ACTIN ASSEMBLY BY LITHIUM IONS

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ABSTRACT The ability of Li^+ to promote the assembly of actin has been compared with the more common cations used in actin assembly assays, K^+, Mg^{2+}, and Ca^{2+}. The principal assay of actin assembly utilized was fluorescence photobleaching recovery (FPR), from which it is possible to determine the fraction of actin protomers incorporated into filaments and the average diffusion coefficients of the filaments. In addition, critical concentrations of actin over a range of concentrations of all of these cations have been determined using an assay that involves sonication and dilution of assembled actin filaments containing trace amounts of pyrene-labeled actin. The results demonstrate that Li^+ is a more potent promoter of actin assembly than is K^+. The more rapid assembly of actin in the presence of Li^+ is attributable to an increased rate of filament elongation. Filaments assembled in equivalent concentrations of Li^+ or K^+ have the same diffusion coefficients, and thus presumably the same average lengths. The critical concentration of actin is about three times less in the presence of Li^+ than in the presence of an equal concentration of K^+. Cytochalasin D accelerates the rate of Li^+-promoted actin assembly and reduces slightly the total fraction of actin assembly. However, cytochalasin D causes less shortening of filaments in the presence of Li^+ than in the presence of K^+ or Mg^{2+}. By the criteria of assembly kinetics and critical concentration, Li^+ is much less potent as a promoter of actin assembly than either Mg^{2+} or Ca^{2+}. These results are discussed in terms of the role of electrostatic forces in the actin assembly mechanism and in terms of possible relationships to therapeutic and toxicity mechanisms for Li^+.

Extensive investigations into the molecular mechanism of cytoplasmic motility have confirmed the central role of actin in contractile assemblies (Pollard and Weihing, 1974; Korn, 1982; Stossel et al., 1985; Pollard and Cooper, 1986). The reversible self-assembly of globular actin (G-actin) into filaments (F-actin) has been the focus of mechanistic investigations for several decades (Frieden, 1985). Actin self-assembly in vitro is manipulated by controlling the concentrations of cations in solution. G-actin has a high-affinity divalent cation-binding site that must be occupied in order to prevent protein denaturation. Recent evidence indicates a dissociation constant for this site in the nanomolar range (Gershman et al., 1986; Carlier et al., 1986*a*, *b*). Under low-salt conditions G-actin is stable as a monomeric protein; upon addition of millimolar divalent cations or up to 100 mM K^+, G-actin spontaneously self-assembles to form F-actin. This process appears to involve about five to nine lower-affinity cation-binding sites with $K_d \sim 0.15$ mM for Ca^{2+} and Mg^{2+} and $K_D \sim 10$ mM for K^+ (Martonosi et al., 1964; Carlier et al., 1986*a*; Strzelecka-Golaszewska et al., 1978). Partial occupation of these sites is sufficient to promote assembly (Barany et al., 1962). Although the mechanism is presumed to involve electrostatic screening, there is also evidence for a specific conformational change in G-actin before assembly by either K^+ (Rouyarenc and Travers, 1981; Pardee and Spudich, 1982*b*) or Mg^{2+} (Boxer and Stossel, 1976; Frieden et al., 1980; Frieden, 1982; Carlier et al., 1986*a*, *b*).

We report here the first study of the self-assembly of actin induced by lithium ion. The primary motivation for these experiments is the elucidation of the role of electrostatic forces in actin self-assembly. Li^+ is a uniquely interesting species for this purpose because its physical and chemical properties place it in an intermediate position among the ions that are most common in living systems. The ionic radius of Li^+ (0.060 nm) is less than half that of K^+ and even less than the radii of Mg^{2+} or Ca^{2+}. In its chemical properties and electrostatic binding behavior, Li^+ generally behaves more like an alkaline earth ion than as an alkali metal. Indeed in the index of "polarizing power," the ratio of charge to ionic radius squared, Li^+ is intermediate between Mg^{2+} and Ca^{2+}.

A second major motivation for this investigation relates to the widespread use of lithium salts as therapeutic agents. Since the 19th century, lithium salts and waters rich in lithium have been prescribed for maladies from gout to hypertension (Birch, 1982). Although a variety of potential medical applications are still being pursued (Rosoff and Robinson, 1980) by far the greatest clinical use of lithium today is in psychiatry.

Following the initial report that lithium salts have beneficial effects in the treatment and prevention of psychotic excitement (Cade, 1949), there has been a steady increase in the acceptance of Li^+ as a psychotherapeutic

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agent. Lithium salts are now the most frequent form of treatment of manic-depressive (bipolar) disorder and are commonly prescribed for the treatment of a general spectrum of manic, depressive, and aggressive behavioral disorders (Birch, 1982; Johnson, 1984; Fieve et al., 1985). However, the efficacy of lithium therapy remains controversial (Cooper et al., 1980; Breggin, 1983). Lithium ion is a toxin with a wide range of reported undesirable side effects (Lazarus, 1986). The dangers of lithium therapy are exacerbated by the fact that therapeutic and toxic plasma Li^+ concentrations are in the same range (mM) (El-Mallakh, 1984; Sashidharan, 1982; Amdisen, 1980).

The molecular basis of Li^+ psychotherapeutic activity and toxicity is not known. Li^+ has already been shown to promote the assembly of tubulin, leading to speculation of a role in the stabilization of microtubules in neurosecretory systems (Bhattacharyya and Wolff, 1976). The role of actin in neurosecretion may be even more central to the issue. It has already been shown that chlorpromazine (the most commonly employed major tranquilizer) and its metabolites alter polymerization and gelation of actin (Elias and Boyer, 1979). It is to be expected that Li^+, like other cations, has some activity in promoting actin assembly. This investigation provides the first characterization of that activity.

MATERIALS AND METHODS

Actin was isolated from commercial acetone powder of rabbit muscle using a slight modification of the method of Pardee and Spudich (1982*b*). Initial purification was accomplished by successive cycles of polymerization and depolymerization. To polymerize G-actin into F-actin, KCl (to 100 mM) and $MgCl_2$ (to 2 mM) were added to the supernatant at room temperature. After 2 h the resulting F-actin was pelleted by centrifugation in a Type 42.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 40,000 rpm for 2 h at 25°C. Depolymerization was performed by dialysis of the homogenized F-actin against buffer G (2 mM Tris base, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.02% NaN_3, adjusted to pH 8 using HCl). The resulting solution was clarified by centrifugation at the same speed for 30 min at 4°C. The final purification was accomplished by size exclusion chromatography using Sephadex G-150. The central fraction was maintained by dialysis against buffer G at 4°C and used for experiments within 3 d of column purification. The concentration of G-actin was determined by absorbance at 290 nm using an extinction coefficient of 0.62 $mg^{-1}ml\ cm^{-1}$.

Actin was labeled with fluorescein using a slight modification of the procedure described by Wang and Taylor (1980). 5 ml of G-actin (2 mg/ml) was dialyzed against 125 ml of buffer F (100 mM boric acid, 100 mM KCl, 4 mM $MgCl_2$, 1 mM Na_2ATP, pH 8.5) at room temperature for 2 h to assemble the actin into filaments. Then 10 mg of 5-iodoacetamido-fluorescein (5-IAF) dissolved in 1 ml of acetone was added to 125 ml of fresh buffer F and the F-actin solution was dialyzed against this medium at room temperature for 6 h with end-to-end agitation. The labeled F-actin was then pelleted, washed, and redissolved by dialysis against buffer G. The labeled G-actin was further purified using first a Sephadex G-25 column and then a G-150 column. From this point the actin was stored in the dark. The concentration of the labeled actin was determined by the Bradford assay (Bradford, 1976), using unlabeled actin as a standard. The extent of labeling was determined from the absorbance of the solution of labeled actin at 495 nm, using an extinction coefficient of $6.0 \times 10^4\ cm^{-1}M^{-1}$.

The pyrene-labeled actin was prepared using modifications of published procedures (Kouyama and Mihashi, 1981; Brenner and Korn, 1983). G-actin (2 mg/ml) was assembled at room temperature for 1 h in buffer F2 (5 mM Tris, 0.1 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.1 mM dithiothreitol, 0.01% NaN_3, pH 8) and dialyzed overnight at 4°C against the same buffer. A suspension of 3 mg of *N*-pyrenyliodoacetamide in 0.2 ml of dimethylformamide was mixed with the F-actin by inversion and the mixture was incubated at 20°C for 10 h. The actin was disassembled and purified on G-25 and G-150 columns as before. The concentration of the pyrene actin was determined by one or both of two Lowry assays (Lowry et al., 1951; Hartree, 1972) using unlabeled actin as a standard. The extent of labeling was determined using an extinction coefficient for the label of $2.2 \times 10^4\ M^{-1}cm^{-1}$ at 344 nm.

Actin assembly was characterized by fluorescence photobleaching recovery (FPR) using instrumentation and methodology that have been described previously (Lanni et al., 1981; Lanni and Ware, 1982, 1984; Ware, 1984). Briefly, a trace quantity of fluorescein-labeled actin is incorporated with native actin. Polymerization is initiated by the addition of salt(s), and the sample is loaded into a microcuvette and placed onto the stage of a fluorescence microscope. For each measurement a striped pattern is photobleached into the specimen, and the contrast of the pattern is monitored as a function of time using a modulation detection scheme (Lanni and Ware, 1982). The decay in the envelope of the modulation reflects the fading of the contrast of the pattern in the specimen as bleached and unbleached species randomize their positions by diffusion. From each FPR record, we determine the relative proportion of rapidly and slowly diffusing actin (G-actin and F-actin, respectively), the diffusion coefficient of the high-mobility fraction, and the average diffusion coefficient of the low-mobility fraction. Successive measurements, always taken from different spatial regions in the specimen, may be recorded about once per minute, permitting kinetic resolution of the parameters determined.

Critical concentrations of actin were determined using the method described by Tobacman et al. (1983). In this method, an increase in the fluorescence yield for trace amounts of pyrene-labeled actin is the indicator for assembly. Solutions of F-actin were sonicated at 50 w for 5 s and then diluted into the medium for which the critical concentration was to be determined. After 2 h the fluorescence intensity was determined at 388 nm using 368-nm illumination in a fluorescence spectrophotometer (model 650-10S; Perkin-Elmer Corp., Instrument Div., Norwalk, CT). Plots of fluorescence intensity versus actin concentration were fitted by least-squares to two lines, the intercept of which is reported as the critical concentration.

RESULTS

The interpretation of the modulation-detected FPR data from actin assembly assays has been discussed at length by Lanni and Ware (1984). In the experiments to be reported here, we found that the recovery traces were well fit as a sum of two components. The fast recovery was an exponential with a time constant consistent with the diffusion coefficient of G-actin. The second component should be regarded as a sum of exponentials corresponding to the diffusion of filaments with a distribution of lengths and hence with a distribution of diffusion coefficients. Since the relative diffusion coefficients of G-actin and F-actin differ by two to three orders of magnitude, it was straightforward and reliable to determine the fraction of the actin that had been incorporated into filaments (called the low-mobility fraction, f_{LM}). The time constant of the low-mobility fraction was fit by the method of Lanni and

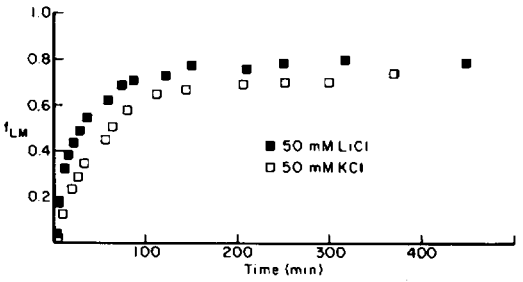

FIGURE 1 The fraction of actin assembled (f_{LM}) after addition of either 50 mM LiCl or 50 mM KCl at $t = 0$. The total actin concentration was 32 μM (5% labeled). $T = 20°C$.

Ware (1984) to the initial slope of the slow recovery, from which one extracts a number-average filament diffusion coefficient. The values obtained were in all cases consistent with reasonable lengths for filaments if one treats the filaments as independent diffusing stiff rods. The correspondence between lengths and diffusion coefficient magnitudes was discussed previously (Lanni and Ware, 1984).

The first set of experiments compared the rate of actin assembly upon addition of LiCl with the rate of assembly observed upon addition of KCl. Sample data are shown in Fig. 1 for assembly of 32 μM actin (5% labeled) at 20°C in the presence of 50 mM salt of either type. The low-mobility fraction (essentially the fraction of actin molecules incorporated into filaments) is plotted as a function of time, where time zero corresponds to the moment of addition of the salt. We observe from this experiment and from others of this type that actin is assembled more rapidly by Li^+ than by K^+ and that the extent of final assembly is also somewhat greater in the case of assembly by Li^+.

Actin assembly under more vigorous (and more standard) conditions is compared in Fig. 2. At 100 mM total salt concentration it is again observed that Li^+ assembles actin more rapidly and to a greater final extent than does K^+. However, the addition of 2 mM $MgCl_2$ to the KCl assembly medium produces much more rapid assembly and leads to a considerably higher final f_{LM} value, which also is achieved at a much earlier time.

The diffusion coefficients determined from the Li^+ and K^+ data of Fig. 2 are shown in Fig. 3. Again the primary

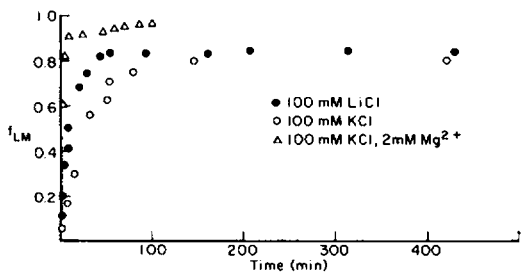

FIGURE 2 The fraction of actin assembled (f_{LM}) as a function of time after addition of salts for three sets of ionic conditions as specified. The total actin concentration in each case was 34 μM; $T = 20°C$.

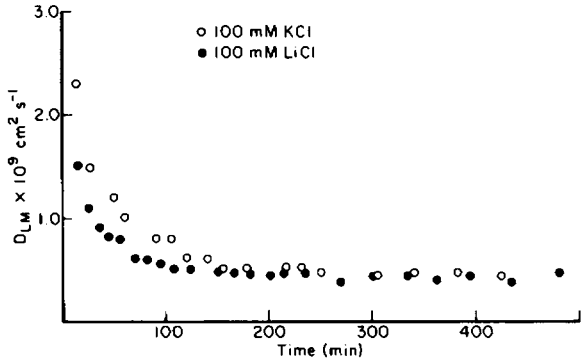

FIGURE 3 The diffusion coefficient of the low-mobility fraction (D_{LM}) as a function of time after addition of either KCl or LiCl. Conditions as in Fig. 2.

effect is kinetic. At short times the filaments assembled by Li^+ have considerably lower diffusion coefficients, probably indicating that they are longer, but at longer times the average diffusion coefficients of filament samples assembled by Li^+ or K^+ are indistinguishable. In recognition of the possibility that slight changes in the concentrations of ATP and ADP may have significant mechanistic implications, the actin assembly assays in the presence of K^+ and Li^+ were repeated using 0.7 mM ATP and 1.4 mM ATP. No significant differences in the kinetics or absolute magnitudes of the parameters determined were observed to be a function of ATP concentration.

Cytochalasin D (CD) has been used extensively as an agent for the study of actin assembly. The activity of cytochalasin D has been reported to be cation-dependent (Maruyama and Tsukagoshi, 1984; Mozo-Villarias and Ware, 1984), and we have pursued an interest in this effect to determine the activity of cytochalasin D in regulation of actin assembly promoted by Li^+. Sample data are shown in Fig. 4. In this assembly assay the actin concentration was 15 μM (13% labeled); other conditions were the same. The effect of 10 μM CD in the presence of 100 mM LiCl was to accelerate the kinetics of assembly and to reduce slightly

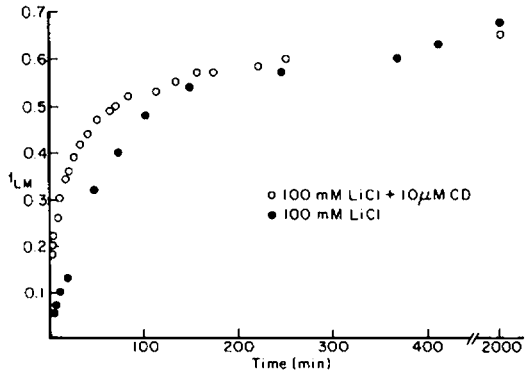

FIGURE 4 The fraction of actin assembled as a function of time after addition of 100 mM LiCl or 100 mM LiCl + 10 μM CD. The total actin concentration was 15 μM (13% labeled); $T = 20°C$.

the final extent of assembly. In these respects the effects of CD are similar to those observed earlier using FPR on actin solutions in the presence of more conventional ionic species (Tait and Frieden, 1982; Lanni and Ware, 1984; Mozo-Villarias and Ware, 1984). We have also examined the effects of cytochalasin D on the diffusion coefficients of actin filaments in a variety of media. The results are tabulated in Table I. The values tabulated were taken ~20 h after initiation of assembly; in most cases these values are, within experimental reproducibility, the same as the steady-state values for the plateau reached within 1–2 h after initiation of assembly. As reported previously (Mozo-Villarias and Ware, 1984), the effect of cytochalasin D is to produce filaments that have a higher diffusion coefficient, presumably because they are shorter. This effect is greatest when Mg^{2+} is present and appears to be least when Li^+ is the assembling species.

In principle the critical concentration of actin under the assay conditions is available from the FPR data. However, in practice the determination of critical concentrations from FPR data is not a reliable method unless the initial actin concentration is quite low. The reason is that the determination requires subtracting from one an f_{LM} value that is very nearly one, leaving an unacceptable relative error in the small magnitude of the difference. We have therefore measured critical concentrations of actin under a variety of ionic conditions using a superior method (Tobacman et al., 1983). The results are tabulated in Table II. In the comparison of critical concentrations for actin in the presence of monovalent ions, it may be important to examine the effect of the strongly bound divalent cation, which for the normal buffers such as those used in the experiments represented in Table II, is generally Ca^{2+}. Therefore we performed assemblies with K^+ and Li^+ also from a buffer analogous to buffer G except that the 0.2 mM $CaCl_2$ was replaced by 0.2 mM $MgCl_2$ Those results are given in Table III.

TABLE I
DIFFUSION COEFFICIENTS OF ACTIN FILAMENTS AFTER ASSEMBLY IN VARIOUS MEDIA*

Polymerizing medium	D_{LM}	D_{LM} with addition of 10 μM CD
1 mM Mg^{2+}	13.0	200.0
100 mM LiCl, 1 mM $MgCl_2$	11.9	23.6
100 mM KCl, 1 mM $MgCl_2$	10.8	23.4
100 mM LiCl	7.5	8.6
100 mM KCl	8.6	18.2

*Units are 10^{-10} cm^2 s^{-1}.

TABLE II
CRITICAL CONCENTRATIONS OF ACTIN IN VARIOUS ASSEMBLY MEDIA PREPARED BY ADDING SALTS TO BUFFER G*

Assembly medium	Critical concentration
mM	*μM*
$MgCl_2$	
0.5	2.2
0.75	0.94
1.0	0.61
1.5	0.32
2.0	0.24
$CaCl_2$	
1.0	2.4
2.0	1.1
3.0	0.70
4.0	0.63
5.0	0.57
KCl	
25	3.2
50	1.8
75	1.26
100	1.15
LiCl	
20	3.0
40	1.0
50	0.76
60	0.61
80	0.46
100	0.30

*0.2 mM $CaCl_2$.

DISCUSSION

The data presented in this paper verify that, in comparison with K^+, Li^+ is a more potent promoter of actin assembly. Accelerated kinetics for Li^+-promoted actin assembly could be explained either by a stronger nucleating activity or by an increased rate of addition of actin protomers to growing filaments. The data for f_{LM} versus time could resolve this issue in principle, but the time required to load the sample after addition of salts and the limited time resolution of the FPR measurement (roughly 1 min for our apparatus) do not permit the construction of a reliable determination of the growth rate (i.e., the time derivative of the data) at sufficiently short times to distinguish between nucleation rate and elongation rate. However, the data for the diffusion coefficients of filaments permit the distinction between the two mechanistic possibilities by two independent lines of reasoning. First we observe that the steady-state diffusion coefficients of filaments assembled in the presence of K^+ or Li^+ are indistinguishable. In the usual nucleation–elongation mechanism of actin assembly it follows that a faster rate of nucleation will produce more filaments, and, since these filaments consume the available actin with equal probability, the result is more filaments of shorter length. Thus the diffusion coefficients observed are not consistent with a significant difference in nucleation rates, from which we may infer that the difference in assembly kinetics must be attributable to an increased rate of elongation. The second and more direct evidence for a difference in elongation rates comes from the data of Fig. 3. At times < 2 h, the diffusion

coefficients of filaments assembled by K^+ are considerably greater than those of filaments assembled by Li^+, from which we infer that in the latter case the filaments are longer. Within the precision permitted by these data it appears that all of the difference in the f_{LM} values can be attributed to the differences in filament lengths. From this we conclude that Li^+ promotes actin assembly to a greater degree than K^+ through an enhanced rate of elongation.

The ability of cytochalasin D to accelerate the rate of assembly kinetics is generally attributed to an enhanced nucleation rate in the presence of this drug. The data of Fig. 4 verify that this activity is also observed when Li^+ is the assembly-promoting cation. The diffusion coefficient data of Table I provide further mechanistic information on the cation dependence of the cytochalasin D activity. As reported previously (Mozo-Villarias and Ware, 1984), the reduction of the lengths of actin filaments by cytochalasin D is much more strongly promoted by Mg^{2+} than by monovalent cations. A comparison of the filament diffusion coefficients for the cases of K^+ and Li^+ reveals that K^+-assembled filaments have a considerably higher diffusion coefficient, and thus are presumably shorter. This may indicate a more potent nucleating activity for cytochalasin D in the presence of K^+ as compared with Li^+, but our kinetic data for these experiments do not confirm that interpretation. The diffusion coefficient for Mg^{2+}-promoted assembly in the presence of CD is much higher than all other values, but the effect is largely eliminated if either 100 mM KCl or LiCl is present. In particular the difference between the values for 100 mM KCl and 100 mM KCl plus 1 mM Mg^{2+}, both in the presence of CD, is smaller than we would have expected from previous work. The diffusion coefficients in Table I are averages of many experiments, including at least three independent actin preparations and two different samples of cytochalasin D. Reproducibility for a given set of diffusion coefficients under nominally identical conditions was $\sim\pm 50\%$ (full range), so detailed interpretations of small differences are not warranted.

The critical concentration data of Table II provide a convenient means for quantifying the differences among the cations in their respective activities for the promotion of actin assembly. As indexed by the ratios of critical concentrations, Li^+ is roughly three times more potent than K^+ and Mg^{2+} is about four times more potent than Ca^{2+}. However, it is obvious that Li^+ is not comparable with either of the divalent cations. Simple electrostatic arguments based on the polarizing power cannot be a major factor in the mechanism for differences among cations in the critical concentration. It is interesting to observe that the threefold difference between K^+ and Li^+ in critical concentration could be explained by a threefold difference in the on-rate constant k_+, which is consistent with the kinetic data shown in Figs. 1–3, if the slope of the assembly curve in the intermediate time range is used to compare k_+ values.

TABLE III
CRITICAL CONCENTRATIONS OF ACTIN IN ASSEMBLY MEDIA*

Assembly medium	Critical concentration
	μM
50 mM KCl	1.1
100 mM KCl	0.53
50 mM LiCl	0.46
100 mM LiCl	0.20

*Media were prepared by adding univalent salts to G-actin in a variant of buffer G in which Mg^{2+} (0.2 mM) was substituted for Ca^{2+} throughout the preparation.

The failure of Li^+ to fall in the proper position as an actin assembly promoter based on electrostatic parameters may be taken as additional evidence for the importance of specific conformational changes in the assembly mechanism. The data of Table III lend support to this notion. It is evident that Mg^{2+}–actin is more readily assembled than Ca^{2+}–actin whether the assembly-promoting monovalent cation is Li^+ or K^+.

The major mechanistic conclusions of this work can be summarized as follows: (*a*) Li^+ is a more potent actin-assembly inducer than K^+. (*b*) The difference in the kinetics of assembly and possibly in the critical concentration for Li^+ and K^+ may be attributed to a faster rate of filament elongation. (*c*) That Li^+ is far less potent than either Mg^{2+} or Ca^{2+} in promoting actin assembly, despite being intermediate between them in polarizing power, indicates that simple electrostatic binding as predicted by this index is not a major element in the mechanism of the promotion of actin assembly by divalent cations.

With regard to the significance of these experiments for the evaluation of any putative actin assembly activity in the mechanism of Li^+ pharmacology, we do not find any supporting evidence. Although slight differences in the critical concentration of actin could be significant in the regulation of actin assembly in vivo, we doubt that the differences in critical concentration that would be obtaind at typical therapeutic levels could be mechanistically important. If there is an actin-related activity of Li^+ that may prove to be of clinical significance, we suggest that it would probably be an effect on one or more of the Ca^{2+}-modulated actin-regulatory proteins. That issue will be the subject of future work in this laboratory.

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