibition half that attained at 1 week is reached within 1 day *in vitro* but only after 3 days *in vivo*. The explanation is surely the difference in time course of $(Li^+)_0$. *In vitro*, $(Li^+)_0$ is abruptly raised to 2 mM at $t=0$ and then remains constant. *In vivo*, for patients on repeated oral Li^+ doses, the time-averaged plasma (Li^+) eventually reached is around 1 mM, and it takes several days of dosing to reach that value.

Figure 3 also compares *in vitro* and *in vivo* conditions with respect to the recovery of counter-

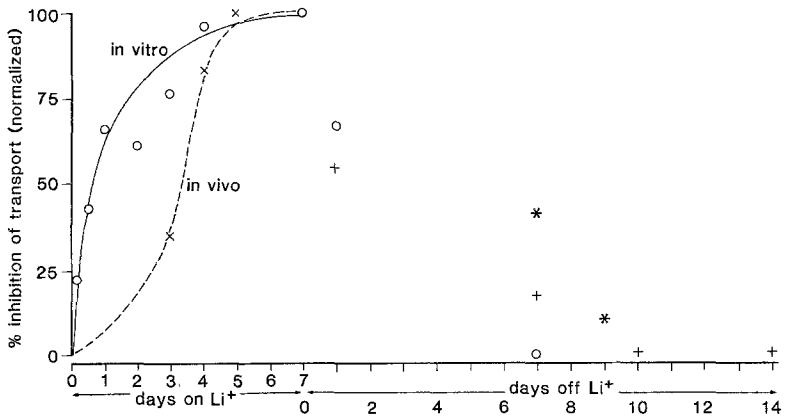

Fig. 3. Comparison of time courses for onset and reversal of countertransport inhibition, *in vitro* (o) and *in vivo* (×, +, *), *In-vitro* results are from the present paper: points for onset are each based on 3–8 batches of RBC incubated in 2 mM Li^+ for the indicated time; points for reversal are each based on 1 or 2 batches of RBC incubated in 2 mM Li^+ for 7 days, then transferred to Li^+-free solution for the indicated time. The *in-vivo* measurements are based on RBC of patients commencing Li^+ therapy (×: Ehrlich et al., 1981), and of patients ceasing Li^+ therapy after many months (*: Rybakowski et al., 1978; +: our unpublished results). *Ordinate:* percent inhibition of transport, normalized to the maximal inhibition attained (after 7 days *in vitro* or after many months *in vivo*). *In-vitro* and *in-vivo* data are normalized separately. The curves through the onset points are drawn by eye and have no theoretical significance

Table 2. Sidedness of Li^+ inhibition

	% inhibition	
	21 hr	44 hr
Li^+ inside	18	18
Li^+ outside	22	21
Li^+ both sides	24	36

Aliquots of the same batch of RBC were incubated for 21 or 44 hr with Li^+ inside, Li^+ outside, Li^+ both sides, or Li^+ on neither side. The numbers are the percent inhibition of countertransport in the former three aliquots, compared to the countertransport rate in RBC that had been incubated without Li^+. (*See* text for details.)

transport from inhibition when Li^+ is removed. Reversal of inhibition is essentially complete within 7 days *in vitro*, and within 10–14 days *in vivo*. Figure 3 does not suffice to prove that reversal is more rapid *in vitro* than *in vivo*, as one might expect from reasoning analogous to that of the preceding paragraph. We did not pursue this question further, because it is hard to find patients halting Li^+ therapy and because accurate measurement of reversal *in vitro* is technically difficult.

Does Li^+ Act from Inside or from Outside the RBC?

The gradual development and decay of inhibition *in vitro* seen in Figs 2 and 3 could be taken to mean that only intracellular Li^+ is capable of inhibiting countertransport. By this interpretation, the time course of inhibition might parallel the time course of rise or fall in $(Li^+)_i$ after Li^+ is abruptly added to or removed from the external solution. This interpretation was checked in one experiment by incubating RBC with Li^+ outside, Li^+ inside, Li^+ both sides, or Li^+ on neither side and measuring countertransport after 21 and 44 hr. The first set of RBC samples (Li^+ outside) was suspended in medium containing 1 mM Li^+ at $t=0$. The second set (Li^+ inside) was quickly loaded to $(Li^+)_i=0.8$ mM by preincubation in medium containing 50 mM Li^+ for 55 min, then suspended in medium without Li^+. The third set (Li^+ both sides) was similarly quickly loaded to $(Li^+)_i=0.8$ mM, then suspended in medium containing 1 mM Li^+. The fourth set (Li^+ neither side) was not loaded at $t=0$ and was suspended in medium without Li^+. Just prior to the measurement of countertransport, $(Li^+)_i$ of all four sets was quickly readjusted as needed to $0.7=0.8$ mM by brief incubation in a Li^+-containing medium.[3]

[3] The third set of cells needed no such readjustment, because $(Li^+)_i=0.8$ mM, $(Li^+)_o=1.0$ mM is close to a steady-state condition and $(Li^+)_i$ scarcely changed by 44 hr. The first set had gained Li^+ up to $(Li^+)_i=0.4$ mM by 44 hr.; the second set had lost Li^+ down to $(Li^+)_i=0.2$ mM at 44 hr; and the fourth set had $(Li^+)_i=0$. Hence these latter three sets were quickly loaded to $(Li^+)_i=0.7$–0.8 mM just before measurement of countertransport. *See also* footnote 2, p. 235, regarding loading. Note also that ideally one would like to maintain the first and second sets with one side constant at 0.8 mM Li^+ and the other side Li^+-free for 21 or 44 hr. However, this is of course impossible because RBC have finite permeability to Li^+, causing $(Li^+)_i$ gradually to rise (first set) or fall (second set) with time.

The result, as shown in Table 2, is that incubation with internal Li^+ does not produce greater inhibition, nor does it inhibit more rapidly, than does incubation with external Li^+. At 21 hr countertransport inhibition is virtually the same in RBC that had been incubated with Li^+ inside, outside, or both sides. (We also obtained this result at 3 hr.) By 44 hr it appears that RBC incubated with Li^+ on both sides show a larger inhibition than RBC incubated with Li^+ on one side alone.

This experiment suggests four conclusions:

1) The time delays seen in Figs. 2 and 3 are not due to time required for Li^+ to accumulate at a site of action inside the RBC.

2) Li^+ outside and Li^+ inside inhibit to similar degrees.

3) For neither Li^+ outside nor Li^+ inside is the inhibition instantaneous.

4) Li^+ present on both sides inhibits to a greater degree than does the same Li^+ concentration on either side alone for the same time.

Discussion

We and several other groups previously showed that RBC exposed *in vivo* to Li^+ exhibited reduced countertransport *in vitro*. However, the present study is the first demonstration that Li^+ exposure *in vitro* has the same effect. We have now shown that, *in vitro* as *in vivo*, chronic exposure of RBC to Li^+ specifically inhibits Li^+/Na^+ countertransport by about 50%, without affecting leak efflux of Li^+. The maximal inhibition attained is as large *in vitro* as *in vivo*. These findings exclude several possible explanations of the slow time course of inhibition.

First, since the inhibition is elicited by treatment of isolated RBC with Li^+, it is not due to Li^+'s releasing elsewhere in the body a humoral factor that acts on the RBC.

Second, the pharmacokinetic delays associated with oral Li^+ dosing *in vivo* certainly affect the initial speed of onset of inhibition (Fig. 3), and may also affect the initial speed of recovery from inhibition. However, pharmacokinetics are far from the whole explanation for the slowness of inhibition, as inhibition is still slow *in vitro*, where the external Li^+ can be presented or removed rapidly.

Instead, the slowness of inhibition involves some slow process in the action of Li^+ on RBC and the sequelae of this action. This slow process is not accumulation of intracellular Li^+ by penetration from the outside: inhibition also develops slowly when RBC are quickly loaded with Li^+. A clue to the identity of the unknown slow process is that internal and external Li^+ appear both to act slowly and to cause similar inhibition, and that both together inhibit more than does either alone. Some caution is required in comparing effects of internal and external Li^+, as $(Li^+)_i$ was changing with time during the incubations with Li^+ initially on only one side. Nevertheless, Table 2 makes unlikely a mechanism whereby Li^+ must interact with an intracellular constituent or with the inner face of the membrane.

At present, the identity of the slow process is entirely speculative. Four possibilities warrant brief mention:

1) Li^+ might alter the rate of choline production from membrane phospholipid (Jope et al., 1978), Jenden, Jope & Fraser, 1980), thereby altering membrane lipid composition and hence countertransport.

2) Li^+ might interact with the membrane to alter the membrane transport mechanism of some other solute. The resulting altered intracellular concentration of that solute might then modify the countertransport mechanism.

3) Li^+ might alter the rate of phospholipid flip-flop across the membrane, thereby again altering membrane lipid composition and hence countertransport.

4) More generally, Li^+ might affect some membrane-bound enzyme, thereby starting a chain of events that affects countertransport.

The identity of this unknown slow process now becomes a matter of much interest.

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