Lithium Ion Inhibits Nerve Growth Factor–induced Neurite Outgrowth and Phosphorylation of Nerve Growth Factor–modulated Microtubule-associated Proteins

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ABSTRACT LiCl (2.5–20 mM) reversibly suppressed nerve growth factor (NGF)-induced neurite outgrowth by cultured rat PC12 pheochromocytoma cells. Similar concentrations of LiCl also reversibly blocked NGF-dependent regeneration of neurites by PC12 cells that had been primed by long-term pre-exposure to NGF and by cultured newborn mouse sympathetic neurons. In contrast, transcription-dependent responses of PC12 cells to NGF such as priming and induction of the NGF-inducible large external glycoprotein, occurred despite the presence of Li^+. SDS PAGE analysis of total cellular phosphoproteins (labeled by 2-h exposure to ^{32}P-orthophosphate) from neurite-bearing primed PC12 cells revealed that Li^+ reversibly inhibited the phosphorylation of a band of M_r 64,000 that was barely detectable in NGF-untreated PC12 cells. However, Li^+ did not appear to affect the labeling of other phosphoproteins in either NGF-primed or untreated PC12 cultures, nor did it affect the rapid increase in phosphorylation of several proteins that occurs when NGF is first added to unprimed cultures. Several criteria indicated that the NGF-inducible phosphoprotein of M_r 64,000 is a microtubule-associated protein (MAP). Of the NGF-inducible phosphorylated MAPs that have been detected in PC12 cells (M_r 64,000, 72,000, 80,000, and 320,000), several (M_r 64,000, 72,000, and 80,000) were found to be substantially less phosphorylated in the presence of Li^+. Neither a phorbol ester tumor promotor nor permeant cAMP analogs reversed the inhibitory effects of Li^+ on neurite outgrowth or on phosphorylation of the component of M_r 64,000. Microtubules are a major and required constituent of neurites, and MAPs may regulate the assembly and stability of neuritic microtubules. The observation that Li^+ selectively inhibits NGF-induced neurite outgrowth and MAP phosphorylation suggests a possible causal relationship between these two events.

Little is understood of the biochemical changes that underlie the outgrowth of neuritic processes in developing neurons. One critical aspect of neuronal differentiation is rearrangement of the neuronal cytoskeleton, leading to specific and directed elongation of microtubules into growing neuritic cytoplasmic extensions. Among the best studied models for examining the mechanism of neurite outgrowth are the neuronal and neoplastic target cells of the polypeptide neurotrophic signal, nerve growth factor (NGF)[1] (1, 2).

[1] *Abbreviations used in this paper:* MAP, microtubule-associated protein; NGF, nerve growth factor; NILE, NGF-inducible large external; PMA, phorbol myristate acetate; TCA, trichloroacetic acid.

One widely studied target cell of NGF is the PC12 rat pheochromocytoma cell line (3). PC12 cells resemble adrenal medullary chromaffin cells, but when exposed to NGF for several days or more, they stop dividing and undergo neuronal differentiation that is manifested by extension of long, branching neurites, onset of electrical excitability, and induction of several neuronal proteins (2, 3).

The mechanism of NGF-promoted neurite outgrowth in PC12 cells, summarized in the priming model (4–7), involves the activation of both transcription-dependent and transcription-independent pathways (4). The former appears to stimulate the relatively slow synthesis and accumulation of a pool

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of material necessary for neurite outgrowth. Sufficient accumulation of this material primes cells for rapid, transcription-inhibitor-resistant, NGF-dependent neurite outgrowth (4–7). The dramatic change in morphology of PC12 cells on exposure to NGF is accompanied by strikingly few compositional changes. It was thus hypothesized that neurite outgrowth from PC12 cells is a consequence not of major biochemical changes but of structural reorganization, mediated by a small number of NGF-induced biochemical alterations. Of relevance to this, priming appears to be accompanied by a significant enhancement in microtubule stability (8) as well as by a large induction of MAP 1.2, a phosphorylated high molecular weight microtubule-associated protein (MAP) (9). The transcription-independent component of NGF-stimulated neurite outgrowth, unlike the priming-related changes, appears to be rapid in onset. Transcription-independent actions of NGF include rapid cell surface changes (10), enhanced growth cone motility (11), selective increases in protein phosphorylation (12), and induction of small molecule uptake (13).

In an attempt to further dissect the steps involved in the NGF mechanism of action with chemical probes, we have examined the effect of Li^+ on NGF-responsive cells. Lithium ion has a variety of effects on the responses of cells to exogenous polypeptide signals, including enhancement of the mitogenic actions of epidermal growth factor and insulin (14, 15) and abrogation of an anti-proliferative effect of interferon (16). We report here that while Li^+ appears to have little effect on NGF-dependent priming, it blocks NGF-promoted neurite outgrowth in PC12 cells and mouse sympathetic neuron cultures and selectively inhibits phosphorylation of several medium molecular weight, NGF-responsive microtubule-associated proteins.

Portions of this work have been previously presented in abstract form (17, 18).

MATERIALS AND METHODS

Cell Culture: PC12 cells were cultured as previously described (3) in 35-mm collagen-coated dishes ($2–5 \times 10^5$ cells/dish) in complete medium consisting of 85% RPMI 1640, 10% horse serum, and 5% fetal calf serum with or without 50 ng/ml of NGF (19) and with 0–20 mM LiCl (added from a 1 M stock solution, as indicated). Cultures were refed every 3 d unless noted otherwise. Cells were primed with NGF for 2–3 wk to produce neurite outgrowth.

Newborn mouse superior cervical ganglia were dissociated and placed into monolayer culture as previously described (20) in complete medium containing NGF and 0–20 mM LiCl. Cells with the distinctive morphology of cultured sympathetic neurons (large, round, and phase-bright) were scored for the presence of neurites.

Neurite initiation from PC12 cells was scored by measuring the percentage of cells bearing processes two or more cell diameters long. For studies involving neurite regeneration, primed PC12 cells were washed three times with NGF-free medium and mechanically dislodged from the culture dishes. The latter treatment shears the neurite from the cell bodies (4). The cells were washed five times by low speed centrifugation and replated on collagen-coated dishes in complete medium containing either 50 ng/ml NGF and 0–20 mM Li^+ or lacking both Li^+ and NGF. Neurite regeneration was then scored 24 h later, as previously described (4).

Characterization of Glycoproteins: PC12 cultures with or without additives as described in the text were labeled for 72 h with 10 μCi/ml [^{3}H]fucose and processed by SDS PAGE and fluorography as previously described (21).

Phosphorylation Studies: Detection of Li^+-sensitive phosphoproteins was achieved by incubation of either primed or unprimed PC12 cultures with 0–20 mM LiCl for 0.5–1 h, followed by labeling at 37°C in a phosphate-free medium (9) containing 50–100 μCi/ml of ^{32}P-orthophosphate and 0–20 mM LiCl for up to 2 h. NGF was added to the appropriate unprimed cultures at the time of addition of ^{32}P-orthophosphate; primed cells were continuously exposed to NGF throughout labeling with ^{32}P-orthophosphate. Cells were harvested in sample buffer, electrophoresed on SDS polyacrylamide gels (7.5–15% gradients, 30-cm long), and the dried gels were autoradiographed as previously described (9).

Phosphorylated PC12 cell MAPs were prepared by minor modifications of previously described procedures (8, 22–24). Briefly, cultures were pretreated for 1 h and then phosphorylated as above, with either 15 mM Li^+, 50 μM nocodazole, or no additive present. The monolayer cultures were then extracted for 10 min at room temperature with a Triton-containing microtubule-stabilizing buffer (100 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM $MgSO_4$, 2 M glycerol, 0.1% Triton X-100, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, and 100 KIU/ml aprotinin) to produce an attached cytoskeletal preparation. After two washes with detergent-free stabilizing buffer, the cytoskeletons were then exposed to a destabilizing buffer (100 mM PIPES, pH 6.8, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 2 mM phenylmethylsulfonyl fluoride, 100 KIU/ml aprotinin) for 10 min at 0°C to selectively solubilize tubulin and MAPs (8, 22–24). The solubilized proteins were mixed with concentrated sample buffer and processed for SDS PAGE and autoradiographed as above.

Tubulin Polymerization and Electron Microscopy: In vitro assembly of microtubules was assayed by published methods (25). For electron microscopic examination of microtubules, primed cultures were treated for 2.5 h with either no additive, 15 mM LiCl, or 50 μM nocodazole. Stabilized cytoskeletons were then prepared as above, fixed, and processed for transmission electron microscopy as previously described (8).

Actinomycin D was obtained from Calbiochem-Behring Corp. (La Jolla, CA). ^{32}P-Orthophosphate and [^{3}H]fucose were obtained from New England Nuclear (Boston, MA). Nocodazole was from Aldrich Chemical Co. (Milwaukee, WI).

RESULTS

Effect of Li^+ on Neurite Generation and Outgrowth

The effect of Li^+ on NGF-dependent neurite generation by unprimed PC12 cells was examined. As illustrated in Fig. 1, PC12 cells do not possess neurites in the absence of NGF, but when exposed to the factor, slowly extend processes so that ~90% of the cells are neurite-bearing by 1 wk of treatment (3). The addition of LiCl to the culture medium along with NGF resulted in cell flattening and in suppression of neurite outgrowth (Fig. 1). Inhibition was consistently detected at concentrations as low as 2.5 mM Li^+ and was maximal at 10–15 mM. Addition of 20 mM NaCl rather than LiCl had minimal or no inhibitory effect.

If 10 mM LiCl was added to the cultures after 5 d of prior treatment with NGF alone, the percentage of cells with neurites decreased 78% in 1 d, and further neurite initiation was suppressed. However, if addition of this concentration of Li^+ was delayed until 10 d of NGF pretreatment, the percentage of neurite-bearing cells decreased by <18% after 4 d of Li^+ exposure. Thus, with increasing time of NGF treatment, preexisting neurites appeared to become increasingly resistant to Li^+.

Effect of Li^+ on NGF-dependent Priming and NGF-inducible Large External Glycoprotein Induction

To test the effect of Li^+ on NGF-dependent priming, cultures treated with NGF and 10 mM Li^+ for 6 d were washed and refed with Li^+-free medium containing NGF. Within 24 h of Li^+ washout, the cells generated large numbers of long neurites so that the extent of neurite outgrowth was comparable to that in control cultures exposed for 7 d to NGF alone. Moreover, as shown in Fig. 1, the rapid outgrowth that occurred after Li^+ removal was not blocked in the presence of 1 μg/ml actinomycin D. In contrast, unprimed cells grown

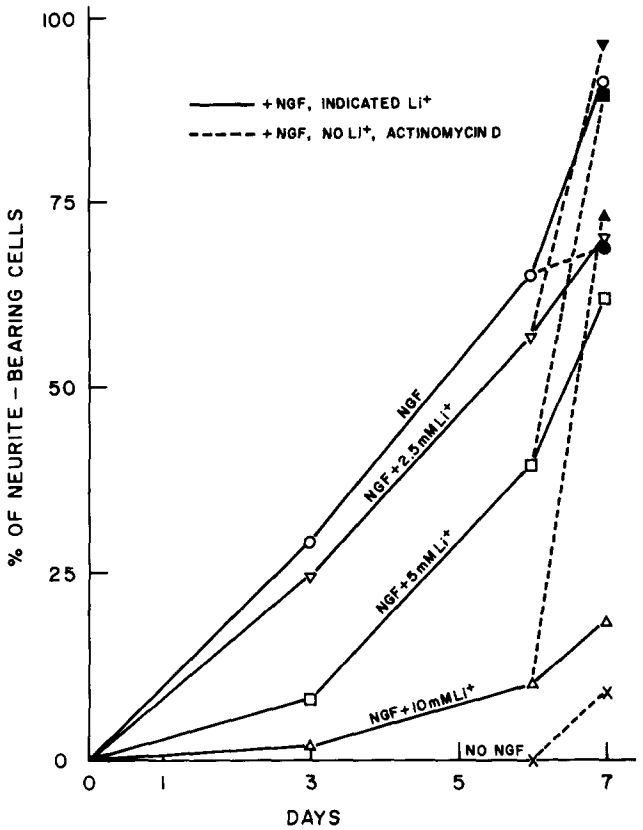

FIGURE 1 Effect of LiCl on neurite initiation and priming in PC12 cultures. PC12 cells were cultured in complete medium with or without 50 ng/ml of NGF and with 0–10 mM LiCl (as indicated in the figure). On day 6, Li^+-containing medium was removed from one set of dishes and replaced with Li^+-free medium containing NGF and 1 μg/ml of actinomycin D. At this time, NGF + 1 μg/ml actinomycin D was also added to a culture that had not previously received NGF. Data for the various Li^+-free, actinomycin-treated cultures are denoted by the dashed lines and solid symbols. In addition, in each case, replicate cultures were maintained under the conditions that prevailed before day 6. At each time-point, the percentage of cells with neurites was scored. At least 150 cells were scored per data point. Cultures exposed to Li^+ concentrations above 2.5 mM for 3 or more d showed cytotoxic changes in <10% of cells.

in the absence (Fig. 1) or presence of 10 mM Li^+ for 6 d, showed little neurite outgrowth when switched to Li^+-free medium containing NGF and 1 μg/ml actinomycin D. Previous studies have demonstrated that NGF-induced neurite outgrowth from unprimed PC12 cells is slow and inhibited by actinomycin D, whereas that from primed cells is rapid and resistant to inhibitors of RNA synthesis (4). Hence, Li^+ appears to block NGF-dependent neurite growth, but has little effect on priming in PC12 cultures.

To test the effect of Li^+ on a transcription-dependent action of NGF other than priming, we examined the induction of the NGF-inducible large external (NILE) glycoprotein by NGF (21). PC12 cultures were exposed either to medium alone, NGF, and NGF plus 7.5–10 mM LiCl for 6 d. [^{3}H]-Fucose was included in the culture during the last 3 d of treatment, and the glycoprotein content of the cultures was then analyzed by SDS PAGE and fluorography. The fluorographs revealed that Li^+ did not block the previously described transcription-dependent induction of NILE glycoprotein by NGF.

Effect of Li^+ on Neurite Regeneration

The Li^+-sensitivity of rapid neurite regeneration by primed PC12 cells was also examined. Primed cells were divested of their processes by mechanical disruption (4) and subcultured into fresh culture dishes with medium containing NGF and various concentrations of LiCl. With NGF alone, neurite regeneration was rapid; most of the cells produced long neurites within 24 h after passaging (Figs. 2 and 3). In the presence of 2.5–20 mM Li^+, neurite regeneration was suppressed; the ID_{50} was ~6.5 mM (Fig. 2*A*). Previous experiments (4) have shown that withdrawal of NGF from primed cells results in a time-dependent loss of priming. When primed cells are subcultured into medium without NGF, reintroduction of the factor after 2 d elicits only a limited degree of neurite production within the next 24 h (4) (Fig. 3). In the present experiments, primed cells were passaged into medium containing NGF plus 10–20 mM Li^+ for 2 d. Washout of the Li^+ after 2 d with continued exposure to NGF resulted in the rapid appearance of long neurites within 24 h (Figs. 2 and 3), indicating that priming had not been lost. These results support the suggestion that Li^+ reversibly suppresses NGF-elicited neurite outgrowth but does not interfere with maintenance of priming by NGF.

Effect of Li^+ on Neurite Outgrowth from Cultured Sympathetic Neurons

Cultures of dissociated newborn mouse sympathetic ganglia were also assessed for their sensitivity to Li^+. The cultures were maintained in the presence of NGF to promote neuronal survival and process outgrowth (1). As illustrated by the experiment shown in Fig. 4, Li^+ effectively suppressed the outgrowth of neurites; after 48 h of culture with 5 mM Li^+, less than half of the neurons bore fibers. This effect was reversible in that washout of Li^+ resulted in the appearance of normal neurites within 1–2 d (Fig. 4). Neurite outgrowth by cultured sympathetic neurons is NGF-dependent, rapid, and resistant to inhibitors of RNA synthesis (26) and therefore is analogous to outgrowth from primed PC12 cells (2, 5–7). Thus, also for neurons Li^+ appears to block NGF-promoted neurite growth, but not maintenance of the primed state. A fundamental difference between sympathetic neurons and PC12 cells is that only the former require NGF for their survival under the culture conditions employed here (RPMI medium plus 15% serum) (1). Visual comparison with non-Li^+-treated controls indicated that there was little or no death of sympathetic neurons in cultures maintained with NGF for at least 4 d in the presence of up to 20 mM Li^+. Since NGF deprivation for only 1 d results in death of neonatal sympathetic neurons (1, 2), it appears that Li^+ did not affect the capacity of NGF to maintain neuronal survival.

Effect of Li^+ on Phosphorylation of PC12 Cell Proteins

Because Li^+ appeared to have little effect on priming and thus on the NGF-activated transcriptional pathway, we explored the possibility that Li^+ might block a transcription-independent pathway activated by NGF. One such action of NGF is rapid alteration in the pattern of protein phosphorylations (12, 27). The most readily observable of such changes in unprimed PC12 cultures, detected by SDS PAGE, is a rapid

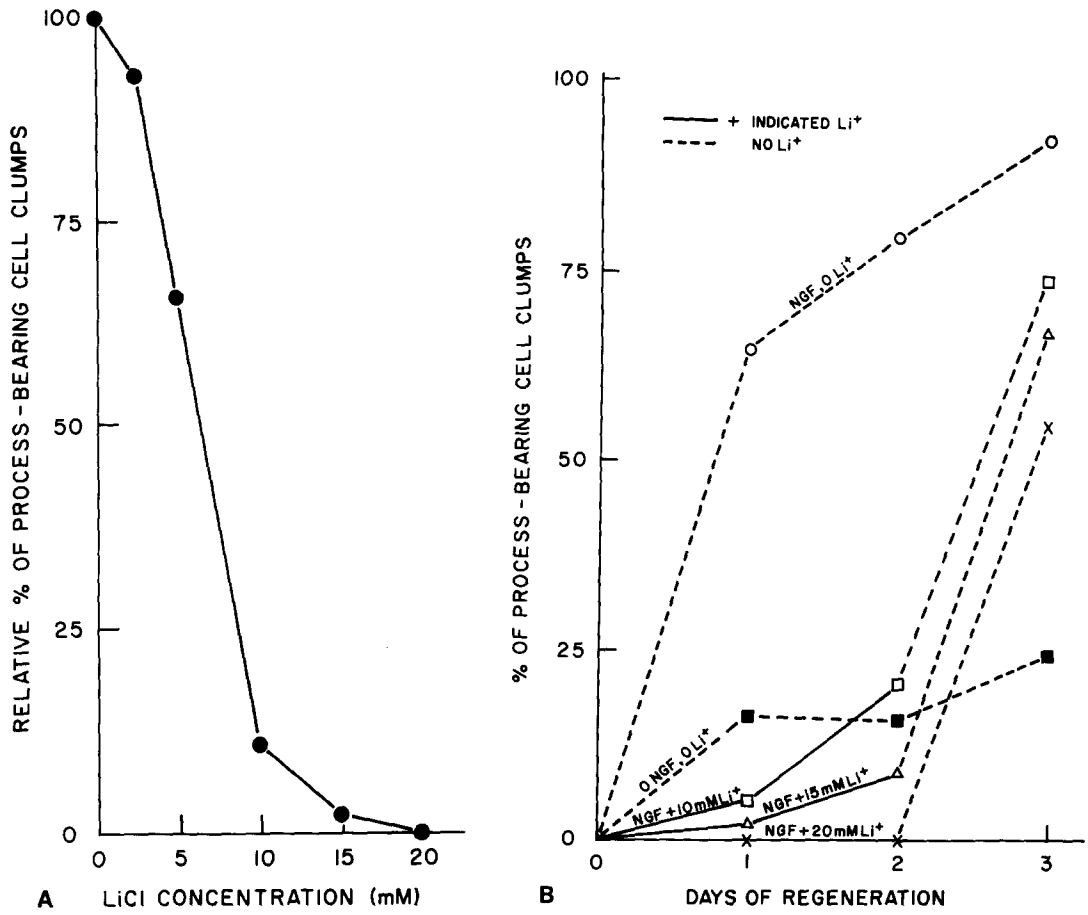

FIGURE 2 Effect of Li^+ on neurite regeneration by primed PC12 cells. (*A*) Inhibition of regeneration: dose-response relationship. Cells were pretreated (primed) for 26 d with NGF, and then dislodged from dishes, washed free of NGF, and replated on collagen-coated dishes in complete medium containing 0–20 LiCl either with or without 50 ng/ml NGF. 1 d later, cell clumps were scored for the presence of neurites. At each concentration of Li^+ tested, background neurite counts from cells passaged into medium lacking NGF but containing the same concentration of Li^+ were substracted from scores of responding cells in NGF-treated cultures. The resulting data are presented as percentages of maximal (i. e., non-Li^+-treated) response. From 68 to 175 cell clumps were scored per dish at each Li^+ concentration. (*B*) Reversibility. Cultures were generated as described in *A* and contained complete medium either with 50 ng/ml NGF and 0–20 mM LiCl or without both LiCl and NGF. 1 and 2 d later, the percentages of cell clusters with neurites were scored. On day 2, cultures were washed with Li^+-free medium and all (including the 0 NGF/0 Li^+ dishes) were refed with medium containing NGF, but lacking the LiCl. The cultures were then rescored for processes on day 3. From 36 to 175 cell clumps were scored per dish for each Li^+ concentration.

increase in incorporation of phosphate into a band of M_r 60,000 that is composed at least in large part of tyrosine hydroxylase (12). As illustrated in Fig. 5, the presence of 15 mM Li^+ for 1 h before, and then during 2 h of exposure to ^{32}P-orthophosphate and NGF, did not block the NGF-enhanced phosphorylation of the band of M_r 60,000 nor of a band of apparent M_r 54,000. Under these conditions, Li^+ also failed to produce consistently observed changes in the baseline pattern of phosphoproteins.

As cells become primed in response to NGF, several selective long-term changes in the phosphoprotein pattern are detectable by SDS PAGE. These include enhanced phosphate labeling of a band of M_r 320,000 identified as MAP 1.2 (9), of a band at M_r 64,000, and of several less prominent bands (see reference 9, and Figs. 6 and 8). Incubation of primed cultures with 15 mM Li^+ for 0.5 h before, and for 1–2 h during exposure to ^{32}P-orthophosphate, produced a marked inhibition of labeling of the band of M_r 64,000 (Figs. 6 and 7). In several experiments, minor bands at apparent M_r of 72,000 and 80,000 were also observed to be induced by longer-term NGF treatment, and phosphorylation of these was also suppressed by Li^+. Under these conditions (Fig. 6), there was no reproducible effect of Li^+ on phosphorylation of NGF-induced MAP 1.2 or of non-NGF-responsive phosphoproteins. A similar inhibitory effect on labeling of the band of M_r 64,000 was obtained in an experiment in which primed cells were preincubated with 15 mM Li^+ for 18 h before short-term labeling with ^{32}P-orthophosphate.

The dose-response relationship for inhibition of phosphorylation of the band of M_r 64,000 by Li^+ was assessed. As shown in Fig. 7, increasing concentrations of Li^+ caused an increasing blockade of the phosphorylation of this protein. As assessed by optical scanning of SDS PAGE autoradiograms, the ID_{50} for this effect was ~3.5 mM.

The effect of Li^+ on phosphorylation of the band of M_r 64,000 was rapidly reversible. When cultures were preincubated with Li^+ for 1.5 h, followed by washout of Li^+ and incubation with ^{32}P-orthophosphate for an additional 1.5 h, incorporation of label into the band of M_r 64,000 was as high as that in control cultures not exposed to Li^+ (data not shown).

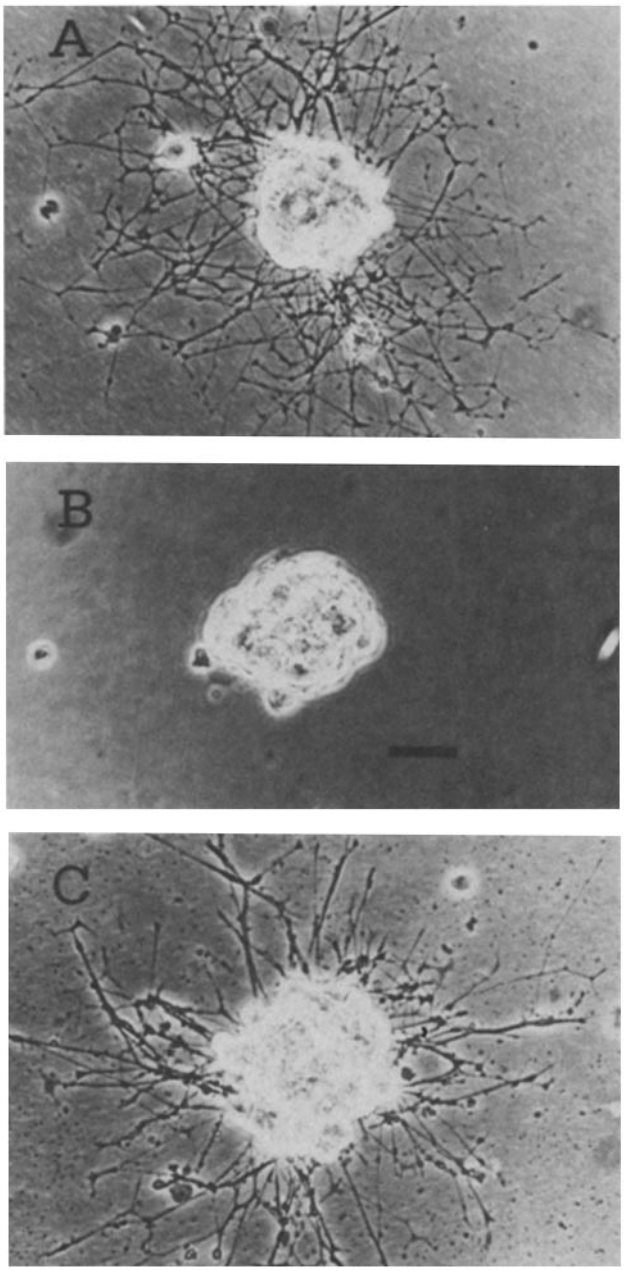

FIGURE 3 Reversibility of the effect of Li^+ on neurite regeneration by PC12 cells. Cells were primed by pretreatment with NGF for 2 wk, passaged as described in the legend to Fig. 2, and then cultured for either 1 d with NGF (*A*), 1 d with NGF plus 15 mM LiCl (*B*), or 1 d with NGF plus 15 mM Li^+ followed by 1 d with NGF alone (*C*). Bar, 50 μm.

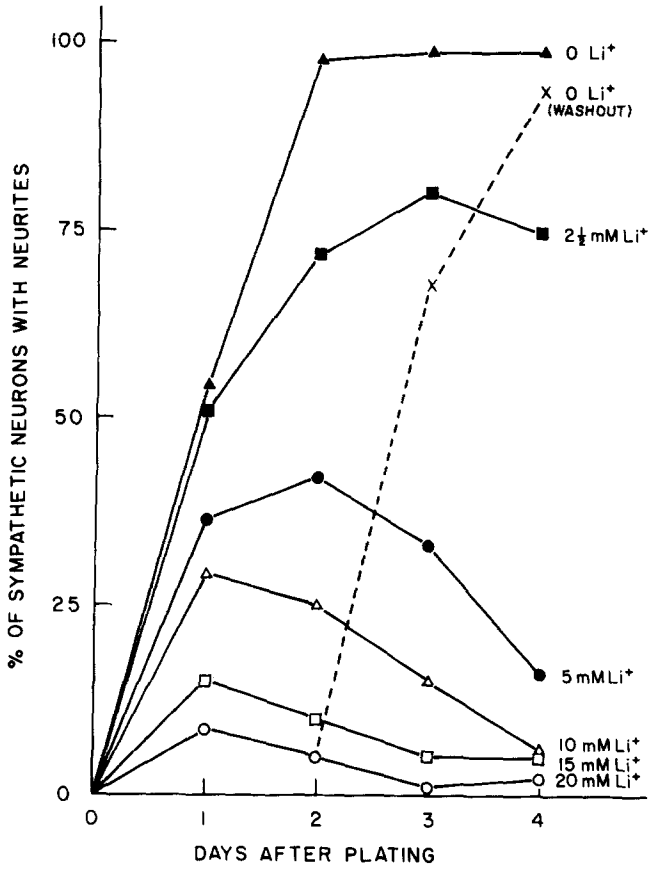

FIGURE 4 Effect of Li^+ on neurite outgrowth by sympathetic neurons. Dissociated newborn mouse sympathetic ganglia were cultured in complete medium containing NGF and 0–20 mM LiCl (as indicated). On each of the next 4 d, neurons were scored for the presence of neurites. On day 2, some of the cultures were washed and then refed with Li^+-free medium. Neurons in these cultures (- - -) were then scored over the next 2 d for the presence of neurites. The reversibility data are shown only for cells treated with 20 mM LiCl; comparable results were obtained for the other concentrations of LiCl as well. At least 100 cells were scored in each culture at each time point.

Subcellular Localization of the Li^+-sensitive Phosphoproteins

The subcellular localization of the lithium-sensitive phosphoproteins was investigated. MAPs have been identified with molecular weights in the size range of the Li^+-sensitive proteins (23, 24, 28), and preliminary studies (Greene, L. A., and P. J. Seeley, unpublished observations) have revealed the presence of M_r 64,000, 72,000, and 80,000 phosphorylated MAPs whose levels increase over long-term exposure of PC12 cells to NGF. To test whether the Li^+-sensitive proteins seen on SDS PAGE of whole cell preparations might be these MAPs, primed PC12 cells were pretreated for 0.5 h with either 15 mM Li^+, 50 μM nocodazole, or no additive (control) and then phosphorylated for 1.5 h in the presence of the same reagents. Nocodazole (29) is an antimicrotubule agent which, at the concentration used, leads to disruption and solubilization of microtubules in primed PC12 cells (Black, M. M., and L. A. Greene, unpublished observations; Brown, K., M. L. Shelanski, and L. A. Greene, unpublished observations; reference 30). After labeling, the cultures were extracted with a Triton-containing microtubule-stabilizing buffer, which leaves cytoskeletons attached to the culture dishes. The cytoskeletons were then extracted with a cold, Ca^{++}-containing destabilizing buffer to selectively solubilize microtubules (8, 22, 23). Solubilized proteins were analyzed by SDS PAGE and autoradiography. The data in Fig. 8 show that this fraction from control primed cells consisted of four major bands (apparent M_r of 55,000, 64,000, 72,000 and 80,000) that were absent from nocodazole-treated cultures. These bands were only barely detectable in preparations from unprimed cultures. Recent evidence (reference 31; and see Discussion) indicates that the phosphoprotein of M_r 55,000 is β-tubulin. The properties of the other phosphoproteins—association with the cytoskeleton, selective solubilization by Ca^{++} and

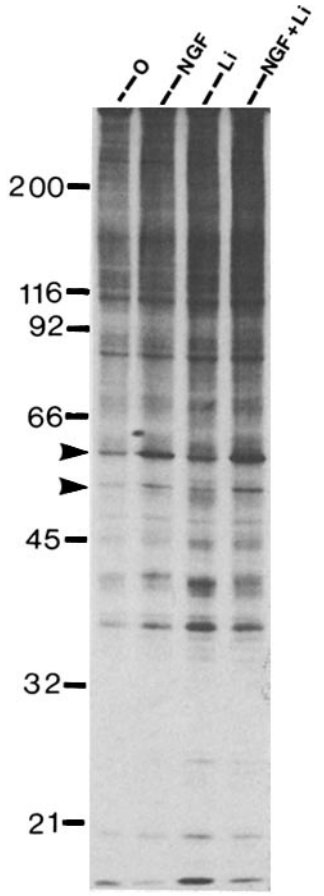

FIGURE 5 Effect of Li^+ on short-term NGF-stimulated phosphorylation of specific proteins in unprimed PC12 cultures. PC12 cultures were transferred to a phosphate-free buffered saline as described in Materials and Methods and then pretreated for 0.5 h as indicated either with no additive or with 15 mM Li^+. After this time, NGF (final concentration 50 ng/ml) was added as indicated and then ^{32}P-orthophosphate (50 μCi/ml final concentration) was applied to all cultures. After incubation for 1.5 h, the cultures were analyzed as described by SDS PAGE and autoradiography. Each lane in the pictured autoradiograph was loaded with 100,000 TCA-precipitable cpm. Arrows show locations of NGF-responsive bands at apparent M_r of 60,000 and 54,000. Numbers indicate the position of marker proteins of known M_r (given $\times 10^{-3}$).

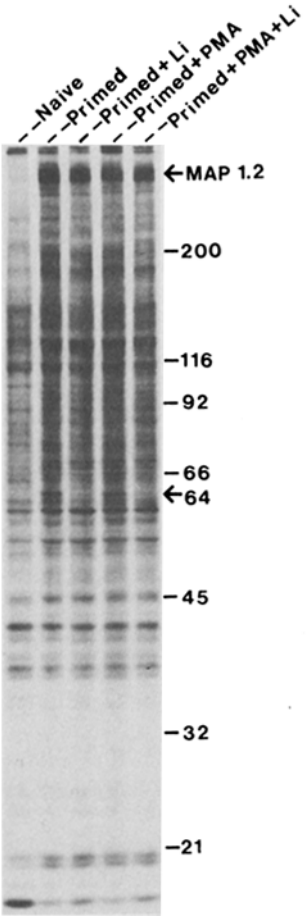

FIGURE 6 Effect of Li^+ (15 mM) and PMA (100 nM) on protein phosphorylation in PC12 cultures. Cultures were pretreated with the indicated additives for 0.5 h and then incubated with ^{32}P-orthophosphate for 2 h in the continued presence of the additives. The naive culture was untreated with NGF; the primed cultures had received 3 wk of NGF treatment. After incubation, the cultures were harvested in sample buffer and equal numbers of TCA-precipitable cpm (100,000) were analyzed by SDS PAGE and autoradiography. The positions of the NGF-inducible MAP 1.2 and bands of M_r 64,000 are indicated by the arrows at the right. The numbers on the right indicate the M_r (given $\times 10^{-3}$) of marker proteins.

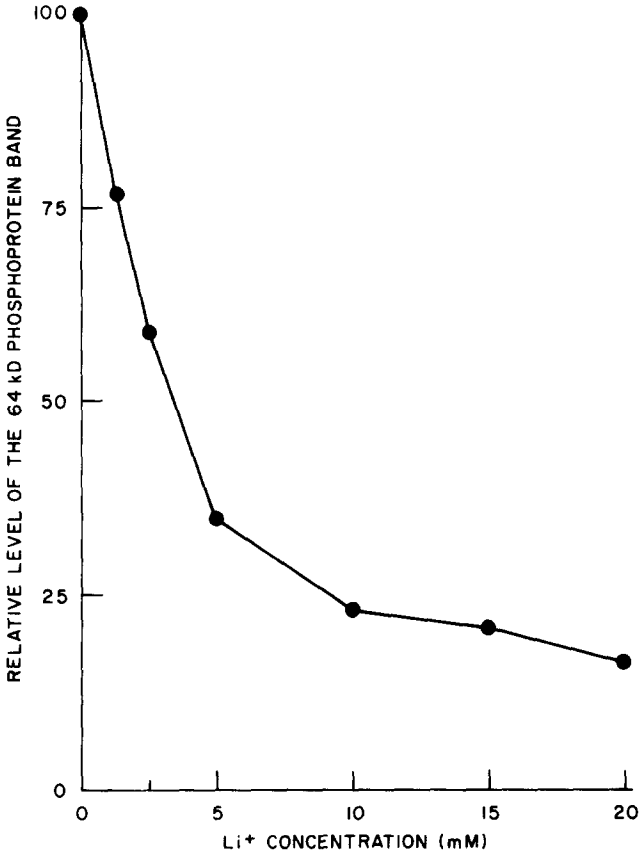

FIGURE 7 Effect of Li^+ concentration on phosphorylation of the phosphoprotein band of M_r 64,000 in primed PC12 cultures. Primed PC12 cultures were preincubated for 0.5 h with various concentrations of Li^+ and then incubated with ^{32}P-orthophosphate in the continued presence of Li^+ for 2 h. The cultures were harvested in sample buffer, and equal numbers of TCA-precipitable cpm (100,000) were subjected to SDS PAGE and autoradiography. The resulting autoradiograph was analyzed with a scanning densitometer. For each sample, the peak height of the band of M_r 64,000 was normalized to an invariant band at M_r 41,000, and then all values were normalized to the value obtained in the absence of added Li^+. Comparable results were obtained in a second independent experiment.

cold, and sensitivity to nocodazole—indicate that they are MAPs.

Examination of the phosphoprotein MAP fraction from the Li^+-treated, primed cultures revealed several marked changes. The M_r 64,000, 72,000, and 80,000 bands were greatly diminished; in contrast, the band of M_r 55,000 was apparently unaffected. Furthermore, there was an apparent increase in the relative abundance of a band of M_r 78,000, which in control cultures, was present as a minor shoulder of the component of M_r 80,000. In several experiments (data not shown), an increase in a band at M_r 78,000 was also apparent in autoradiograms derived from unfractionated whole cell extracts of primed cells treated with Li^+ for 2 h.

Possible Mechanisms of the Inhibitory Action of Li^+

One potential action of Li^+ could be to prevent turnover of protein phosphate groups (e.g., by inactivating a phosphatase), thereby saturating sites with unlabeled phosphate, blocking labeling by subsequent addition of ^{32}P-orthophosphate. To test this possibility, NGF-primed PC12 cell cultures were

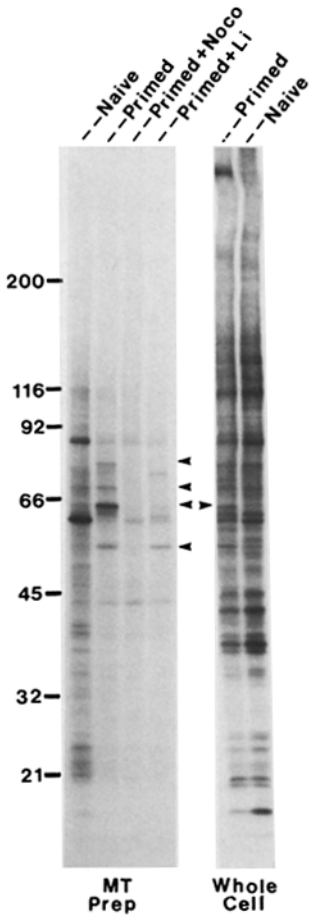

FIGURE 8 The effect of Li^+ (15 mM) on microtubule-associated phosphoproteins. Primed PC12 cultures (i.e., treated with NGF for 18 d) were pretreated for 0.5 h with either 50 μM nocodazole (*Noco*), 15 mM Li^+, or no additive and then incubated for 2 h with ^{32}P-orthophosphate in the presence of the same additives. Naive (i.e., NGF-untreated) cultures were also phosphorylated for the same period of time. The cultures were then treated as described in the text so as to produce microtubule (MT) preparations. These, as well as ^{32}P - orthophosphate - labeled whole primed and naive cells were analyzed by SDS PAGE and autoradiography. The total numbers of TCA-precipitable cpm loaded in each sample were: naive cell microtubule preparation, 25,000; primed cell microtubule preparations, 10,000; and primed and unprimed whole cells, 100,000. The arrowheads pointing to the left indicate the positions of the various phosphorylated microtubule-associated proteins present in the primed cells that are absent after treatment with nocodazole. The arrowhead pointing to the right indicates the position of the NGF-induced phosphoprotein band at M_r 64,000. Numbers on the left indicate the M_r (given × 10^{-3}) of marker proteins.

pretreated with ^{32}P-orthophosphate for 1.5 h and then, in the continued presence of label, with 15 mM Li^+ for an additional 1.5 h. Under these circumstances, SDS PAGE revealed (data not shown) that labeling of the species of M_r 64,000 was suppressed relative to non-Li^+-treated controls to the same degree as in sister cultures pre-treated with Li^+ for 0.5 h before and during labeling. This finding appears to rule out stabilization of incorporated phosphate groups as a mechanism for the presently observed effects of Li^+.

Evidence from other experimental systems indicates the Li^+ can interfere with activation of the phospholipid/Ca^{++}-dependent protein kinase (protein kinase C) by interfering with the ligand-stimulated generation of diacylglycerol (32–35). To test whether this property of Li^+ was responsible for its inhibitory effects on NGF actions, we examined the effect of the tumor promotor, phorbol myristate acetate (PMA) on Li^+ inhibited cultures. In at least several systems studied, PMA can replace diacylglycerol and directly activate kinase C (36). If Li^+ acts in PC12 cells by blocking diacylglycerol-mediated activation of kinase C, then PMA should reverse the effect of Li^+. However, PMA, at a concentration (100 nM) that activates PC12 cell kinase C (Müller, T. H., and L. A. Greene, unpublished data), reversed neither the inhibition by Li^+ of neurite outgrowth (data not shown) nor phosphorylation of the band of M_r 64,000 (Fig. 6). PMA itself did not inhibit either NGF-induced regeneration or phosphorylation of the band of M_r 64,000.

Li^+ has also been reported to interfere with the activation of adenylate cyclase (37–39). If the effect of Li^+ on neurite outgrowth and MAP phosphorylation was due to blockade of endogenous production of cAMP by adenylate cyclase, then addition of permeant cAMP analogs such as dibutyryl- or 8-bromo-cAMP should overcome the blockade. This did not occur; these cAMP analogues (1 mM) did not reverse the effects of 15 mM Li^+ on neurite regeneration or on phosphorylation of the phosphoprotein of M_r 64,000 in the presence of NGF (data not shown), nor did these analogues alone inhibit the action of NGF on neurite outgrowth.

Several other possible modes of action of Li^+ can apparently be ruled out. As assessed by methods described elsewhere (40), LiCl at concentrations up to 20 mM had no effect on the capacity of PC12 cells to bind or internalize ^{125}I- NGF. Also, the effect of Li^+ did not appear to be due to general suppression of protein synthesis since incorporation of [^{35}S]methionine into trichloroacetic acid (TCA)-precipitable counts by PC12 cells over 2 h was unaffected by 5–10 mM LiCl. Furthermore, LiCl (up to 50 mM) did not interfere with in vitro assembly of bovine microtubules nor, as judged by light microscopic and ultrastructural examination, did 2.5 h of exposure to 20 mM Li^+ affect the structural integrity of neurites or of microtubules in PC12 neurites or cell bodies. Hence, direct physico-chemical interference by Li^+ with microtubule formation, such as occurs with several other agents that block neurite outgrowth (41), seems unlikely.

DISCUSSION

We report here than Li^+ reversibly inhibits NGF-induced neurite outgrowth and regeneration from PC12 cells and mouse sympathetic neurons. Such inhibition appears to be specific in that several other responses to NGF are not affected. For instance, both NGF-induced transcription-dependent priming and transcriptional-dependent induction of NILE glycoprotein occurred in the presence of concentrations of Li^+ that suppressed neurite production. Also, Li^+ did not alter transcription-independent, NGF-stimulated phosphorylation of the M_r 60,000 (tyrosine hydroxylase) and 54,000 components. In other experiments (Connolly, J. L., D. Burstein, and L. A. Greene, unpublished observations), Li^+ did not suppress the rapid changes in PC12 cell surface architecture that are triggered by NGF (10). Furthermore, although Li^+ blocked neurite outgrowth in sympathetic neuron cultures, it did not interfere with the capacity of NGF to maintain viability of these cells.

Li^+ specifically and reversibly inhibited the short-term incorporation of phosphate into three phosphoproteins, the most heavily labeled of which has an apparent M_r of 64,000. These phosphoproteins are barely detectable in unprimed PC12 cells labeled with ^{32}P-orthophosphate, and labeling markedly increases as NGF-exposed cells undergo neuronal differentiation. On the basis of several previously defined criteria, we have classified the PC12 phosphoproteins of M_r 64,000, 72,000, and 80,000 as MAPs. These species remained in the cytoskeleton after extraction under microtubule-stabilizing conditions (but not under destabilizing conditions; Greene, L. A., unpublished observations), and were solubilized from stabilized cytoskeleton by Ca^{++}. In previous studies, MAPs of similar size range have been found in cultured PC12 cells (23, 28) and sympathetic neurons (24).

Some comments may be made regarding the relationship of the phosphoproteins of M_r 55,000–80,000 to previously described species. The position of the M_r 55,000 species on

two-dimensional isoelectric focusing/SDS PAGE (Aletta, J. M., and L. A. Greene, unpublished observations) and its recognition by an appropriate monoclonal antibody (Butler, M., J. M. Aletta, L. A. Greene, and M. L. Shelanski, unpublished observations) strongly indicate its identity with β-tubulin. This is consistent with recent findings (31) that β-tubulin becomes phosphorylated in murine neuroblastoma cells under conditions that favor neurite outgrowth and/or assembly of microtubules. Although similar in size to the well-described tau proteins, the phosphoproteins of M_r 64,000–80,000 appear to be distinct from this class of MAPs. Two-dimensional isoelectric focusing/SDS PAGE positions of these proteins (reference 23; Black, M. M., J. M. Aletta, and L. A. Greene, unpublished observations) are different from those of the tau proteins and are essentially identical to those of a set of non-tau MAPs recently described in cultured rat sympathetic neurons (24, 42). Also, it is of interest to note that in vitro experiments (43) indicate that phosphorylated tau MAPs have decreased microtubule-assembling activity compared with dephosphorylated taus.

The presently described effects of Li^+ on neurite outgrowth and MAP phosphorylation may be viewed in light of prior findings with the PC12 line. Although NGF appears to trigger a required transcriptional pathway in eliciting process outgrowth (4–6), comparison of the proteins synthesized by PC12 cells before and after long-term exposure to NGF has revealed few changes in the relative abundances of many major components (21, 44). Thus, NGF-induced alterations in protein levels that occur may be among species of relatively low abundance whose actions would regulate the assembly or organization of more abundant structural elements. One such likely target for regulation is the tubulin-microtubule system. Microtubules are a major component of the neurite cytoskeleton and hence regulation of microtubule assembly may be a critical step in the mechanism of neurite outgrowth (45). Although NGF causes little change in the relative abundance of tubulin within PC12 cells (21, 44), long-term treatment with the factor elicits the formation of parallel arrays of microtubules within their processes (46), brings about a large increase in the stability of microtubules (as judged by resistance to depolymerization by colchicine) (8), and results in a shift in the proportion of cellular tubulin that is polymerized (47, 48). The prospect was therefore raised (8, 9) that NGF might bring about such changes in microtubules by affecting the level of MAPs—a class of proteins for which evidence suggests a major role in regulation of microtubule formation and stability (49). Support for this has come from the findings that NGF induces the relative levels of, or modifies, at least several MAPs in PC12 cells: MAP 1.2 (9), the phosphoproteins of M_r 64,000–80,000 (present data), tau MAPs (48), and a MAP of M_r 35,000 (50). An attractive interpretation of the present findings is therefore that Li^+, either by interfering with the phosphorylation or by promoting the dephosphorylation of specific NGF-regulated MAPs, may interfere with their function. This in turn may adversely affect either microtubule assembly and stability or linkage of microtubules to other cellular components, thereby resulting in inhibition of neurite outgrowth. Of relevance to this hypothesis, Pallas and Solomon (23) reported that phosphorylation of MAPs in neuroblastoma and PC12 cells similar in molecular weight to the Li^+-sensitive MAPs reported here is correlated with their incorporation into assembled microtubules. On this basis, these authors also suggested that MAP phosphorylation could play a causal role in microtubule formation and neurite outgrowth. It remains to be determined whether the MAPs that fail to become labeled with ^{32}P-orthophosphate in Li^+-treated PC12 cells (Fig. 8) associate with the microtubules.

One alternative interpretation of the present findings is that the effect of Li^+ on neurites might be the cause rather than the consequence of the observed effects of Li^+ on MAP phosphorylation. This appears less likely, however, since neither microscopic changes in neurites nor ultrastructural effects on neuritic microtubules were observed during the time period in which Li^+ blocks MAP phosphorylation.

There are several possible mechanisms by which Li^+ could affect MAP phosphorylation and function in PC12 cells: (*a*) Li^+ could accelerate the degradation of the polypeptide moieties of these phosphoproteins. This seems unlikely in view of the rapid reversibility of the effect of Li^+ on phosphorylation. (*b*) Li^+ could directly interfere with the kinase(s) for which the MAPs are a substrate, or (*c*) activate a phosphatase that dephosphorylates these proteins. (*d*) Li^+ could interact directly with MAPs, altering their accessibility to kinase or phosphatase activity, perhaps by causing MAPs to detach from the microtubules. However, in a preliminary experiment, exposure to primed PC12 cytoskeletal preparations to 15 mM Li^+ did not solubilize phosphorylated MAPs. An additional mechanistic question raised by the present study is whether or not NGF regulates the specific Li^+-affected pathway that results in rapid phosphorylation of these NGF-modulated MAPs, or whether modulation of these proteins by NGF occurs only via activation of other pathways.

The present data indicate that the dose-response relationships between Li^+ concentration and inhibition of either neurite outgrowth or of phosphorylation of the band of M_r 64,000 do not precisely correspond. The half effective concentration for the former was 6.5 mM, while that for the latter was ~3.5 mM. One interpretation of these findings that is consistent with the above hypothesis is that substantial interference with neurite outgrowth by Li^+ is apparent only when a large proportion of MAP phosphorylation is inhibited.

The capacity of Li^+ to affect microtubule-mediated cellular actions is not without precedent. It was recently shown that Li^+ at similar concentrations to those used here reversibly inhibited microtubule-dependent motility of demembranated spermatozoa (51). The present study points to a possible explanation for this effect, namely, interference with the phosphorylation-dependent activation of specific MAPs.

Lithium ion is used clinically in the treatment of certain affective disorders. The effective concentrations of Li^+ found in this study exceed the threshold of clinical toxicity (1.5–2.0 mM in serum) (52). It is thus of interest to speculate whether neuropathic complications of lithium overdose (53–56) might be related to the effects on neurites reported here. In particular, we have shown that effects on neuronal cytoskeletal phosphoproteins occur at concentrations only slightly higher than that of the toxic threshold of Li^+.

In summary, it was shown that Li^+ blocks NGF-induced neurite outgrowth, but has little effect on acquisition or maintenance of transcription-dependent priming. As such, Li^+ appears to spare one set of NGF-promoted actions, while effectively interfering with another. Li^+ appears to be useful both as a probe of NGF's mechanism of action, and as a probe of microtubule and MAP structure and function. Identification of rapid, specific, and reversible action of Li^+ on three microtubule-associated phosphoproteins at concentra-

tions that interfere with neurite outgrowth may define a functional subset of potential cytoskeletal regulatory proteins, and may focus the ongoing search for the critical intracellular molecular targets of NGF.

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