LITHIUM HOMEOSTASIS IN XENOPUS OOCYTES: IMPLICATIONS FOR THE STUDY OF SIGNAL TRANSDUCTION

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Summary

The Xenopus oocyte has been shown to be a useful model for the study of signal transduction pathways. The present study investigated whether or not the oocyte could be used to study the effects of lithium on signal transduction mechanisms by comparing the dynamics of lithium homeostasis in the oocyte and a human immortalized hippocampal cell line using Flame Atomic Emission Spectroscopy (FAES). A biphasic pattern of lithium uptake was observed in the oocyte in the presence of 5 mM extracellular lithium. The late phase of lithium uptake, which started after 30 minutes of incubation time, was sensitive to phloretin, an inhibitor of Na⁺/Li⁺ counter-transport. Differences in lithium efflux kinetics further characterized the two observed phases of accumulation and also suggested that lithium might be distributed in different pools within the oocyte, including one sequestered in organelles or associated with cytosolic proteins. An analogous sequestered pool was not, however, observed in the hippocampal cell line indicating that lithium is distributed differently in these cell types. This suggests that the Xenopus oocyte might not be a suitable model for evaluating the effects of lithium on signal transduction pathways because of the unknown contribution of the sequestered pool on predicting relevant physiological effects.

Key Words: lithium, homeostasis, Xenopus oocytes

Lithium salts have been the primary treatment for bipolar disorder for many years. While the precise mechanisms underlying the therapeutic actions of lithium are not fully understood, lithium is known to affect several signal transduction systems (1). Lithium has, however, a narrow therapeutic window, specifically, extracellular (i.e. serum) concentrations in the 0.8-1.2 mM range are most effective in treating bipolar disorder (2). This narrow range not only supports the role of enzymatic regulation by lithium (1), but also raises the question of whether the specificity of lithium for bipolar disorder may depend on some aspect of lithium homeostasis.

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Lithium homeostasis varies across cell types, depending on features like membrane transporter characteristics and ion channel activity (3). Transport systems that regulate the concentrations of other ions are also thought to be involved in regulating lithium homeostasis (3). Extracellular concentrations of lithium salts have been monitored extensively to prevent adverse side effects associated with potential toxicity (2). However, intracellular concentrations are more critical for predicting physiological effects. Studies have estimated average intracellular concentrations in the brain around 1 mM (4). This means that lithium concentrations in the brain are not in equilibrium since the negative resting potential of neurons creates an electrochemical gradient that would serve to concentrate lithium intracellularly. Synaptic activity could exploit this driving force, creating local heterogeneity in lithium concentrations perhaps associated with a targeting effect of possible significance for the therapeutic use of lithium (5).

The presence of relatively low levels of intracellular lithium suggests that efflux mechanisms must dominate lithium homeostasis. One proposed mechanism for lithium extrusion is via a phloretin-sensitive Na+/Li+ counter-transport, previously described in Xenopus oocytes and human skin fibroblasts (6,7). This system has also been shown to be sensitive to phloretin in human red blood cells (3). Although it is perhaps the best studied lithium efflux mechanism, a Na+/Li+ exchange mechanism alone is not likely to account for all of the non-equilibrium processes regulating lithium efflux. Other components of lithium efflux in human erythrocytes may be partially inhibited by lanthanum chloride and ouabain (8).

Previous studies have used the Xenopus oocyte to study lithium homeostasis (6,9) and mechanisms of signal transduction (10,11). Therefore it becomes relevant to address the question of whether the Xenopus oocyte is a useful model for studying the specific effects of lithium on signal transduction systems. This is a critical question given the potential use of the oocyte as an expression system for neurotransmitter receptors involved in signal transduction in the brain. In the present study, we characterize lithium transport in Xenopus oocytes at the high end of the physiologically relevant range (5 mM), and then compare lithium efflux data from the oocyte with that obtained from a human immortalized hippocampal cell line (HN33 cells). We report a different lithium distribution between the two cell types, indicating that the Xenopus oocyte might not be a suitable model for evaluating the effects of lithium on signal transduction systems in the brain.

**Methods**

**Solutions:**
Lithium (5 mM LiCl) was partially substituted for sodium in frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 1 μM atropine, 10 mM HEPES) used to incubate the oocytes. In addition, high potassium Ringer was prepared by substituting potassium for sodium in the lithium Ringer (5 mM LiCl, 62.5 mM NaCl, 50 mM KCl, 1.8 mM CaCl2, 1 μM atropine, 10 mM HEPES). This Ringer solution was used in the experiments where oocytes were depolarized. Transport inhibitors were added to the Ringer solution to evaluate their effects on lithium uptake: lanthanum chloride (1 mM), ouabain (100 μM) and phloretin (100 μM). Previous studies have shown that these concentrations affect the transport of other monovalent cations in addition to lithium (6,7,8). Ethanol controls were used to compare the effects of ouabain (1%) and phloretin (0.5%); standard lithium Ringer (5 mM LiCl) was used to assess the effects of lanthanum chloride.

**Preparation of Xenopus oocytes:**
Ovarian lobes were surgically removed from adult female frogs and then cut open to expose the oocytes. The ovarian tissue was then treated with collagenase in calcium-free Barth's solution (88 mM NaCl, 10 mM HEPES, 0.33 mM MgSO4, 0.1 mg/ml gentamicin sulfate, pH 7.6) for two hours at room temperature (12).

**Preparation of the immortalized hippocampal cell line (HN33 cells):**
HN33 cells were isolated by standard hypoxanthine-aminopterin-thymidine selection. Cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 5% dialyzed fetal bovine serum. The cells were kept in culture in multiwell plates for 24 hours (13).
Oocyte sample collection:
The method was adapted from Aoshima (9). Oocytes were incubated in the experimental solution. After incubation, each oocyte was taken by a pipette and rapidly washed twice with 1 ml of lithium-free Ringer solution in a 5 ml plastic cup. Excess solution was removed with a wick formed from lab tissue (Kimwipe). The oocytes were then incubated in 5 ml of 0.02 N HCl overnight. The resulting mixture was filtered using a manifold vacuum filtration system (Millipore model 1225) through filter paper (2.4 cm in diameter). This solution was pooled with an additional 5 ml of 0.02 N HCl washed through the filter to ensure the collection of any residual lithium. Samples were evaluated for lithium content using FAES.

Cell line sample collection:
HN33 cells were exposed to 5 mM lithium media for 24 hours. After removing LiCl, standard DMEM media was added for various time intervals. Cells were pelleted at 1500 rpm for 6 minutes and were collected in medium lacking LiCl. The medium was removed and pellets were suspended in 200 μl of 0.02 N HCl overnight. Samples were then collected using the manifold vacuum filtration system described above. Lithium measurements were normalized to sample pellet weights, calculated by subtracting the weight of dry eppendorf tubes from the weight of tubes containing the cell samples. Thus, lithium values were expressed as mM Li/mg packed cells, where 1 mg of packed cells corresponded to 40 μg of protein (14).

Lithium measurements:
Measurements were taken using a R955 photomultiplier (Hamamatsu Corp, Middlesex, NJ), mounted in a cooled housing (Model TE-177RF, Products for research, Danvers, MA). A transimpedance amplifier (Model SR 570, Stanford Research Systems, Palo Alto, CA) was used to amplify and filter (1 Hz low pass filter, 12dB/octave) the photocurrent. Atomic absorption grade acetylene and research grade air were used, both filtered through 7 μM in-line filters after pressure regulation. An acetylene pressure of 12 psi and an air pressure of 46 psi were used for all measurements. Mass flow controllers (Model 5850C, Brooks Instrument Division, Hatfield, PA and Model CM4, Porter Instruments Division, Hatfield, PA) were used to maintain constant gas flows. The flame emission signal at 670.7 nm was recorded on a strip chart recorder using 1 volt as full scale and sensitivity in the range of 0.1 to 10 μA. Samples were aspirated directly into the acetylene-air flame at a flow rate of about 3 ml/min. Standard curves of lithium flame emission signals were used to convert the photocurrents (μA) into lithium concentrations (mM). Lithium standards were prepared by adding 2 μl (the approximate volume of the oocyte) of lithium Ringer (0.25-2 mM) to 10 ml of 0.02 N HCl. Fig. 1 shows a sample standard curve with its linear equation estimated by Cricket Graph (Computer Associates International, version 3.1). Similar standard curves were made for each set of experiments, and the corresponding equations were used to calculate lithium concentrations from the photocurrent measurements.

Electrophysiological measurements:
Resting potentials of individual oocytes were measured using a Warner Instruments OC-725 oocyte amplifier. Oocytes were placed in a Lucite recording chamber with a total volume of 0.5 ml and were perfused with the corresponding incubation solution. Voltage electrodes were filled with 3 M KCl and had resistances of 1-3 MΩ (12).

Data presentation:
Unless otherwise noted, results are presented as means ± S.E.M of individual batches of four oocytes. Comparisons were made using two-tailed t-tests and were regarded as significantly different at p<0.05.

Results
Characterization of endogenous lithium transport in Xenopus oocytes.
We observed two distinct phases of lithium uptake when oocytes were incubated in 5 mM lithium Ringer (Fig. 2). In an early phase, intracellular lithium levels reached 0.29 ± 0.04 mM within 1 minute of incubation. This concentration did not increase further in the next 4 minutes. However, lithium levels decreased significantly after 30 minutes of incubation (0.15 ± 0.02 mM). At later time points, a new phase of accumulation was observed, reaching a maximum level of 0.71 ± 0.02
mM within 4 hours. This new equilibrium concentration remained constant for up to 24 hours of incubation. In parallel experiments, we checked for cell viability by measuring the resting potentials of oocytes exposed to 5 mM lithium for 24 hours. We found no significant difference between the resting potentials of these oocytes (-30.0 ± 2.0 mV) and those that were incubated in control Ringer solution (-33.6 ± 2.2 mV).

The dynamics of lithium efflux were evaluated in both the early and late phases of lithium accumulation. The lithium pool that accumulated during the early phase was rapidly extruded. Specifically, intracellular lithium approached baseline levels when oocytes, previously exposed to 5 mM lithium for 1 minute, were returned to control Ringer for 30 seconds (Fig. 3). In contrast, lithium efflux in the late phase was biphasic (Fig. 4). In oocytes that had been previously exposed to 5 mM lithium for 24 hours, we initially observed a significant decrease in lithium levels from 0.79 ± 0.06 to 0.49 ± 0.08 mM within 2 hours of incubation in control Ringer. However, intracellular lithium levels then remained constant in these oocytes for up to 24 hours (0.48 ± 0.08 mM).

Our observations suggested the presence of a sequestered pool of intracellular lithium. Similar results were previously reported by Busch et al (6). We investigated whether this sequestered pool could be loaded to levels higher than 0.5 mM, if cells were initially exposed to higher levels of extracellular lithium. Treatment of oocytes for 4 hours in 115 mM lithium Ringer produced intracellular levels of 3.50 ± 0.10 mM. When these oocytes were transferred to control Ringer for 24 hours we observed that the sequestered lithium pool loaded with 115 mM extracellular lithium Ringer was significantly increased (1.13 ± 0.08 mM) compared to the pool loaded with 5 mM lithium Ringer (0.48 ± 0.08 mM).

Effects of transport inhibitors on lithium uptake in the oocyte.
Table 1 and Figure 5 summarize the effects of lanthanum chloride (1 mM), ouabain (100 μM) and phloretin (100 μM) on lithium uptake in the early and late phases of lithium accumulation respectively. Although we did not find significant differences at the inhibitor concentrations tested in the early phase of accumulation, we observed a significant effect of phloretin in the late phase. Intracellular lithium levels in oocytes incubated in the lithium/phloretin bath for 24 hours were about twice as high as those incubated in the lithium/ethanol control (Fig. 5). Resting potentials of oocytes exposed to lithium and phloretin were only a few mV (-7.3 ± 2.4 mV). This effect was specific for an interaction between phloretin and lithium since the resting potentials of oocytes with
Top: Lithium uptake as a function of time in the presence of 5 mM extracellular LiCl. Oocytes were incubated in 5 mM Li⁺ Ringer for 0.33, 1, 5, 30, 120, 240 and 1440 min. Values are means ± S.E.M of 3 batches oocytes (n=12). Bottom: Examples of FAES responses from oocytes incubated in 5 mM Li⁺ Ringer for 5 minutes, 30 minutes, or 24 hours. Fast transients in the waveforms were blanked. The dashed line indicates the level of background signal.

Comparable levels of intracellular lithium following exposure to high lithium (115 mM Li) were significantly higher (-40.3 ± 2.5 mV). In addition, resting potentials of oocytes incubated in lithium-free/phloretin baths (-36.3 ± 1.9 mV) did not significantly differ from lithium-free/ethanol controls (-38.7 ± 2.1 mV).
Lithium efflux after 1 min in 5 mM LiCl. Oocytes were incubated in 5 mM Li⁺ Ringer for 1 min. and then transferred to control Ringer for 10, 15, 20 and 30 sec. Data points are means ± S.E.M of eight measurements from two batches of oocytes (n=8).

Lithium efflux after 24 hours in 5 mM LiCl. Oocytes were incubated in 5 mM Li⁺ Ringer for 24 hours and then transferred to control Ringer for 30, 60, 120 and 240 min. Data points are means ± S.E.M of twelve measurements from three batches of oocytes (n=12).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SEM (mM Li)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.203 ± .063</td>
</tr>
<tr>
<td>Ouabain 100 µM</td>
<td>0.218 ± .079</td>
</tr>
<tr>
<td>Phloretin 100 µM</td>
<td>0.185 ± .047</td>
</tr>
<tr>
<td>Control</td>
<td>0.154 ± .072</td>
</tr>
<tr>
<td>LaCl 1 mM</td>
<td>0.171 ± .086</td>
</tr>
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To evaluate the long term effects of lithium/phloretin exposure on oocyte viability, we measured resting potentials of oocytes exposed to the lithium/phloretin bath for 24 hours as well as those subsequently transferred to lithium-free Ringer for another 24-hour period. We found that there was no significant difference between resting potentials of the oocytes immediately after the lithium/phloretin treatment (-2.75 ± 1.3 mV) and resting potentials of those after the recovery period (-2.5 ± 1.2 mV). Thus, resting potentials of oocytes exposed to lithium and phloretin did not recover.
Effects of ouabain and phloretin on lithium uptake after 24 hours in 5 mM LiCl. Oocytes were incubated in 5 mM Li⁺ Ringer with either ouabain (100 µM) or phloretin (100 µM). Values are means ± S.E.M of five batches of oocytes (n=20).

To test whether the increased lithium accumulation after prolonged phloretin exposure was a consequence of depolarization, high potassium Ringer (50 mM K, 62.5 mM Na, 5 mM Li) was used to induce oocyte depolarization. Oocytes exposed to high potassium had significantly less lithium (p<0.01) than those exposed to the lithium/phloretin group (Fig. 6). In addition, the high level of potassium did not reverse the lithium/phloretin effect, as the intracellular lithium level in this group was comparable to that observed in the lithium/phloretin group (Fig. 6). Resting potentials of oocytes exposed to lithium and phloretin (-2.9 ± 1.5 mV) did not recover while resting potentials of KCl-depolarized oocytes did (-28.3 ± 1.8 mV) after 24 hours.

Comparison of lithium/phloretin treatment with oocytes that were experimentally depolarized. Oocytes incubated in 5 mM Li⁺ Ringer with 100 µM phloretin for 24 hours (Li/P) were compared with others incubated in high potassium Ringer (5 mM LiCl, 62.5 mM NaCl, 50 mM KCl) with (Li/K/P) or without 100 µM phloretin (Li/K) for 24 hours. Values are means ± S.E.M of eight measurements from two batches of oocytes (n=8).
Lithium efflux in the immortalized hippocampal cell line (HN33 cells).

We wished to determine if the oocyte might be a useful model for lithium homeostasis in a mammalian system. We compared late phase efflux data from the oocyte to analogous conditions in a mammalian hippocampal cell line (HN33 cells). The HN33 cells were exposed to 5 mM LiCl for 24 hours then most of the accumulated lithium was extruded after five minutes of incubation in lithium-free media (Fig. 7). Specifically, intracellular lithium levels decreased from 1.97 ± 0.04 to 0.30 ± 0.03 mM/mg packed cells. Furthermore, this lithium pool was different from the one that had been observed in the late phase in the oocyte as no lithium could be detected after 2 hours of incubation in lithium-free media (i.e. there was no sequestered pool).

Discussion

Endogenous lithium transport in Xenopus oocytes.

Since lithium uptake into Xenopus oocytes may be easily measured using flame atomic emission spectroscopy, the Xenopus oocyte has been proposed as a model for the study of lithium homeostasis (6, 9). Aoshima et al., reported that lithium uptake increased proportionately with incubation time when oocytes were exposed to 116 mM LiCl (9). Busch, et al. (6) described similar observations in oocytes exposed to 65 mM LiCl. Although important in evaluating lithium transport in the oocyte, these studies did not examine the question of lithium uptake in the presence of lower and more therapeutically relevant concentrations (i.e. 1-5 mM Li). In the present study, we described the endogenous transport systems maintaining lithium homeostasis in the oocyte at the high end of this physiologically relevant range (5 mM Li).

Our results indicate that there is a biphasic pattern of lithium uptake (Fig. 2). In an early phase, intracellular lithium levels peaked around 0.3 mM after one minute, and remained constant for the next five minutes. At this point, an efflux system appeared to be activated in the oocyte as lithium levels decreased significantly in spite of the electrochemical gradient driving lithium inward. This period of induced efflux was followed by a late phase of accumulation that began after 30 minutes. The late phase peaked at around 0.7 mM after 4 hours and remained constant for up to 24 hours of incubation. It is important to note that 5 mM lithium Ringer had no effect on resting membrane potential and therefore on the electrochemical gradient driving lithium inward (data not shown).

Fig. 7

Lithium efflux in HN33 cells after 24 hours in 5 mM LiCl. HN33 cells were incubated in 5 mM LiCl for 24 hours and then transferred to lithium-free media for 5, 15, 60 and 120 min. Lithium measurements were normalized to sample pellet weights and were expressed as mM Li/mg packed cells, where 1 mg of packed cells corresponded to 40 μg of protein. Values are means ± S.E.M of eight measurements (n=8).
Our findings suggest that lithium may be distributed in three different pools within the oocyte. The pool observed in the early phase of accumulation could be readily available for fast extrusion (i.e. a "diffusable" pool). The other two pools would be taken up after prolonged lithium exposure: one could involve lithium sequestering, perhaps in organelles or bound to cytosolic proteins, while the other could store lithium temporarily before further release. Alternatively, the pool could be identical to the diffusable pool but display much slower kinetics. The latter extrusion, in turn, would be mediated by a transport system different from the one used for the diffusable pool given the differences in efflux kinetics (compare Figs. 3 and 4). This hypothetical distribution of lithium within the oocyte raised the critical question of whether or not an analogous distribution exists in mammalian cells.

The pharmacology of transport systems of the Xenopus oocyte.
We saw a late phase of lithium accumulation that was apparently sensitive to phloretin (Fig. 5). The observed increase of intracellular lithium may have reflected an inhibition of Na⁺/Li⁺ counter-transport, an efflux mechanism that has been shown to regulate lithium homeostasis in human red blood cells and skin fibroblasts (3,7). Similar effects had been described previously in Xenopus oocytes (6), although the precise contribution of phloretin in those experiments was uncertain since the efflux media also contained ouabain (6). Since, ouabain and lanthanum chloride did not affect lithium uptake in our experiments, our results might suggest that phloretin mediated-inhibition of Na⁺/Li⁺ counter-transport is one of the main mechanisms maintaining lithium homeostasis in the oocyte. However, the finding that resting potentials of oocytes exposed to lithium and phloretin were on the order of only a few mV, raised the issue of whether complicating factors might exist that generally compromised the health of the oocytes. Nonetheless, if the oocytes were in fact effectively killed by the treatment, one would expect intracellular concentrations to be around 5 mM, as the cell would become completely equilibrated with the extracellular lithium, which was not the case (Fig. 5).

Moreover, our experiments did not support depolarization as a possible mechanism for the observed change in intracellular lithium concentration since oocytes that were experimentally depolarized had significantly less intracellular lithium than those exposed to the lithium/phloretin treatment (Fig. 5) even though both had comparable resting potentials prior to recovery. This suggests that depolarization alone was not responsible for the increase in intracellular lithium observed after lithium/phloretin exposure and that a complicated interaction exists between phloretin, lithium, the regulation of lithium homeostasis, and the ionic balance associated with the maintenance of resting potential.

It may be noted that Busch et al. reported a H-Li exchange system in the oocytes (6), that could be potentiated by pre-acidification of the oocyte. We could not reproduce this effect (data not shown). However, Busch et al. also reported changes in lithium homeostasis that occurred with increased time post surgery, perhaps representing a progressive loss of factors associated with the oocyte follicular cells. While the study by Busch et al. primarily used cells immediately after harvesting, we exclusively used cells that had been incubated in Barth’s solution for at least 1 day after surgery.

The Xenopus oocyte as a model for lithium’s effects on signal transduction systems.
The Xenopus oocyte has been explored as a potential model for evaluating signal transduction (10, 11, 15). We specifically set out to determine the validity of oocyte as a model to predict the physiological effects of lithium on signal transduction pathways. Comparing lithium efflux after 24-hr lithium exposure in both the oocyte and the mammalian hippocampal cell line, we found no evidence for a pool of sequestered lithium in the hippocampal cell line, as observed in the oocyte (compare Figs. 4 and 7). This suggests that lithium is distributed differently in those cell types. Thus, our result indicates that although the oocyte presents an accessible model for the study of second messenger effects mediated by lithium, it should be used with caution because of the unknown contribution of the sequestered pool on predicting relevant physiological effects.
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