Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8455–8459, August 1996
Developmental Biology

This contribution is a part of the special series of Inaugural Articles by members of the National Academy of Sciences elected on April 25, 1995.

A molecular mechanism for the effect of lithium on development

PETER S. KLEIN* AND DOUGLAS A. MELTON†

*Howard Hughes Medical Institute and Department of Medicine (Hematology–Oncology) and Institute on Aging, University of Pennsylvania, Philadelphia, PA 19104; and †Howard Hughes Medical Institute and Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138

Contributed by Douglas A. Melton, May 6, 1996

ABSTRACT **Lithium, one of the most effective drugs for the treatment of bipolar (manic-depressive) disorder, also has dramatic effects on morphogenesis in the early development of numerous organisms. How lithium exerts these diverse effects is unclear, but the favored hypothesis is that lithium acts through inhibition of inositol monophosphatase (IMPase). We show here that complete inhibition of IMPase has no effect on the morphogenesis of *Xenopus* embryos and present a different hypothesis to explain the broad action of lithium. Our results suggest that lithium acts through inhibition of glycogen synthase kinase-3β (GSK-3β), which regulates cell fate determination in diverse organisms including *Dictyostelium*, *Drosophila*, and *Xenopus*. Lithium potently inhibits GSK-3β activity (K_i = 2 mM), but is not a general inhibitor of other protein kinases. In support of this hypothesis, lithium treatment phenocopies loss of GSK-3β function in *Xenopus* and *Dictyostelium*. These observations help explain the effect of lithium on cell-fate determination and could provide insights into the pathogenesis and treatment of bipolar disorder.**

Lithium has a profound effect on the development of diverse organisms, including *Dictyostelium*, sea urchins, zebrafish, and *Xenopus* (1–5). In *Dictyostelium*, lithium alters cell fate by blocking spore cell and promoting stalk cell development (1, 2). In *Xenopus*, lithium causes an expansion of dorsal mesoderm, leading to duplication of the dorsal axis or, in extreme cases, entirely dorsalized embryos lacking identifiably ventral tissues (3). Lithium also mimics insulin by stimulating glycogen synthesis (6) and is the most effective treatment for bipolar disorder (7, 8).

Several hypotheses have been advanced to explain the mechanism of lithium action, yet its molecular target in cell-fate determination and glycogen metabolism, as well as in the treatment of bipolar disorder, remains unclear (7–10). The most widely accepted model, the inositol depletion hypothesis, is based on the observation that lithium inhibits inositol monophosphatase (IMPase) and could thereby deplete the cell of an endogenous source of inositol (9, 11). Cells would become unable to generate inositol 1,4,5 trisphosphate (InsP_3) in response to extracellular signals and thus InsP_3-dependent responses would be blocked. In support of this hypothesis, microinjection of myo-inositol can prevent the effect of lithium in *Xenopus* (12), although this requires the coinjection of a high concentration of myo-inositol (0.3 M) with lithium (also at 0.3 M). However, the inositol depletion hypothesis does not explain why lithium alters cell fate in *Dictyostelium discoideum*, since mutants lacking the gene for phospholipase C, and thus unable to generate InsP_3 in response to extracellular signals, develop normally (13). It also does not explain simply the effect of lithium on glycogen synthesis or on cells that have an exogenous source of inositol.

A class of bisphosphonate compounds that are ≈1000-fold more potent than lithium in inhibiting IMPase has recently become available (14, 15). Using these inhibitors, we have reexamined the role of IMPase in the early development of *Xenopus*. We show here that complete inhibition of IMPase has no discernible effect on the morphogenesis of *Xenopus* embryos, ruling out this enzyme as the target of lithium-induced teratogenesis.

An alternative target of lithium is suggested by the close similarity between lithium action and the effect of ectopic expression of *wnt* genes in *Xenopus* embryos (3, 16). We have focused on a protein that appears to antagonize *wnt* signaling, the enzyme glycogen synthase kinase-3β (GSK-3β; ref.17), which plays a central role in the development of diverse organisms including *Drosophila* (18, 19), *Dictyostelium* (20), and *Xenopus* (21–23). In this paper, we show that lithium directly inhibits GSK-3β *in vitro* and propose that GSK-3β is the endogenous target of lithium in these diverse systems.

MATERIALS AND METHODS

Materials. Purified, bacterially expressed GSK-3β, casein kinase II (CKII), and phosphatase inhibitor 2 (I-2) were purchased from New England Biolabs. Glutathione *S*-transferase–extracellular signal-related kinase 1 (ERK-1) was a gift of Sandro Allesandrini and Ray Erickson (Harvard University). Protein kinase A (PKA) was from Sigma. L-690,330 was from Tocris Cookson (St. Louis). Tritium-labeled inositol-1-phosphate (1.0 Ci/mmol, 25 μCi/ml; 1 Ci = 37 GBq) was from American Radiolabeled Chemicals (St. Louis). [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham.

Embryos and Microinjection. Eggs were fertilized *in vitro* according to well-established protocols (24). Microinjections were done at 4–32 cell stages with 5–10 nl volumes. For lithium treatment, embryos were transferred to 0.3 M lithium chloride (LiCl) in 0.1× modified Ringer's solution (MMR; ref. 24) for 6 min, then washed in 0.1× MMR as described (3) or were microinjected into ventral vegetal blastomeres (C tier) at the 32-cell stage with 5 nl of 0.3 M LiCl (12). L-690,330 was dissolved in water at 0.1–10 mM, and 5–10 nl was injected into the ventral vegetal region of 4, 8, 16, and 32 cell embryos. All four cells of 4-cell stage embryos were also injected in separate experiments.

IMPase Activity. Thirty embryos at the 32-cell stage were washed in 1 ml of 50 mM Tris (pH 7.8), 250 mM KCl, and 3 mM $MgCl_2$ (IMPB) and then lysed in 300 μl of the same buffer. Lysates were centrifuged for 5 min at 20,000 × *g* at 4°C and supernatant was recovered. Water (2.5 μl), LiCl, or L-690,330 was added to 25 μl of lysate, which was then incubated at room temperature for 5 min. ^{3}H-inositol-1-phosphate (1 μl) was

Abbreviations: GSK-3β, glycogen synthase kinase-3β; IMPase, inositol monophosphatase; LiCl, lithium chloride; PKA, protein kinase A; CKII, casein kinase II; MAP, mitogen-activating protein; ERK-1, extracellular signal-related kinase 1.

added and the incubation was continued for 25 min. Samples were then diluted with 1.0 ml of 0.1× MMR, boiled for 5 min, and applied to Dowex columns as described (25). Inositol was present in the flow-through and inositol-1-phosphate was eluted by application of 3 ml of 0.1 M formic acid/1.5 M ammonium formate (25). Assays were repeated 2–4 times with similar results. LiCl (20 mM) also completely inhibited *in vitro Xenopus* IMPase activity (not shown). For *in vivo* measurement of IMPase activity, ^{3}H-inositol-1-phosphate was concentrated to 250 μCi/ml and mixed 1:1 with water, 10 mM L-690,330, or 0.6 M LiCl. Then 10 nl was injected into ventral-vegetal blastomeres at the 8- to 16-cell stage. After 10 min, 20 embryos were harvested by lysis in 1.0 ml of boiling 0.1× MMR with 20 mM LiCl. Lysates were prepared and applied to Dowex columns as above.

GSK-3β Activity. GSK-3β was obtained from New England Biolabs and assayed according to the supplier's protocol using 0.5–1.0 unit of GSK-3β per assay. The peptide substrate (GS-2) was present at 25 μM and incorporation of ^{32}P was measured by binding to P81 paper according to previously described methods (26). The peptide was synthesized with phosphate incorporated into the serine closest to the C terminus and had the sequence RPASYPPSPSLSRHSSPHQS(P)EDEEE (27). I-2 was present at 50 μg/ml and τ was 25 μg/ml. PKA, ERK-1 [mitogen-activating protein (MAP) kinase], and CKII were assayed under similar conditions as for GSK-3β except that

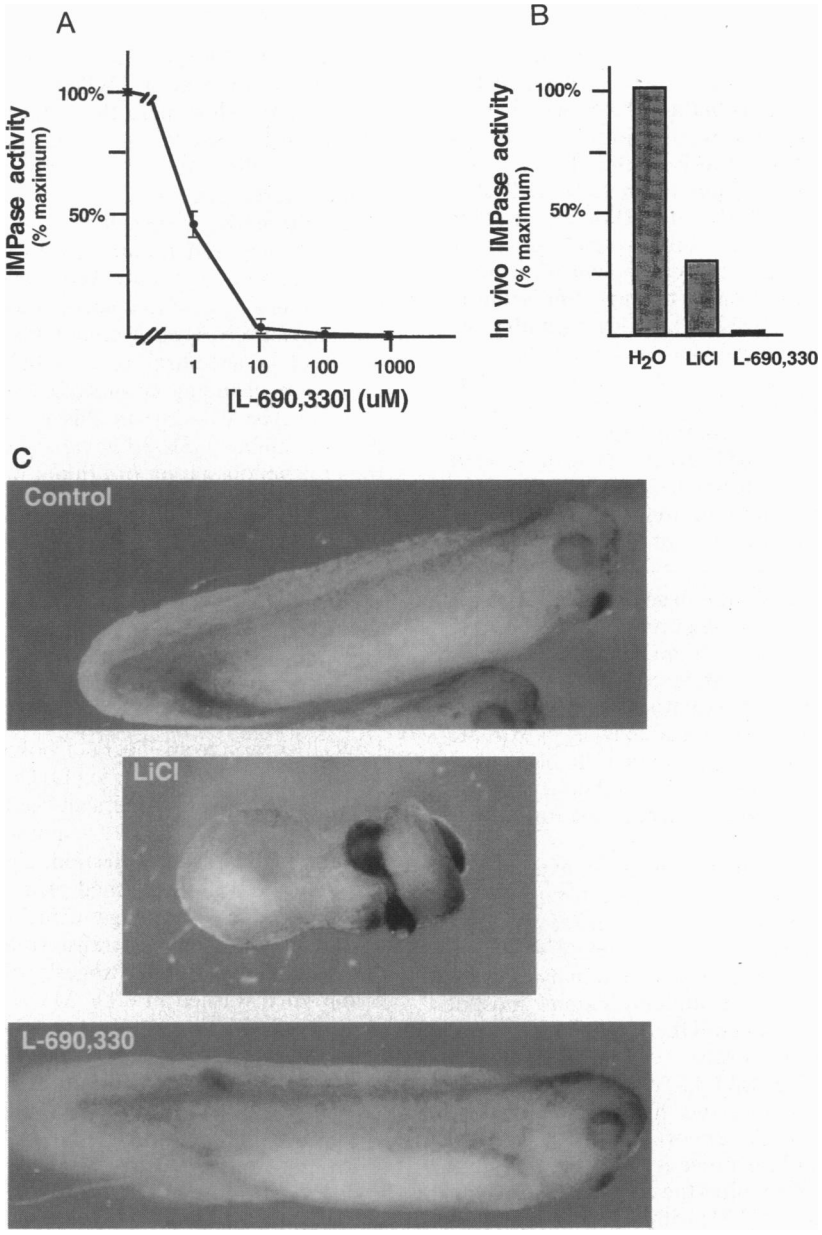

FIG. 1. Inhibition of IMPase does not dorsalize *Xenopus* embryos. (*A*) IMPase activity was measured as the amount of ^{3}H-inositol produced by hydrolysis of ^{3}H-inositol-1-phosphate in the presence of increasing concentrations of the bisphosphonate L-690, 330 in lysates of *Xenopus* embryos harvested at the 32-cell stage. (*B*) *In vivo* IMPase activity was measured by injecting a single ventral–vegetal blastomere at the 8- to 16-cell stage with 10 nl of ^{3}H-inositol-1-phosphate plus water, 5 mM L-690,330, or 0.3 M LiCl. Embryos were incubated for 10 min and then conversion to ^{3}H-inositol was measured. (*C*) Control, stage-30 embryo injected at 32-cell stage with water. LiCl, stage-30 embryo treated with 0.3 M lithium at the 32-cell stage, showing profound dorsalization with concentric cement gland, expanded anterior neural structures, and absent ventral and posterior structures. L-690,330, stage-30 embryo injected with 1 mM L-690,330 into a ventral–vegetal blastomere at the 16-cell stage (n = 55). Similar results were seen when ventral–vegetal blastomeres were injected at the 4-, 8-, or 32-cell stage or when each cell at the 4-cell stage was injected. L-690,330 was also injected at 10 mM. In no case was any evidence of dorsalization observed after injection of the IMPase inhibitor L-690,330.

2-mercaptoethanol was included in the PKA assays. For PKA assays, kemptide was 50 μM. For ERK-1 assays, myelin basic protein was present at 0.5 μg/μl. For CKII assays, casein was present at 50 μg/ml. (GSK-3β phosphorylation of I-2, but not GS-2 peptide or τ protein was partially inhibited by 20 mM KCl or NaCl, data not shown.)

RESULTS AND DISCUSSION

To test further the role of IMPase in *Xenopus* development, we have used a recently described competitive inhibitor of IMPase, the bisphosphonate L-690,330, which is ≈1000-fold more potent than lithium in inhibiting IMPase (14). As shown in Fig. 1, L-690,330 effectively inhibits IMPase activity from *Xenopus* embryos *in vitro* (50% inhibition at ≈1 μM). L-690,330 microinjected into blastomeres at the 4-cell, 8- to 16-cell, and 32-cell stages also completely inhibits IMPase activity *in vivo* (Fig. 1*B*). However, microinjection of L-690,330, in contrast to lithium, does not dorsalize embryos (Fig. 1*C*; n = 55) and has no discernible effect on development (at the highest injected doses mild nonspecific toxicity was observed, data not shown). These data show that inhibition of IMPase does not explain the effect of lithium on *Xenopus* development.

Because dorsalization by lithium is remarkably similar to ectopic expression of wingless/int-1 related genes (*wnt*) in *Xenopus* embryos (16), we have tested whether lithium might act on a component of the *wnt* signaling pathway. This pathway, which appears to be well conserved between vertebrates and invertebrates (21–23, 28), is inhibited in *Drosophila* by zeste white 3/shaggy (*zw3/sgg*), the homologue of the mammalian enzyme GSK-3β. Although GSK-3β was first described as an inhibitor of glycogen synthase (17, 29), it is likely that it regulates distinct substrates in other signal transduction pathways (17), including the *wnt* pathway. A central role has also been demonstrated for this gene in the development of diverse organisms including *Dictyostelium* and *Xenopus* (20–23). Therefore, we wished to test whether lithium acts by inhibiting GSK-3β directly, as this would be predicted to mimic *wnt* signaling.

To test our hypothesis, purified GSK-3β was assayed by using a peptide substrate (27, 30) and was found to be inhibited by lithium chloride with 50% inhibition (IC_{50}) at 2 mM (Fig. 2*A*). Little inhibition of phosphorylation was seen with NaCl, KCl, NH_4Cl, or CsCl at 20 mM (Fig. 2*B*), while lithium acetate showed inhibition nearly identical to LiCl (Fig. 2*B* and data not shown), indicating that the inhibition is mediated specifically by lithium ion and not by other monovalent cations or by chloride. LiCl inhibited GSK-3β-mediated phosphorylation of protein substrates, including protein phosphatase inhibitor-2 (31) and τ protein (32, 33) with an IC_{50} also near 2 mM (Fig. 3*A*, data not shown). Inhibition of GSK-3β is observed at concentrations well within the therapeutic range for lithium (0.5–1.5 mM) in the treatment of mania. In *Xenopus*, the effective internal concentration after lithium treatment is not known precisely but reaches a maximum of ≈8–9 mM (12).

The specificity of lithium inhibition was tested by assaying other protein kinases, including PKA, ERK-1/MAP kinase, and CKII, under similar conditions. Thus, at 20 mM lithium, PKA shows full activity toward kemptide (Fig. 3*B*) and casein (not shown). Similarly, lithium shows minimal inhibition of ERK-1/MAP kinase-mediated phosphorylation of myelin basic protein and CKII phosphorylation of casein (Fig. 3). These data indicate that lithium is not a general inhibitor of protein kinases in this dose range.

The kinetic nature of the inhibition of GSK-3β by lithium was investigated by measuring initial velocity as a function of substrate concentration at several concentrations of LiCl. The data, presented as double reciprocal plots (Fig. 4), show that the greatest effect is on the apparent V_{max}, and the plot of $1/V_{max}$ (y-intercept) versus lithium concentration is linear (not shown), suggesting that lithium acts as an uncompetitive inhibitor of GSK-3β. (K_i for LiCl = 2.1 ± 0.6 mM). Thus, inhibition of GSK-3β by lithium should not be overcome by increasing substrate concentration. The inhibition of IMPase by lithium is also uncompetitive (K_i = 0.8 mM), and it has been argued that this is an important feature for its therapeutic utility (9, 11) since the degree of inhibition is proportional to the concentration of substrate.

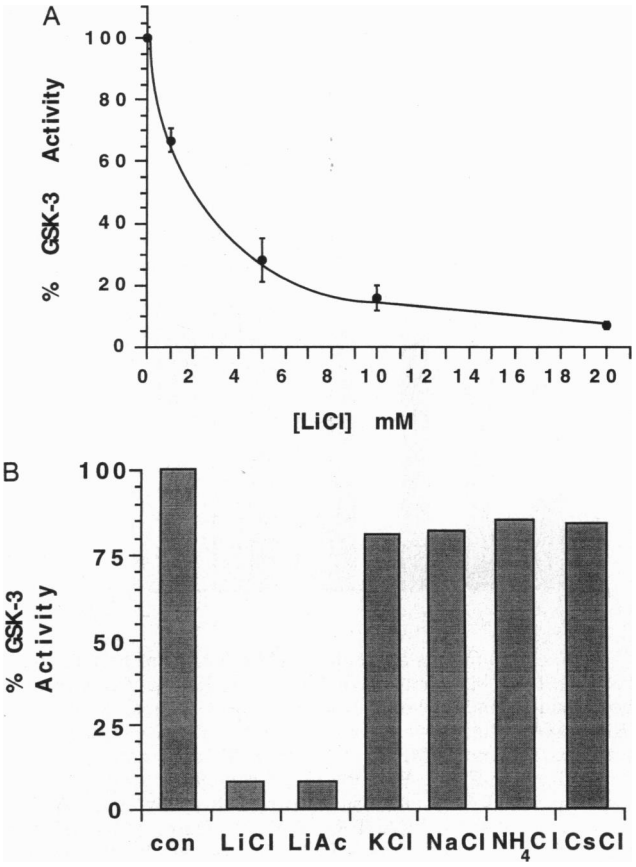

FIG. 2. GSK-3β activity is inhibited by lithium. (*A*) Purified GSK-3β was assayed by its ability to phosphorylate a peptide with a sequence derived from glycogen synthase (GS-2) in the presence of increasing concentrations of lithium chloride. Each data point represents the average of duplicate samples taken from three independent experiments and normalized to maximum GSK-3β activity. (*B*) GSK-3β was assayed as above without addition of monovalent cation (control) or in the presence of 20 mM monovalent cations as indicated in the figure. GSK-3β activity was normalized to the activity seen without added monovalent cations.

The hypothesis that GSK-3β is the endogenous target of lithium action is supported by genetic data as well as *in vivo* biochemical data. Thus inhibition of GSK-3β activity through expression of a dominant negative mutant of GSK-3β leads to dorsalization of *Xenopus* embryos, similar to lithium treatment (3, 21–23) and consistent with lithium inhibiting GSK-3β. In addition, disruption of GSK-3β in *Dictyostelium* (*gskA*) diverts cells normally fated to form spores to adopt the stalk-cell lineage (20), and this phenotype is also duplicated by lithium treatment (1). GSK-3β was first identified in mammals as the protein kinase responsible for the inhibitory phosphorylation of glycogen synthase (17, 29). Insulin is known to inhibit GSK-3β (30, 34), which in turn (together with activation of protein phosphatase) leads to increased glycogen synthesis. Lithium mimics insulin action by stimulating glycogen synthesis (6), and while the mechanism remains unknown, we suggest that lithium mimics insulin by inhibiting GSK-3β.

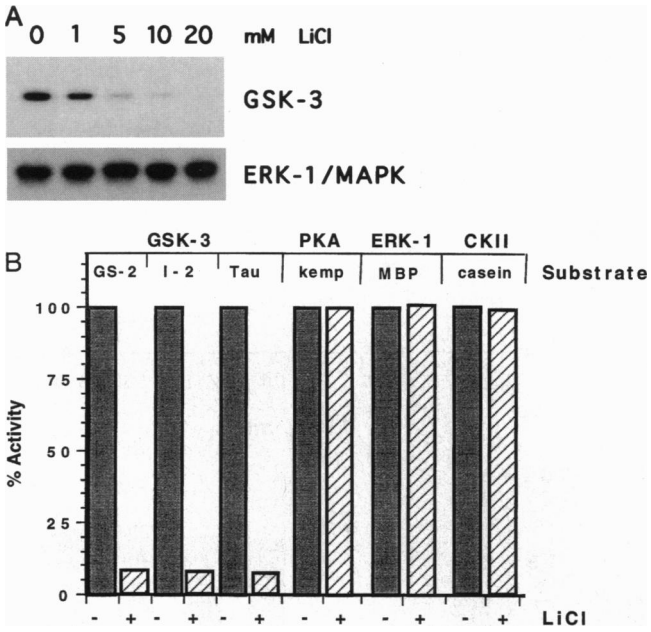

FIG. 3. Lithium does not inhibit cAMP-dependent protein kinase (PKA), ERK-1/MAP kinase, or CKII. (*A*) GSK-3β was assayed as above using protein phosphatase inhibitor-2 (I-2) as a substrate. ERK-1/MAP kinase was assayed under similar conditions using myelin basic protein (MBP) as substrate. Concentration of LiCl is indicated in the figure. Phosphorylated protein substrates were resolved by SDS/PAGE and visualized by autoradiography. (*B*) The activities of GSK-3β, PKA, ERK-1/MAP kinase, and CKII were measured in the absence or presence of 20 mM LiCl. Incorporation of ^{32}P was measured as in Fig. 1 (for GS-2 and kemptide) or by exposure of polyacrylamide gels to a Fuji BioImager and was normalized to incorporation in the absence of lithium. The substrates for GSK-3β were GS-2, I-2, and τ protein. For ERK-1, PKA, and CKII, the substrates were MBP, kemptide, and casein, respectively.

Our hypothesis offers a simple explanation for the effect of lithium on diverse systems and allows specific predictions, as well. For example, *wnt* signaling has been proposed to act through inhibition of GSK-3β (*zw3/sgg*) in *Drosophila* (28) and in *Xenopus*. If this pathway is conserved in other organisms, then lithium treatment should mimic *wnt* signaling in those systems as it does in *Xenopus* and zebrafish. In support of this, *wnt-4* has been shown to be required for the induction of mesenchymal condensation in the formation of renal epithelium in the mouse and ectopically expressed *wnt-1* can also induce mesenchymal condensation (35, 36). These effects of *wnts* are recapitulated by treating explanted renal mesenchyme with lithium (37), which also induces mesenchymal condensation. Furthermore, *wnts* were first identified in mammals by their ability to stimulate cell division and induce tumors in mammary cells (38); lithium can also stimulate cell division in primary mammary cell lines (39). It is not known how lithium exerts these effects in renal mesenchyme or mammary cell lines, but the correlation with *wnt* is compelling, given the other similarities of lithium to *wnt* actions.

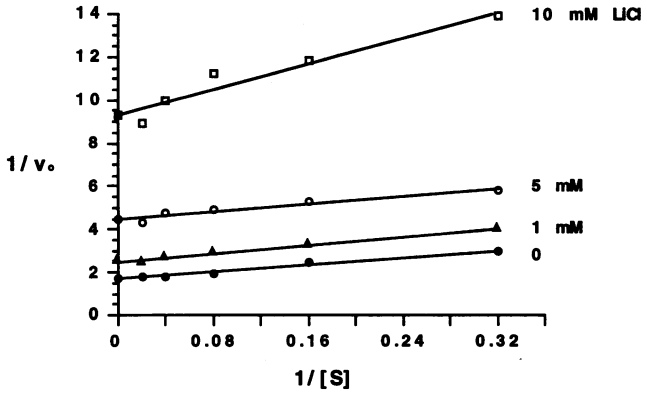

FIG. 4. Lithium is an uncompetitive inhibitor of GSK-3β. Initial velocity of GSK-3β phosphorylation at 3.125, 6.25, 12.5, 25, and 50 μM GS-2 peptide was measured in the presence of LiCl at 0, 1, 5, and 10 mM. The data are shown as a double-reciprocal plot. *Y*-intercepts ($1/V_{max}$) were extrapolated by linear regression. Reaction velocity was linear for up to 8 min and time points were taken at 5 min. The experiment was repeated four times with similar results. In addition, a plot of $1/V_{max}$ as a function of LiCl concentration was linear and allowed a calculation of the $K_i = 2.1 \pm 0.6$ mM (average from four separate experiments). However, it should be noted that kinetic analysis may be complicated by the fact that the peptide actually contains multiple GSK-3β phosphorylation sites that have different rates of phosphorylation (41).

Lithium has effects on numerous other organisms and it may have multiple physiological targets. However, the mechanism of lithium action in bipolar disorder remains uncertain. We propose that GSK-3β, which is abundantly expressed in brain (40), may serve a role in signal transduction in the brain, and that lithium inhibition of this pathway could explain its actions in manic-depressive illness in addition to its effects on development and its insulin-like activity.

We wish to thank Leslee Conrad for superlative technical assistance, Sandro Allesandrini for advice and reagents including glutathione *S*-transferase–ERK-1, Brian Anton for his advice on assaying GSK-3, and Betty Belmonte and Skip Brass for their advice on assaying IMPase. We thank Joseph Avruch and Albert Mildvan for very helpful discussions, and Min Ku and Dan Kessler for critically reading the manuscript. P.S.K. wishes to express special thanks to Betsy Wilder and to Min Ku for their ideas and experimental help.

1. Maeda, Y. (1970) *Dev. Growth Differ.* **12,** 217–227.
2. Van Lookeren Campagne, M. M., Wang, M., Spek, W., Peters, D. & Schaap, P. (1988) *Dev. Genet.* **9,** 589–596.
3. Kao, K. R., Masui, Y. & Elinson, R. P. (1986) *Nature (London)* **322,** 371–373.
4. Stachel, S. E., Grunwald, D. J. & Myers, P. Z. (1993) *Development (Cambridge, U.K.)* **117,** 1261–1274.
5. Livingston, B. T. & Wilt, F. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3669–3673.
6. Bosch, F., Gomez-Foix, A. M., Arino, J. & Guinovart, J. J. (1986) *J. Biol. Chem.* **261,** 16927–16931.
7. Price, L. H. & Heninger, G. R. (1994) *N. Engl. J. Med.* **331,** 591–598.
8. Goodwin, F. K. & Jamison, K. R. (1990) *Manic-Depressive Illness* (Oxford Univ. Press, New York).
9. Berridge, M. J., Downes, C. P. & Hanley, M. R. (1989) *Cell* **59,** 411–419.
10. Avissar, S., Schreiber, G., Danon, A. & Belmaker, R. H. (1988) *Nature (London)* **331,** 440–442.
11. Hallcher, L. M. & Sherman, W. R. (1980) *J. Biol. Chem.* **255,** 10896–10901.
12. Busa, W. B. & Gimlich, R. L. (1989) *Dev. Biol.* **132,** 315–324.
13. Drayer, A. L., Van der Kaay, J., Mayr, G. W. & Van Haastert, P. J. (1994) *EMBO J.* **13,** 1601–1609.
14. Atack, J. R., Cook, S. M., Watt, A. P., Fletcher, S. R. & Ragan, C. I. (1993) *J. Neurochem.* **60,** 652–658.
15. Atack, J. R., Prior, A. M., Fletcher, S. R., Quirk, K., McKernan, R. & Ragan, C. I. (1994) *J. Pharmacol. Exp. Ther.* **270,** 70–76.
16. McMahon, A. P. & Moon, R. T. (1989) *Cell* **58,** 1075–1084.
17. Woodgett, J. R. (1991) *Trends Biochem. Sci.* **16,** 177–181.
18. Siegfried, E., Perkins, L. A., Capaci, T. M. & Perrimon, N. (1990) *Nature (London)* **345,** 825–829.
19. Bourouis, M., Moore, P., Ruel, L., Grau, Y., Heitzler, P. & Simpson, P. (1990) *EMBO J.* **9,** 2877–2884.
20. Harwood, A. J., Plyte, S. E., Woodgett, J., Strutt, H. & Kay, R. R. (1995) *Cell* **80,** 139–148.
21. Pierce, S. B. & Kimelman, D. (1995) *Development (Cambridge, U.K.)* **121,** 755–765.

22. He, X., Saint-Jeannet, J.-P., Woodgett, J. R., Varmus, H. E. & Dawid, I. B. (1995) *Nature (London)* **374,** 617–622.
23. Dominguez, I., Itoh, K. & Sokol, S. Y. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8498–8502.
24. Peng, H. B. (1991) in *Methods in Cell Biology*, eds. Kay, B. K. & Peng, H. B. (Academic, San Diego), pp. 657–662.
25. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212,** 473–482.
26. Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C. & Cohen, P. (1992) *EMBO J.* **11,** 3985–3994.
27. Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W. & Roach, P. J. (1987) *J. Biol. Chem.* **262,** 14042–14048.
28. Siegfried, E., Wilder, E. L. & Perrimon, N. (1994) *Nature (London)* **367,** 76–80.
29. Cohen, P., Yellowlees, D., Aitken, A., Donella-Deana, A., Hemmings, B. A. & Parker, P. J. (1982) *Eur. J. Biochem.* **124,** 21–35.
30. Sutherland, C., Leighton, I. A. & Cohem, P. (1993) *Biochem. J.* **296,** 15–19.
31. Hemmings, B. A., Resnick, T. J. & Cohen, P. (1982) *FEBS Lett.* **150,** 319–324.
32. Hanger, D. P., Hughes, K., Woodgett, J. R., Brion, J.-P. & Anderton, B. H. (1992) *Neurosci. Lett.* **147,** 58–62.
33. Mandelkow, E. M., Drewes, G., Biernat, J., Gustke, N., Van, L. J., Vandenheede, J. R. & Mandelkow, E. (1992) *FEBS Lett.* **314,** 315–321.
34. Welsh, G. I. & Proud, C. G. (1993) *Biochem. J.* **294,** 625–629.
35. Stark, K., Vainio, S., Vassileva, G. & McMahon, A. P. (1994) *Nature (London)* **372,** 679–683.
36. Herzlinger, D., Qiao, J., Cohen, D., Ramakrishna, N. & Brown, A. M. (1994) *Dev. Biol.* **166,** 815–818.
37. Davies, J. A. & Garrod, D. R. (1995) *Dev. Biol.* **167,** 50–60.
38. Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K. & Varmus, H. E. (1984) *Nature (London)* **307,** 131–136.
39. Ptashne, K., Stockdale, F. E. & Conlon, S. (1980) *J. Cell. Physiol.* **103,** 41–46.
40. Woodgett, J. R. (1990) *EMBO J.* **9,** 2431–2438.
41. Fiol, C. J., Wang, A., Roeske, R. W. & Roach, P. J. (1990) *J. Biol. Chem.* **265,** 6061–6065.