meats commonly eaten in the United States after treatment with sizable amounts of nitrite (*7*), that is, amounts considerably larger than commonly used in the processing of such foods (*4*), is noteworthy. This may be due to the fact that nitrite is preferentially bound to myoglobin (*8*). Second, the mutagenic activity found in nitrosated fish might be relevant to the question of the etiology of human gastric cancer.

Our observation that ascorbic acid prevents the formation of mutagenic activity in nitrite-treated fish is in agreement with earlier findings of the protective effect of vitamin C in other systems (*9*). It also supports the reported inverse association of the consumption of foods rich in ascorbate and gastric cancer (*5*). Thus, vitamin C and foods rich in this essential micronutrient may be useful as an easily available means of prevention of this cancer.

Further work is needed to isolate the mutagenic principles.

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## Lithium Ion Entry Through the Sodium Channel of Cultured Mouse Neuroblastoma Cells: A Biochemical Study

Abstract. *Lithium ion entry at low concentrations (1 to 5 m*M*) into an electrically active adrenergic clone of mouse neuroblastoma cells was stimulated by veratridine; and this stimulation was blocked by tetrodotoxin. These data provide biochemical evidence that lithium ions enter by way of the sodium channel which may be a major pathway for entry of this ion into electrically active cells.*

The efficacy of lithium salts for treating affective illness (mania and depression) has been well established since Cade (*1*) first used them for this purpose in 1949. Although many investigators have sought to determine the biochemical effects of $Li^+$ which might lead to a better understanding of these psychiatric disorders, the mechanism of action of $Li^+$ remains uncertain (*2*).

Interest has focused recently on the entry of $Li^+$ into cells. In studies with pharmacologic concentrations of $Li^+$, many investigators have used red blood cells (*3*, *4*) rather than nervous tissue, the probable site of lithium's therapeutic effects. The red blood cell is easily accessible, and electrolyte transport by its membrane is similar to that of a neuron (*3*). However, electrolyte transport by a membrane of a neuron is different from that of a red cell in that the neuronal membrane has properties that allow the initiation and propagation of an action potential. The basis of one of these properties is thought to be sodium channels or ionophores which gate the flow of sodium ions to make the membrane electrically excitable (*5*).

Electrophysiological studies with very high concentrations of $Li^+$ (usually total $Na^+$ replacement by $Li^+$) have shown that $Li^+$ will support an action potential for a brief time (*6*). Such results suggest that $Li^+$ enters through the sodium channel. Whether $Li^+$ enters through the sodium channel at therapeutic concentrations and in the presence of normal concentrations of $Na^+$ is unknown.

The sodium channel may be studied biochemically in cultured cells with the use of the alkaloid veratridine (*7*) which selectively increases the permeability of electrically excitable membranes to $Na^+$ (*8*). With these biochemical techniques, Catterall and Nirenberg (*9*) showed that veratridine-stimulated $Na^+$ uptake occurred only in cells that were electrically excitable. These results were confirmed by others (*10*). Using similar pharmacologic tools, we have obtained biochemical evidence for the entry of $Li^+$ into mouse neuroblastoma cells through the sodium channel at low concentrations of $Li^+$ and high concentrations of $Na^+$. The entry of $Li^+$ into these cells by way of the sodium channel occurs more rapidly and to a greater extent than the entry of $Li^+$ into the resting cell.

We used a clone of mouse neuroblastoma cells, N1E-115, as a model for the adrenergic neuron (*11*). These cells have electrically excitable membranes (*12*), enzymes that participate in the synthesis and catabolism of catecholamines (*11*), and muscarinic acetylcholine receptors that are blocked by certain psychotropic drugs (*13*).

The time course for the entry of $Li^+$ into cultured neuroblastoma cells was linear for approximately 30 minutes in the absence or presence of veratridine (Fig. 1). This alkaloid, however, caused a stimulation of $Li^+$ entry at all time points. The intracellular volume of the cells was determined by using [$^{14}$C]inulin and [$^3$H]OH as external and internal volume markers, respectively; this volume was 8.1 $\mu$l per milligram of cell protein or 5.6 $\mu$l per $10^6$ cells. Thus, the cell to medium ratio of $Li^+$ at 30 minutes was ten- and fourfold with and without veratridine, respectively.

The magnitude of this stimulation of $Li^+$ entry by veratridine was dependent on the concentration of veratridine bathing the cells (Fig. 2A). At the highest concentration tested (0.5 m*M*), veratridine stimulated $Li^+$ entry more than six times above control levels. In electrophysiological studies with this clone (*14*), 50 $\mu M$ veratridine, a concentration that caused a threefold stimulation of lithium ion entry (Fig. 2A), caused a reversal of polarization of the transmembrane potential in the absence of any electrical stimulus.

Tetrodotoxin (*8*) inhibited the veratridine-stimulated $Li^+$ entry (Fig. 2B). Tetrodotoxin, which blocks the action potential by blocking the "fast" sodium channel, inhibited by about 50 percent the veratridine-stimulated $Li^+$ entry at a concentration of about $10^{-7}M$. Tetrodotoxin completely blocked the sodium-dependent phase of the action potential in this clone at 3 $\mu M$, a concentration that would nearly completely block the veratridine-stimulated $Li^+$ entry (Fig. 2B) (*14*). Ouabain (5 m*M*), an inhibitor of $Na^+$- and $K^+$-dependent adenosinetriphosphatase, had no effect on $Li^+$ entry

alone or in the presence of veratridine (data not shown).

Although for these experiments we used 5 m*M* $Li^+$, a concentration that would be highly toxic to a human being, we obtained similar results with 1 m*M* $Li^+$, a concentration that would be in the middle of the therapeutic range. Thus veratridine (0.1 m*M*) caused a sixfold stimulation in the rate of $Li^+$ entry when the $Li^+$ concentration was 1 m*M*. However, at this concentration, $Li^+$ entry in the absence of veratridine at early time points was only slightly greater than nonspecific (zero time) values; whereas with 5 m*M* $Li^+$, this entry was at least twice the nonspecific values. Hence, most experiments were done at the higher concentration of $Li^+$.

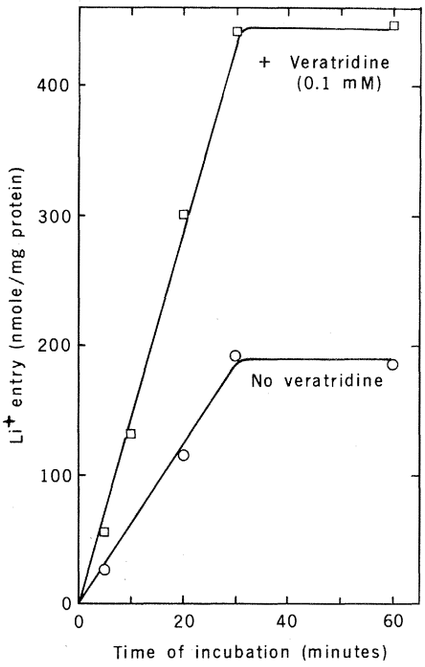


Fig. 1. Time curve for the entry of $Li^+$ into cultured mouse neuroblastoma cells. Cells (subculture 14) were grown to confluency in Falcon flasks (75 $cm^2$/250 ml) with a Dulbecco-Vogt modification of Eagle's medium (DMEM, high glucose, no pyruvate; Gibco) supplemented with 10 percent (by volume) fetal calf serum (Colorado Serum) in an atmosphere of 10 percent $CO_2$ and 90 percent humidified air at 37°C. Cells were then subcultured into Linbro wells (model FB16-24TC, Bellco Glass) with a modified Puck's $D_1$ solution (*16*). For cells in wells, medium (1 ml) was changed twice daily to enhance cohesiveness of this clone to the surface of the well. After 2 days, cells were used for assays performed at 37°C in 250 $\mu$l of phosphate-buffered saline (PBS) containing the following: 110 m*M* NaCl; 5.3 m*M* KCl; 1.8 m*M* $CaCl_2$; 1.0 m*M* $MgCl_2$; 2.0 m*M* $Na_2HPO_4$; 25 m*M* glucose; 50 m*M* sucrose; and 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) titrated to *p*H 7.4 with 1*N* NaOH. The osmolality was 335 to 340 mosmole. The assay technique is described in detail elsewhere (*17*). Briefly, growth medium was removed by aspiration and cells were washed once with 0.5 ml of PBS. At zero time, lithium chloride (Baker) at a final concentration of 5 m*M* with or without 0.1 m*M* veratridine (Aldrich) was added to the cells. At the various time points the incubation medium was aspirated and the cells were washed twice with 0.5 ml of ice-cold PBS. Cell protein and lithium were quantitatively recovered from the wells with the addition of 0.5 ml of distilled water, trituration, and two washes with 0.5 ml of water. The solution of cells and $Li^+$ was brought to a total volume of 2.0 ml and a concentration of approximately 8 percent in trichloroacetic acid; it was then vortexed vigorously and placed in an ice bath for 10 minutes prior to being centrifuged (Damon/IEC; CRU-5000) at 2500 rev/min for 5 minutes. The supernatant was then assayed for lithium by means of a Jarrel-Ash flame spectrophotometer set at a wavelength of 670.4 nm. Nonspecific recovery of lithium was determined from a zero time point value and subtracted from all data points. All points were determined in duplicate for the amount of lithium and protein, and the number of cells per well. Protein was determined by a modification of the method of Lowry *et al.* (*18*) with bovine serum albumin as standard. Cell counts were determined with a Coulter counter model $Z_F$. For this experiment the average number of cells per well was $2.8 \times 10^5$ and the average amount of protein per well was 170 $\mu$g.

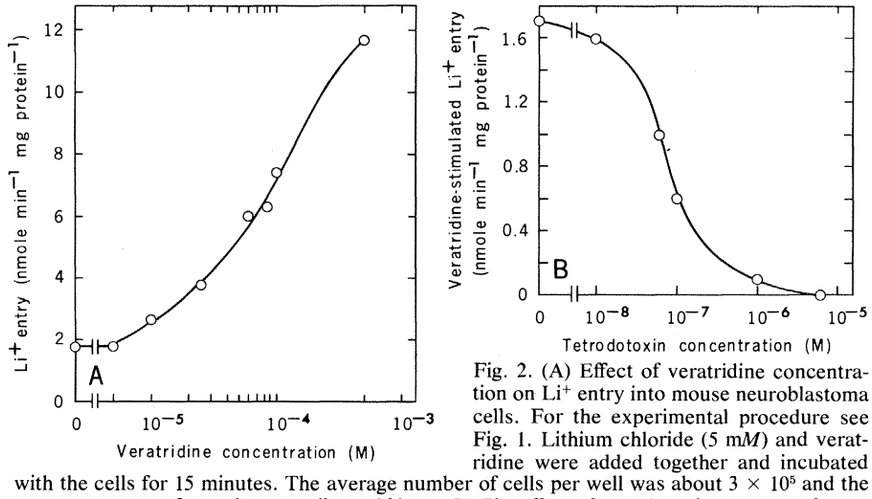


Fig. 2. (A) Effect of veratridine concentration on $Li^+$ entry into mouse neuroblastoma cells. For the experimental procedure see Fig. 1. Lithium chloride (5 m*M*) and veratridine were added together and incubated with the cells for 15 minutes. The average number of cells per well was about $3 \times 10^5$ and the average amount of protein per well was 280 $\mu$g. (B) The effect of tetrodotoxin concentration on the veratridine-stimulated entry of $Li^+$ into mouse neuroblastoma cells. Tetrodotoxin (Sigma) was added to the cells (to give the indicated final concentrations) just prior to the additions of lithium chloride and veratridine (0.1 m*M*). The average number of cells per well was $2.5 \times 10^5$ and the average amount of protein per well was 300 $\mu$g. In the absence of veratridine and tetrodotoxin $Li^+$ entry was 1.6 nmole per minute per milligram of protein, and this value has been subtracted from the experimental data to give the veratridine-stimulated values.

These results provide biochemical evidence that $Li^+$ enters through the sodium channel of an electrically active clone of mouse neuroblastoma cells at low concentrations of $Li^+$ and high concentrations of $Na^+$. This pathway of entry of $Li^+$ into a nerve cell may be the most important one with respect to the therapeutic effects of lithium salts in the treatment of mania. Such entry may inhibit the entry of $Ca^{2+}$ during the action potential and thereby inhibit the stimulus-coupled release of norepinephrine (*15*). In addition, this study suggests that the sodium channel in electrically active cultured cells may more conveniently be studied with $Li^+$ than with radioactive sodium which emits gamma radiation.


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