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# THE TIGHTLY BOUND DIVALENT CATION REGULATES ACTIN POLYMERIZATION

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The polymerization characteristics of $Ca^{++}$-actin and $Mg^{++}$-actin were studied by measuring initial rates of polymerization upon addition of phalloidin-stabilized nuclei and neutral salt. Under conditions where the effects of divalent cation exchange were minimized, $CaCl_2$ and $MgCl_2$ were found to be equally effective in polymerizing actin. $Mg^{++}$-actin was found to nucleate and polymerize more readily than $Ca^{++}$-actin, having a forward rate constant about twice that of $Ca^{++}$-actin under a variety of polymerizing conditions. The critical concentration for $Ca^{++}$-actin is approximately 20 times that for $Mg^{++}$-actin under equivalent conditions. These data imply that the polymer of $Mg^{++}$-actin must be more stable than that of $Ca^{++}$-actin, having a depolymerization rate constant about 10 fold lower. Since $Mg^{++}$ is probably the tightly-bound cation *in vivo*, whereas $Ca^{++}$-actin has been more widely studied *in vitro*, it would appear that actin in its physiological state is probably more polymerizable and more stable in the polymer form than previously considered.

The polymerization of actin is usually initiated by the addition of neutral salt. G-actin then undergoes a rapid conformational change (1-3) before aggregating into nuclei (4-7) which then elongate by first order addition of monomer units onto both polymer ends with a forward rate constant $k^+$. The reverse rate constant $k^-$ represents depolymerization at both polymer ends. The critical actin concentration, $C_\infty$, is the monomer concentration at polymerization equilibrium, and is equal to the ratio $k^-/k^+$.

Actin contains one mole of tightly bound divalent cation per mole of monomer unit (8-10). The binding of $Mg^{++}$ to the divalent cation site in G-actin apparently induces conformational changes in the protein which can be reversed by the addition of $Ca^{++}$ (11). Indeed, the properties of actin containing bound $Ca^{++}$ ($Ca^{++}$-actin) are reportedly different from those of actin containing bound $Mg^{++}$ ($Mg^{++}$-actin)

Abbreviation: EGTA, ethyleneglycol-bis (β-amino-ethyl) N,N tetraacetic acid

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(4,8,12-16). However, a systematic study of the polymerization kinetics of actin of known divalent cation composition has not been reported.

In the work presented here, the polymerization kinetics of $Mg^{++}$-actin and $Ca^{++}$-actin are studied. Measurement of initial polymerization rates largely averts the effects of bound divalent cation exchange. Use of phalloidin-stabilized nuclei ("seeds") (17) circumvents the problems of spontaneous nucleation. The results obtained indicate that the tightly-bound divalent cation exerts marked effects on the polymerization of actin.

## MATERIALS AND METHODS

All reagents were of analytical grade. ATP and EGTA were purchased from Sigma Chemical Co., phalloidin from Boehringer Mannheim, N-(1-pyrenyl)-iodoacetamide (N-P) from Molecular Probes. All solutions were prepared with sterilized double-distilled water.

Actin was purified as previously reported (17), except 0.2 mM ATP was used throughout and 0.01% $NaN_3$ was present except in the initial extraction. Actin was labelled with N-P as will be described elsewhere. Briefly, the actin pellets collected after the 0.8 M KCl incubation were homogenized in 0.1 M KCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.02 mM $CaCl_2$, 2mM Tris, pH 7.8, and 0.01% $NaN_3$, (18) and reacted overnight in the dark with equimolar N-P dissolved in 0.1% dimethylformamide (final concentration) at 25°C and with gentle stirring. The labelled F-actin was then collected by ultracentrifugation, dialyzed, sonicated and column purified as previously reported (17), except a Sephacryl S-300 column was employed. This actin was 90-100% labelled with N-P.

$Mg^{++}$-actin was prepared from stock actin (containing 0.02 mM $Ca^{++}$) by adding $MgCl_2$ and EGTA each to 0.05 mM and incubating for 6 min. Replacement of the actin-bound $Ca^{++}$ by $Mg^{++}$ was essentially complete as evidenced by bound cation measurements, using $^{45}Ca$ and flame photometry, which showed that the stock actin contained bound $Ca^{++}$ at a mole ratio of approximately 1:1, whereas following Mg/EGTA treatment, bound $Mg^{++}$ was present at approximately 1:1 mole ratio with little residual $Ca^{++}$ detectable. Control experiments (not shown) on $Mg^{++}$-actin rapidly chromatographed on Sephadex G-50 to remove the EGTA and any residual $Ca^{++}$ demonstrated no significant deviations in polymerization characteristics from $Mg^{++}$-actin which had not been chromatographed on G-50. Thus, in the experiments presented below, chromatography of $Mg^{++}$-actin to remove EGTA and residual $Ca^{++}$ was not performed. $Ca^{++}$-actin was prepared for polymerization experiments by adding EGTA to 0.05 mM and $CaCl_2$ to 0.07 mM (i.e. an additional 0.05 mM) and incubating for 6 minutes. Thus, $Mg^{++}$-actin and $Ca^{++}$-actin were handled equivalently prior to polymerization.

A stock solution of phalloidin-stabilized actin nuclei ("seeds") was prepared by polymerizing 25 μM unlabelled column-purified actin with equimolar phalloidin for 24 hours at 25°C in the absence of salt (17). Ten-fold diluted aliquots of the stock "seed" solution were sonicated for two 30 sec periods and stored on ice for at least 3 h before use.

Protein was measured by the method of Bradford (19) or by ultraviolet absorption (17) after correcting for the absorption of N-P at 290 nm (=0.33 x $A_{344}$). Fluorescence was measured on 2 ml samples in a thermostated cell at 20°C with an Aminco-Bowman spectrophotofluorometer using an activation wavelength of 350 nm and emission wavelength of 380 nm. The relationship between fluorescence and actin concentration was non-linear above an actin concentration of

10 μM, apparently due to the high percentage of fluorescent labelling. This non-linearity only affected the results in Fig. 3. Therefore, the polymerization rates in Fig. 3 are expressed in μM/min based on the calibration curve for fluorescence vs. actin concentration. Initial polymerization rates were calculated as the average rate over the first 30 seconds of the polymerization time-course.

## RESULTS AND DISCUSSION

Figure 1A shows the time course of polymerization for $Ca^{++}$-actin and $Mg^{++}$-actin polymerized by addition of neutral salt to final concentrations of 1 mM $CaCl_2$, 1 mM $MgCl_2$ or 0.1 M KCl. For each polymerizing salt, the nucleation of $Mg^{++}$-actin is seen to be faster than for $Ca^{++}$-actin. Indeed, the initial polymerization

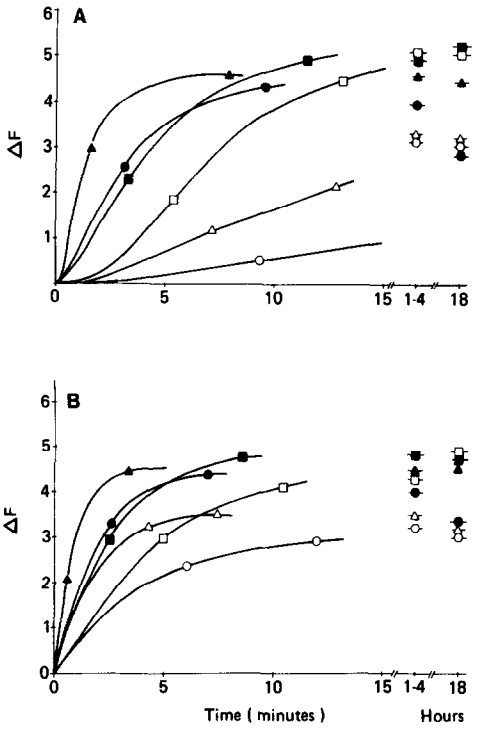


Figure 1. Time course of polymerization of $Ca^{++}$-actin and $Mg^{++}$-actin with neutral salt. The fluorescence intensity change of N-pyrenyl-labelled $Mg^{++}$-actin (filled symbols) and N-P labelled $Ca^{++}$-actin (open symbols) was followed over the initial time course and at 1-4 h and 18 h after initiation of polymerization. The curves are tracings of continuous chart recordings with symbols added for identification. The symbols at 1-4 h and 18 h represent data points. The 18 h data have been corrected for an as yet unexplained uniform 5-10% increase in fluorescence intensity of N-P actin stored at 25°C over this time period. N-P actin concentration, 5 μM. In addition to polymerizing salt and actin, all solutions contained 0.2 mM ATP, 0.01% $NaN_3$, 2mM Tris, pH 7.8, 0.05 mM EGTA and 0.07 mM $CaCl_2$ (open symbols), 0.02 mM $CaCl_2$ plus 0.05 mM $MgCl_2$ (filled symbols).
(A) Spontaneous polymerization following addition at time t = 0 of 1 mM $CaCl_2$ (circles) 1 mM $MgCl_2$ (squares) and 100 mM KCl (triangles).
(B) Induced polymerization following addition of 50 μl phalloidin-stabilized nuclei at time t = 0 with polymerizing salt (same symbols as for A).

rate for $Mg^{++}$-actin is non-zero, suggesting that nuclei are present at the initiation of the experiment. The polymerization of $Ca^{++}$-actin by $MgCl_2$ initially starts slowly, then accelerates so that the maximum polymerization rate at 5-6 min is quite similar to that for $Mg^{++}$-actin polymerized by $MgCl_2$. This is most likely the result of the exchange of actin-bound $Ca^{++}$ for $Mg^{++}$ with subsequent rapid nucleation and polymerization of the $Mg^{++}$-actin produced. A similar occurrence is not seen when $Mg^{++}$-actin is polymerized with $CaCl_2$; probably the highly polymerizable $Mg^{++}$-actin polymerizes before significant exchange of the monomer-bound cation can occur. However, the data at 18 h suggest that exchange ultimately does occur; the final extent of polymerization is greatest when $Mg^{++}$ is the predominant divalent cation present.

In the experiments of Fig. 1B the effects of nucleation are circumvented by addition of phalloidin-stabilized seeds with the polymerizing salt. Note that the initial rate of polymerization of $Mg^{++}$-actin with 1mM $CaCl_2$ is essentially the same as that with 1 mM $MgCl_2$ and that the rate with 0.1 M KCl is about 3 times faster than the rate with either $MgCl_2$ or $CaCl_2$. The results for $Ca^{++}$-actin are similar, but the polymerization rates are clearly lower than for $Mg^{++}$-actin. From the data of Fig. 1B, after correction for polymerization due to spontaneous nucleation from Fig. 1A, the ratio of the forward polymerization rate for $Mg^{++}$-actin vs that for $Ca^{++}$-actin, $k^+_{Mg}/k^+_{Ca}$, is approximately 2.6 in 1 mM $CaCl_2$, 2.7 in 1 mM $MgCl_2$, and 1.6 in 0.1 M KCl. These values are in good agreement with similar determinations from computer fits to polymerization curves (4).

The experiments of Fig. 1 suggest that barring the effects of divalent cation exchange, $CaCl_2$ or $MgCl_2$ are equally effective as polymerizing agents. This finding is consistent with the reported similarity in binding of $Ca^{++}$ and $Mg^{++}$ to the low affinity sites of actin (10), which is the presumed mechanism by which these cations polymerize actin (20). More importantly, the experiments of Fig. 1 show that both the rate and extent of polymerization of actin depend upon the species of tightly bound cation, $Mg^{++}$-actin being more polymerizable than $Ca^{++}$-actin. The experiments of Fig. 2 were performed to further demonstrate

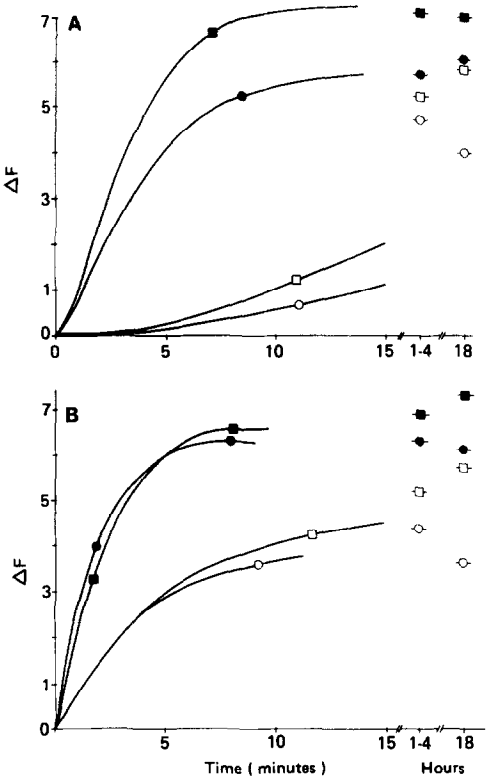


Figure 2. Time course of polymerization of $Ca^{++}$-actin and $Mg^{++}$-actin with $CaCl_2/MgCl_2$. Experimental procedure as for Fig. 1. Actin concentration, 10 μM. Polymerization initiated by addition of KCl to 10 mM at time t = 0 to all samples along with $CaCl_2$ and/or $MgCl_2$ to final concentrations as shown: ○, $Ca^{++}$-actin with 0.42 mM $CaCl_2$; □, $Ca^{++}$-actin with 0.22 mM $CaCl_2$ + 0.20 mM $MgCl_2$; ●, $Mg^{++}$-actin with 0.22 mM $CaCl_2$ + 0.20 mM $MgCl_2$; ■, $Mg^{++}$-actin with 0.02 mM $CaCl_2$ + 0.40 mM $MgCl_2$.
(A) Spontaneous polymerization
(B) Induced polymerization following addition of 50 μl phalloidin-stabilized nuclei at time t = 0 with polymerizing salt.

these points, with conditions chosen to minimize early monomer divalent cation exchange and to provide identical polymerizing conditions for $Ca^{++}$-actin and $Mg^{++}$-actin. Figure 2A shows that in 0.22 mM $CaCl_2$, 0.20 mM $MgCl_2$ and 10 mM KCl, $Mg^{++}$-actin (filled circles) nucleates and polymerizes more rapidly than $Ca^{++}$-actin (open squares). However, essentially the same extent of polymerization was attained by these samples after 18 hrs. Thus, it appears that a re-equilibration of the tightly bound divalent cation occurs with time. The experiments of Fig 2B were performed similarly but with phalloidin-stabilized seeds also added at time t=0. The difference between the initial polymerization rates for $Mg^{++}$-actin and $Ca^{++}$-actin is clearly evident. With correction for self-nucleation

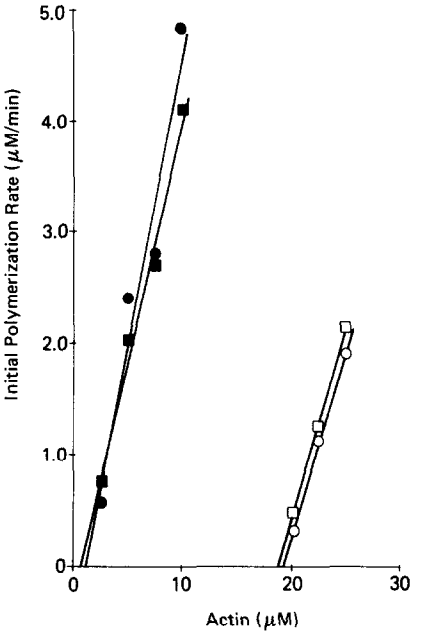


Figure 3. Actin concentration dependence of initial rates of polymerization. Initial polymerization rates were calculated from fluorescence intensity increases of N-P labelled $Ca^{++}$-actin (open symbols) and N-P labelled $Mg^{++}$-actin (filled symbols) polymerized by the addition of 50 μl phalloidin-stabilized seeds at time t = 0 along with $CaCl_2$ and/or $MgCl_2$ to the same final concentrations as for Fig. 2 (same symbols as for Fig. 2). Polymerizing solutions did not contain KCl but did include 0.2 mM ATP, 0.01% $NaN_3$, 0.05 mM EGTA, 2mM Tris, pH 7.8.

from the data of Fig 2A, the ratio $k^+_{Mg}/k^+_{Ca}$ under these conditions was found to be 2.5.

In Fig. 3 are plotted, as a function of actin concentration, the initial polymerization rates for $Ca^{++}$-actin and $Mg^{++}$-actin induced by phalloidin stabilized nuclei and further additions of $MgCl_2$ or $CaCl_2$. The experiment was initially conceived to validate the "initial-slope" method by demonstrating linearity of polymerization rate with increasing actin concentration, and in this it succeeds. However, the experiment produces additional useful information. The slope of each line is proportional to $k^+$; thus for the polymerizing condition 0.22 mM $CaCl_2$ plus 0.20 mM $MgCl_2$, the ratio $k^+_{Mg}/k^+_{Ca}$ may be calculated from the slopes of the appropriate two lines (solid circles, open squares) and is found to be 1.6. Furthermore, Fig. 3 demonstrates a kinetic method for measurement of the critical concentrations for $Mg^{++}$-actin and $Ca^{++}$-actin - a method which does not require long-term equilibrium measurements. By this method, the critical

concentration, $C_\infty$, is that actin concentration at which the initial polymerization rate extrapolates to a rate of zero. It is seen that under these conditions the critical concentration depends markedly on the tightly-bound cation and little on the polymerizing salt. Fig. 3 indicates a $C_\infty$ for $Mg^{++}$-actin and $Ca^{++}$-actin of approximately 1.0 μM and 19.0 μM, respectively. Since $C_\infty = k^-/k^+$, these data may be used to calculate a ratio of depolymerization rate constants for $Mg^{++}$-actin and $Ca^{++}$-actin. For these conditions, this ratio is found to be $k^-_{Mg}/k^-_{Ca} = 0.08$. This result implies that the polymer of $Mg^{++}$-actin is significantly less depolymerizable than that of $Ca^{++}$-actin.

In summary, we have demonstrated that $Mg^{++}$-actin nucleates and polymerizes more readily than $Ca^{++}$-actin. When the effects of bound divalent cation exchange are minimized, $CaCl_2$ and $MgCl_2$ have been shown to be equally effective polymerizing agents for actin. From measurements of initial rates of polymerization under a variety of solvent conditions, the ratio of polymerization rate constants for $Mg^{++}$-actin and $Ca^{++}$-actin, $k^+_{Mg}/k^+_{Ca}$, is in the range of 1.6 to 2.7. Studies in 0.22 mM $CaCl_2$ and 0.20 mM $MgCl_2$ using a novel kinetic method for determining $C_\infty$ indicate that the ratio $k^-_{Mg}/k^-_{Ca}$ is about 0.1; thus the polymer of $Mg^{++}$-actin must be significantly more stable (less depolymerizable) than that of $Ca^{++}$-actin. These differences in polymerization characteristics between $Mg^{++}$-actin - the most likely species *in vivo* (20) - and $Ca^{++}$-actin - the species most studied *in vitro* - may be important to our understanding of the physiological role of actin.

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