Inhibition by Lithium of Cyclic GMP Formation without Inhibition of Nitric Oxide Generation in the Mouse Neuroblastoma Cell (N1E-115)

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We investigated the effects of lithium ion (Li^+) on muscarinic receptor-mediated nitric oxide (NO) generation, and guanylate cyclase (GCase) activation using the mouse neuroblastoma clone, N1E-115. The levels of released NO were determined by measuring the levels of nitrite/nitrate in the incubation medium, and the activity of GCase was measured with an assay for cellular cyclic [^3H] GMP levels. We determined that Li^+ had no effects on muscarinic receptor-activated elevation of nitrite/nitrate levels, which were significantly inhibited

by 100 μM L- N^{G} -monomethylarginine, although it has been reported that Li^+ inhibits muscarinic receptor-activated cyclic GMP formation in the cells. In addition, Li^+ inhibited the cyclic GMP formation induced by an NO donor, sodium nitroprusside (SNP), in both intact cells and a crude cellular homogenate; thus, the inhibition by Li^+ of muscarinic receptor-mediated cyclic GMP synthesis appeared to be at the level of GCase, but not NO synthase. [Neuropsychopharmacology 11:119–124, 1994]

KEY WORDS: *Lithium chloride; Nitric oxide; Cyclic GMP; N1E-115 cells; Muscarinic receptor; Sodium nitroprusside*

Lithium (Li^+) is often prescribed for patients with manic depressive illness (MDI), and has a preventive effect for recurrence of MDI (Cade 1949; Schou 1968). The precise mechanisms underlying the effects of Li^+ in such psychiatric illnesses as MDI remain unknown. Hokin and Hokin (1953) reported that activation of muscarinic receptors stimulated the breakdown of phosphatidylinositol (PI), and it has been shown that Li^+ caused the accumulation of the product of this breakdown, myo-inositol-1-phosphate, in the brain through inhibition of myo-inositol-1-phosphatase (Hallcher and Sherman 1980; Berridge 1984). Although many investigations have focused on the PI cycle as the site of therapeutic action of this ion, no consensus as to the basic mechanism of Li^+ actions has yet been established.

We have already reported that Li^+ significantly inhibited cyclic GMP formation induced by stimulation with carbamylcholine, a muscarinic receptor agonist, by using a mouse neuroblastoma clone: N1E-115 cells (Kanba and Richelson 1984; Kanba et al. 1986). In addition, when sodium channel was activated, Li^+ had an increased potent intracellular effect (Kanba et al. 1991). We hypothesized that the inhibitory action of Li^+ on cyclic GMP formation may be associated with its clinical efficacy and toxicity, and that the action may become

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increasingly potent in pathologically-active neurons underlying manic-depressive disorder.

Conversely, in many types of cells, guanylate cyclase (GCase) is activated by nitric oxide (NO), which is produced by nitric oxide synthases (NOS) from L-arginine (Knowles et al. 1989; Garthwaite 1991). Activation of the muscarinic receptors on N1E-115 cells can also induce production and release of NO by mediating the activation of NOS (Arroyo and Forray 1991). Despite our results and those of others, we hypothesized that the inhibitory effects of Li^+ on cyclic GMP formation are due to inhibition of NO synthesis, or GCase activation. The present studies were designed to investigate the mechanism by which Li^+ inhibits the muscarinic receptor-mediated cyclic GMP formation. First, to assess the possibility that Li^+ inhibits receptor-mediated NO synthesis, we examined if Li^+ inhibits muscarinic receptor-mediated NO generation in N1E-115 cells by measuring the nitrite/nitrate levels in the incubation medium. Second, in order to determine if Li^+ directly inhibits activation of guanylate cyclase, we determined the guanylate cyclase activity in both intact cells and a crude homogenate of N1E-115 cells containing several concentrations of Li^+ by measuring the levels of cyclic GMP elicited by SNP.

METHODS

Cell Culture

N1E-115 cells (passages 12–14) were cultured in Dulbecco's MEM as described previously (Kanba and Richelson 1984). The cells were used for the experiment when they had reached a state of confluence in 25 cm² culture flasks (Corning).

Assay of Nitrite/Nitrate Levels in the Incubation Medium of N1E-115 Cells

After the cells had been washed three times with 3 ml of Hanks' solution (137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl_2 , 0.8 mM MgSO_4 , 0.338 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 5.6 mM glucose, 4.2 mM NaHCO_3 , pH 7.4), they were preincubated at 37°C for 30 min in 3 ml of Hanks' solution. Five-hundred microliters of the incubation medium were collected, and 2.5 μl of 100 mM L-NG-monomethylarginine (L-NMMA), for inhibition of NOS, or vehicle was added. The cells were then stimulated with carbamylcholine to achieve 0.01, 0.03, 0.1, 0.3, 1, or 3 mM as a final concentration. Five-hundred microliters of the incubation medium were collected again at 30 seconds after stimulation.

In another series of experiments, after the cells had been washed three times as described previously, they were preincubated at 37°C for 30 minutes in 3 ml of Hanks' solution containing various concentrations of

lithium chloride (LiCl), sodium chloride (NaCl), or ammonium chloride (NH_4Cl). Five-hundred microliters of the incubation medium were collected immediately before and 30 seconds after stimulation with 1 mM carbamylcholine. Two and one half microliters of 100 mM L-NMMA, or vehicle were added immediately before stimulation.

When the series of experiments had been completed, the numbers of cells were counted after scraping. Nitrite/nitrate concentrations in the incubation medium were quantitated with an automated system based on the Griess reaction (Hishikawa et al. 1992). The values obtained by subtracting nitrite/nitrate levels before stimulation from those obtained after stimulation were regarded as nitrite/nitrate derived from the cells.

Assay of Cyclic GMP Levels in Intact N1E-115 Cells

N1E-115 cells were cultured as described previously. Detached and washed N1E-115 cells were suspended in 3 ml of physiological iso osmolar phosphate buffer containing saline with glucose and sucrose (PBS; 110 mM NaCl, 5.3 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 25 mM glucose, 2 mM Na_2HPO_4 ; pH adjusted to 7.35; osmolality adjusted to 335 ± 5 mOsmol with sucrose) at 37°C ($3\text{--}5 \times 10^6$ cells/ml). The cells were transferred to a 25-ml Erlenmeyer flask and 30 μCi of [³H]guanine was added. The cells were placed in a shaker bath at 37°C and incubated for 45 min. After labeling with [³H]guanine, the cells were centrifuged gently, and the supernatant containing excess radioactivity was aspirated. The pelleted cells were then resuspended in PBS at 37°C to about 3 to 4×10^5 cells/ml, and were distributed into the wells of a multiwell plate in 320- μl aliquots. Several concentrations of LiCl, NaCl, and NH_4Cl were then added in a 100- μl aliquot, and the cells were further incubated at 37°C for 30 minutes. The cells were stimulated with sodium nitroprusside (SNP; an 80- μl aliquot), to achieve 10, 25, 50, 100, 200, 300, or 400 μM as the final concentration, for 30 seconds. The assay was stopped with the addition of 50 μl of 50% trichloroacetic acid. The levels of cyclic [³H]GMP were then determined as described previously (Richelson et al. 1978).

Assay of Guanylate Cyclase Activity in Homogenates of N1E-115 Cells

Detached and washed cells were suspended in 50 mM Tris-HCl buffer (pH 7.6 at 37°C) to about 2 to 3×10^6 cells/ml. The cells were then sonicated for 30 seconds (setting no. 3, Ultrasonic Cell Disrupter, Kontes Glass Co., Vineland, NJ) on ice. The assay of GCase activity was begun with the addition of 50 μl of the cell homogenate to the reaction cocktail. The final volume of the

Figure 1. Nitrite/nitrate levels in the incubation medium of intact N1E-115 cells following 30 sec carbamylcholine stimulation (○). Carbamylcholine enhanced the levels of nitrite/nitrate in a dose dependent manner, and the elevation of nitrite/nitrate levels was significantly inhibited by preincubation with 100 μ M L-NG-monomethylarginine (●). Circles and vertical bars represent means and SEM, respectively, of five independent experiments. Circles without bars indicate that the size of the circle is greater than the length of the bar.

assay was 150 μ l and consisted of the following: 50 mM Tris-HCl (pH 7.6); 5 mM $MgCl_2$; 0.5 mM 3-isobutyl-1-methylxanthine; 2.5 mM cyclic GMP; 5 mM creatine phosphate; 0.6 U of creatine phosphokinase; 0.25 mM GTP; 3 to 4 $\times 10^5$ dpm of [3H]GTP; several concentrations of LiCl and SNP. Incubations were carried out at 37°C for 5 minutes and reactions were stopped by the addition of 30 μ l of 50% trichloroacetic acid. The cyclic [3H]GMP formed was determined as described previously (Richelson et al. 1978).

Figure 2. Effect of 30 min preincubation with LiCl, NaCl, or NH_4Cl on 300 μ M SNP-activated cyclic GMP formation in intact N1E-115 cells. The ordinate represents the percentage for cyclic GMP levels elicited by 300 μ M SNP in the absence of cations. LiCl inhibited the elevated cyclic GMP formation in a dose-dependent manner (○). NaCl (●) and NH_4Cl (□) showed no significant inhibitory effects. For circles and vertical bars, see legend in Figure 1.

RESULTS

Effects of LiCl on Muscarinic Receptor-activated Nitrite/Nitrate Derived from N1E-115 Cells

Carbamylcholine enhanced the levels of nitrite/nitrate in the incubation medium in a dose dependent manner, and the elevation of nitrite/nitrate levels was

Table 1. The Effects of LiCl, NaCl, and NH_4Cl of Concentrations on Muscarinic Receptor-activated Nitrite/Nitrate Levels in the Medium

Concentration (mM)	Nitrite/Nitrate (pmol/ 10^6 cells)			100 μ M L-NG-monomethyl-arginine		
	LiCl	NaCl	NH_4Cl	LiCl	NaCl	NH_4Cl
2.5	303 $\pm$ 25	298 $\pm$ 31	289 $\pm$ 37	60 $\pm$ 6	59 $\pm$ 9	62 $\pm$ 8
5	300 $\pm$ 30	301 $\pm$ 25	294 $\pm$ 29	57 $\pm$ 9	62 $\pm$ 10	50 $\pm$ 15
10	290 $\pm$ 38	295 $\pm$ 28	305 $\pm$ 30	61 $\pm$ 4	58 $\pm$ 8	56 $\pm$ 6
20	295 $\pm$ 28	304 $\pm$ 35	300 $\pm$ 30	59 $\pm$ 7	65 $\pm$ 12	54 $\pm$ 9
30	272 $\pm$ 23	269 $\pm$ 28	280 $\pm$ 20	50 $\pm$ 9	53 $\pm$ 9	51 $\pm$ 12
50	245 $\pm$ 32	241 $\pm$ 35	250 $\pm$ 35	45 $\pm$ 8	45 $\pm$ 13	41 $\pm$ 10

The data are mean $\pm$ SEM of quadruplicate independent experiments. After the cells had been incubated with each concentration of LiCl, NaCl, or NH_4Cl for 30 minutes, they were stimulated with 1 mM carbamylcholine. Immediately before stimulation, 100 μ M L-NMMA, or vehicle were added. The levels of nitrite/nitrate in the incubation medium, that was collected 30 seconds after stimulation, were determined using the Griess reaction.

Figure 3. Effect of 10 (●), 25 (□), and 50 (■) mM LiCl on several concentrations of SNP-activated cyclic GMP formation in intact cells. Cells not exposed to LiCl (○) responded maximally to 300 μM SNP. For circles and vertical bars, see legend in Figure 1.

significantly inhibited by preincubation with 100 μM L-NMMA (Figure 1). In intact N1E-115 cells, LiCl, as well as NaCl and NH₄Cl, exerted no significant inhibitory effects on carbamylcholine (1 mM)-stimulated elevation of nitrite/nitrate levels (Table 1).

Effects of LiCl on SNP-induced Cyclic GMP Levels in Intact N1E-115 Cells

In intact cells, elevation of the cyclic GMP levels elicited by 300 μM SNP was inhibited by pretreatment with several concentrations of LiCl for 30 minutes in a dose-dependent manner (Figure 2). IC₅₀ was 10 ± 2 mM ($n = 4$). NaCl and NH₄Cl had no significant inhibitory effects, but did appear to suppress the response at levels higher than 30 mM because of high osmolarity (Figure 2). Elevation of the cyclic GMP levels elicited by 300 μM SNP was maximal in intact cells. Concentrations of SNP higher than 400 μM decreased this response (Figure 3). LiCl inhibited cyclic GMP formation induced by various concentrations of SNP, and this inhibition by LiCl was not restored by SNP doses as high as 300 μM (Figure 3).

Effects of LiCl on Cyclic GMP Levels in a Crude Homogenate of N1E-115 Cells

In a crude homogenate of N1E-115 cells, LiCl inhibited SNP (from 10^{-8} to 10^{-5} M)-activated cyclic GMP formation in a dose-dependent manner (Figure 4). Up to 10^{-5} M SNP did not restore the inhibition by 10, 25, or 50 mM LiCl, though a concentration higher than 10^{-4} M did appear to restore this inhibition.

Figure 4. Effect of 0 (○), 10 (●), 25 (□), and 50 (■) mM LiCl on several concentrations of SNP-activated cyclic GMP formation in a cellular homogenate of N1E-115 cells. For circles and vertical bars, see legend in Figure 1.

DISCUSSION

We investigated the effects of Li⁺ on muscarinic receptor-mediated NO generation and activation of GCase using a mouse neuroblastoma clone, N1E-115. We previously reported that Li⁺ inhibits muscarinic receptor-activated cyclic GMP formation in N1E-115 cells (Kanba and Richelson 1984). There is agreement that activation of soluble GCase after receptor activation is a consequence of receptor mediated Ca²⁺ influx and activation of NOS (Dunnwiddie and Hoffer 1982; Knowles et al. 1989). Although the mechanism by which Li⁺ inhibits cyclic GMP formation remains speculative, Li⁺ is known to increase rather than decrease cellular Ca²⁺ levels by affecting transmembrane Na⁺/Ca²⁺ exchange processes (Aldenhoff and Lux 1984). Consequently, we hypothesized that Li⁺ may exert its inhibitory actions by inhibiting the activation of either NOS or GCase.

Because carbamylcholine enhanced the levels of nitrite/nitrate in a dose-dependent manner and the increase in these levels was significantly inhibited by preincubation with 100 μM L-NMMA (Figure 1), it was suggested that the elevated nitrite/nitrate levels reflect NO production/release from L-arginine following the activation of muscarinic receptors. Because incubation with LiCl for 30 minutes, which appeared to be enough time to inhibit receptor-mediated cyclic GMP formation (Kanba et al. 1986), did not reduce nitrite/nitrate levels in the incubation medium (Table 1), it was suggested that Li⁺ does not affect receptor-mediated activation of NOS and that interference with the activation of NOS cannot explain the described inhibitory effects of Li⁺ on cyclic GMP formation.

It is known that certain cofactors including tetrahydrobiopterine, calcium-calmodulin, flavin mononucleotide, flavin adenine dinucleotide, and heme are necessary for NO synthase to be activated (Nathan 1992). Considering the effects of Li^+ on Ca^{2+} influx (Aldenhoff and Lux 1984), it was expected that Li^+ might change the activity of NOS by affecting activation of the calcium-calmodulin system. However, our results indicate that Li^+ does not affect the receptor-mediated activation of NOS. We cannot explain why Li^+ did not exert the expected action on NO synthesis, despite its effects on Ca^{2+} influx.

The IC_{50} of LiCl for cyclic GMP formation induced by NO donor SNP in intact cells was consistent with that for carbamylcholine-induced cyclic GMP formation in intact cells, which we previously reported (Figure 2) (Kanba and Richelson 1984). Because dose-dependent inhibition by LiCl was observed in both the intact cells and a crude cellular homogenate (Figure 3, Figure 4), we speculate that Li^+ is capable of inhibiting cyclic GMP formation without mediating receptor activation. SNP is known to activate GCase by producing NO enzymatically in the cells. Several oxidizing agents, such as methylene blue and $\text{KFe}(\text{CN})_6$, also inhibit SNP-induced cyclic GMP accumulation by converting NO to higher nitrogen oxides (Waldmann and Murad 1987). Li^+ does not share these oxidizing properties and may act via other mechanisms; therefore, these findings suggest that LiCl acts directly on GCase to inhibit the NO-mediated activation.

Inhibition of cyclic GMP formation by LiCl was not restored by stimulation with 300 μM SNP in intact cells. Because of SNP's inherent cytotoxicity in intact cells, we did not examine if this response was restored at concentrations of SNP higher than 400 μM . It did appear to be restored by higher concentrations of SNP in the experiments using cell homogenates. The reason for the biphasic inhibition curve is unknown. It is possible that Li^+ acts directly at the catalytic subunit of GCase by modifying substrate binding at lower levels and that Li^+ affects the activation subunit by interfering with reactions of NO at higher levels. In addition, because SNP is known to activate soluble GCase through multiple catalytic sites with different K_m values (Lewicki et al. 1982), inhibition of GCase activity may occur at different catalytic sites depending upon the concentration of Li^+ .

The clinically effective concentration of Li^+ is much lower than the GCase inhibiting dose observed in this study; however, a recent report showed that therapeutic concentrations of Li^+ inhibit SNP-induced accumulation of cyclic GMP in human lymphocytes (Schubert et al. 1991). Jope et al. (1992) reported that cyclic GMP production was reduced by acute and chronic administration of a therapeutic concentration of Li^+ . Furthermore, methylene blue which also in-

hibits SNP-induced cyclic GMP formation has been proven to have a prophylactic effect in manic-depressive disorder like Li^+ (Naylor et al. 1986); thus, it is interesting to know if other cyclic GMP inhibiting agents would have a similar clinical effect.

In summary, it was suggested that Li^+ directly inhibits GCase activation without inhibition of receptor-mediated NO generation.

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