Effects of Magnesium on the Dynamic Instability of Individual Microtubules†

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ABSTRACT: We investigated the effect of magnesium ion (Mg) on the parameters of dynamic instability of individual porcine brain microtubules. Rates of elongation and rapid shortening were measured by using video-enhanced DIC light microscopy and evaluated by using computer-generated plots of microtubule length vs time. Increasing [Mg] from 0.25 to 6 mM increased the second-order association rate constant for elongation about 25% at each end. At plus ends, this resulted in a 1.5–2-fold increase in elongation rates over the tubulin concentrations explored. Rapid shortening rates were more dramatically affected by Mg. As [Mg] was increased from 0.25 to 6 mM, the average rate of rapid shortening increased about 3-fold at plus ends and 4–5-fold at minus ends. The ends had roughly equivalent average rates at low [Mg], of 30–45 μm/min. At any Mg concentration, rates of disassembly varied from one microtubule to another, and often an individual microtubule would exhibit more than one rate during a single shortening phase. Individual rates at 6 mM Mg varied from 12 to 250 μm/min. Over the concentration range explored, Mg affected the frequencies of transition from elongation to shortening and back only at minus ends. Minus ends were relatively stable at low [Mg], having 4 times the frequency of rescue than at high [Mg], and a lower frequency of catastrophe (particularly evident at low tubulin concentrations). Plus ends, surprisingly, were highly unstable at all Mg concentrations investigated, having about the same transition frequencies as did the least stable (high Mg) minus ends. Our results have implications for models of the GTP cap, again emphasizing that GTP caps cannot build up in proportion to elongation rate, and must be constrained to the tips of growing microtubules.

It is well established that individual microtubules, both in vitro and in vivo, alternate between distinct, persistent phases of slow elongation and more rapid disassembly (Mitchison & Kirschner, 1984; Horio & Hotani, 1986; Walker et al., 1988; Schultz & Kirschner, 1988; Sammack et al., 1988; Cassimeris et al., 1988). This behavior, comprising both the alternating phases and the transitions between them, is termed dynamic instability (Mitchison & Kirschner, 1984; Walker et al., 1988). The rate constants for the elongation (assembly) phase, rapid shortening (disassembly) phase, and the concentration dependence of the transition frequencies between phases have been determined in vitro (Walker et al., 1988), and this information has fueled speculation about the mechanisms that must underlie dynamic instability, particularly the mechanisms that result in the transitions between phases (Walker et al., 1988; Bayley et al., 1990).

While the data from Walker et al. (1988) have provided an important base line of microtubule behavior, only one factor (tubulin concentration) was explored in that study. Left unanswered was the question of how different solution conditions might affect microtubule behavior, and how changes in one parameter of dynamic instability might correlate with changes in the others. For example, if a condition were found that increased the rate of elongation for a given tubulin concentration, would the rate of disassembly and the frequency of catastrophe be reduced?

Indications that dynamic instability might be able to be modulated come from a number of different observations. For instance, the data reported in Walker et al. (1988) do not correspond perfectly to the limited data that have been derived from living cells. Elongation rates in interphase cells were higher than expected relative to the estimated tubulin concentration and frequency of catastrophe observed [see Walker et al. (1988) and Cassimeris et al. (1988) for a discussion]. These differences could derive from differences in the respective tubulins, or in the solution conditions present during observation. Another example of modulation of dynamic instability might be the change in the turnover rate and general "dynamic state" of microtubules at the interphase–mitosis transition (Saxton et al., 1984; Vandre et al., 1984; Cassimeris et al., 1987), a change that could not be due to differences in the tubulin involved. We were therefore interested not only in how a change in buffer components would affect the various parameters of dynamic instability but also in how these changes might correlate with dynamic instability in vivo.

In a recent study of the effects of various buffers and nucleotides on the assembly and stability of pure microtubules, we noted that the usual effect of an agent that promoted assembly, such as the nonhydrolyzable GTP analogues, ATP, glycerol, and glutamate, was to stabilize the polymer against disassembly after depletion of free GTP (O'Brien & Erickson, 1989). The lone exception to this rule was magnesium ion (Mg). Magnesium increased the rate of initial assembly, yet also increased the rate of disassembly after the free GTP had been consumed. We wondered whether these effects could be observed on the individual microtubule level and which of the parameters of dynamic instability were affected.

Our approach was essentially the same as described earlier for investigating the assembly of individual microtubules with video-enhanced DIC light microscopy (Walker et al., 1988), with the addition of a flow cell to change buffers without changing axoneme nucleation centers, and to extend the range of tubulin concentrations we could explore (Berg & Block, 1984). We show that magnesium affects all parameters of dynamic instability except for plus-end catastrophe frequency.

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Surprisingly, plus-end catastrophe frequency remained constant even though elongation rates increased 2–3-fold with increased [Mg]. Magnesium increased the general "dynamic state" of microtubules: increasing both the assembly rate during the elongation phase and the disassembly rate during rapid shortening.

Materials and Methods

We investigated the effects of Mg concentration on dynamic instability using two similar methods. For concentrations of tubulin between 10 and 16 μM, we added axoneme fragments and tubulin in a given buffer at a working volume of 30–40 μL, put a 5-μL sample on a slide, and then covered, sealed, and recorded the assembly behavior of individual microtubules using video-enhanced DIC light microscopy, as described previously (Walker et al., 1988). At these tubulin concentrations, microtubules could nucleate from the axoneme seeds with sufficient frequency to obtain data, yet did not grow beyond our field of view in the time required to begin observation of a normal slide/coverslip preparation. Axoneme fragments were present at a final concentration of about 5×10^{-14} M. If each fragment nucleated nine microtubules from each end, and each was 10–20 μm long, about 0.5–2% of the tubulin present would be in the polymer form (given 1630 subunits/μm). In general, fewer than 18 microtubules were nucleated from each axoneme fragment at the tubulin concentrations used, and experiments rarely continued after microtubules were more than 20 μm long, since the growing ends of longer polymers would be lost from view. Slides were observed from about 5 min after they were prepared up to about 30 min, and two to three different reaction mixtures per slide preparation were used to generate data for any one data point (i.e., any particular tubulin concentration). For low tubulin concentrations, sometimes more slides would be necessary to evaluate since few microtubules were nucleated per viewing area. During the observation time, the limited number of seeds kept the reaction well below the steady-state polymer concentration, and maintained the GTP–tubulin concentration essentially at "initial conditions" throughout the observation time. This is discussed further below, and in Walker et al. (1988).

To obtain data from higher and lower concentrations of tubulin, we used a miniature flow cell (Berg & Block, 1984). In this way, we could follow the early growth of rapidly growing microtubules by prewarming the flow cell, focusing on axoneme fragments, and then flowing in high tubulin concentrations. Because of the rapid growth and a gradual buildup of self-nucleated microtubules after 15–20 min at tubulin concentrations >18 μM, experiments at high tubulin concentrations were observed for only 5–10 min each. At low tubulin concentrations, microtubules nucleated very rarely and then grew only a short distance before undergoing a catastrophe. With the flow cell, many microtubules could be nucleated from axonemes at moderately high tubulin concentrations and then the tubulin concentration reduced to the desired concentration and the slow elongation rates monitored.

It has been calculated that 6 s of continuous flow exchanges approximately 90% of the solution near the glass surface (Walker and Salmon, unpublished results). For the present work, we used two flushes of 6 s with a 3–5-s pause in between to further ensure complete exchange. This increased the working volumes of tubulin used per data point from about 30 to 200 μL. The large volumes made pipetting error less likely a significant factor in the rates observed. Thus, the fit of the data generated with and without the flow cell served as a convenient check on the pipetting of the smaller volumes used at low concentrations of tubulin. The data are quite consistent between flow cell and "stationary" slide preparations (10–16 μM tubulin was done using the stationary slide; above and below those concentrations, the flow cell was used), with $r = 0.97$ and 0.98 for the regression lines through the plus- and minus-end elongation data (Figure 1). The perfusion chamber also allowed us to determine the polarity of axonemes at one Mg concentration and then alter the [Mg] and assess the polarity of growth at the same initiation sites. This provided assurance that high [Mg] did not cause a reversal of the character of the two ends, and indeed, as [Mg] was changed, slower growing ends remained slower.

Mg concentrations are reported as the total present, to be consistent with our earlier study (Walker et al., 1988). The concentrations of GTP, Pipes, and EGTA were also kept the same as that study (1 mM GTP, 100 mM Pipes, and 2 mM EGTA, pH 6.9). The calculated free Mg concentration at 0.25, 1, and 6 mM added was 0.038, 0.29, and 4.6 mM, respectively, using a program modified from Robertson and Potter (1984), and stability constants from Martell and Smith (1974). Video DIC light microscopy and analysis were carried out as before, and tubulin was freed of associated proteins by using phosphocellulose chromatography and a subsequent cycle in 1 M glutamate, as before (Walker et al., 1988).

A trivial explanation of our results might be that increasing [Mg] increased the concentrations of GTP–tubulin. Mg is known to be required for GTP binding to tubulin (Huang et al., 1985; Correia et al., 1987). We believe this is not a factor in our experiments. GTP was bound to tubulin under high [Mg] (5–6 mM) conditions and then brought to a lower [Mg] by passage through a desalting column. Under these conditions, without GDP present and in the presence of excess GTP, GTP should remain bound to tubulin unless a substantial amount of GDP can accumulate. Since (as mentioned above) at any one time we assemble only a small percentage of the tubulin present, and observe assembly for only 5–30 min, negligible GDP can accumulate relative to the 1 mM GTP present. This is also reflected in the constant elongation rates seen over the time of observation: no detectable slowing of elongation was seen during even the longest observation period, implying a negligible buildup of GDP–tubulin over the time of our experiments. Further, assembly of GTP–tubulin with no free nucleotide present gave the same qualitative results as with GTP present, i.e., slower elongation and disassembly with low [Mg] and higher rates with increasing [Mg] (data not shown). Finally, although a complete data set was not obtained for these concentrations, we did investigate the rates of elongation at 2.5, 5, 7.5, 10, 12.5, and 15 mM Mg [reported in O'Brien et al. (1989)]. Elongation rate constants were increased not only from 0.25 to 6 mM Mg but from also from 2.5 to 5 mM, and slightly further at 7.5 mM or above, well beyond the concentration of Mg wherein limited Mg might significantly decrease the availability of GTP–tubulin. As a final control were tested the axonemes, at about twice the normal concentration for about twice the normal time of an experiment (60 min), at 37 °C, to see if they alone or with 20 μM tubulin could produce significant GDP (i.e., did the axonemes contain any phosphatase activity on their own). As measured by HPLC (O'Brien et al., 1987), there was no increase in GDP over 1 h with axonemes only, and about 20 μM GDP evolved with tubulin present.

As discussed in Walker et al. (1988), the transitions from elongation to rapid shortening (catastrophe) and back (rescue) appeared to be stochastic. In the present study, we again found that there was no way to predict when a microtubule would

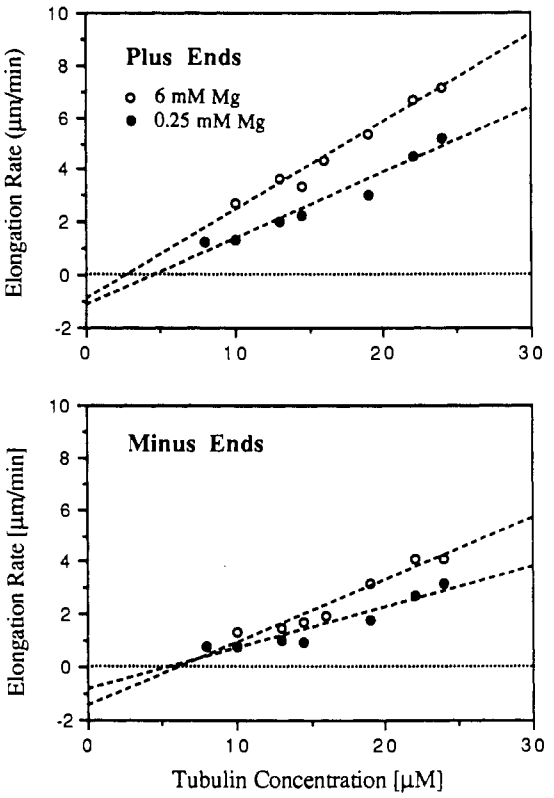

FIGURE 1: Mean elongation rates vs tubulin concentration. The rate of elongation was estimated by the slope of the least squares regression lines of plots of microtubule length vs time (life history plots, see Figure 2). Rates for one tubulin and Mg concentration were tabulated and then sorted into plus and minus ends on the basis of growth rate and relation to other microtubules at the same axoneme as discussed in Walker et al. (1988). Dashed lines are least-squares regression lines.

make a transition from one state to the other, or which microtubule in a group would make a transition. No microtubule appeared to be especially prone to catastrophe or rescue. To calculate transition frequencies, therefore, we totalled the number of catastrophies or rescues observed for all microtubules observed at a particular tubulin concentration and polarity and divided by the total time that all microtubule spent either elongating or shrinking under those same conditions. We did not select only those microtubules that underwent a transition to calculate transition frequencies.

RESULTS

Effect of Magnesium on Elongation Rates. Plots of elongation rate vs tubulin concentration are shown in Figure 1. The figure shows plus- and minus-end mean rates of elongation for 10–30 individual microtubules per data point, at 0.25 and 6 mM added magnesium. We also obtained data at 1 mM to compare with those obtained at other Mg concentrations, and the rates obtained were very close to those of our earlier study (Walker et al., 1988), and intermediate between the results at 0.25 and 6 mM. In general, increasing [Mg] increased rates of elongation at both ends at all tubulin concentrations. These increases were primarily a result of increases in the slopes of the least-squares regression lines, while the effects on y intercepts were less consistent. [As discussed in Walker et al. (1988), the slope of these lines corresponds to the second-order rate constant for tubulin association to microtubule ends, and the y intercept to the first-order dissociation rate during elongation.] At plus ends, the slope increased from 0.25 to 0.33 μm/(μM·min) as [Mg] was increased from 0.25 to 6 mM, while the y intercept decreased from −1.13 to −0.89 μm/min. The critical concentration for elongation [equivalent to the K^e_{Eq} for the subunit binding during elongation and corresponding to the x intercept, as discussed in Walker et al. (1988)] is calculated to be 4.54 μM at 0.25 mM Mg and 2.67 μM at 6 mM Mg. At minus ends, the slope increased from 0.16 to 0.24 μm/(μM·min) as [Mg] was increased, but the magnitude of the y intercept also increased, from −0.87 to −1.49, resulting in very little change in the equilibrium constant (5.54 μM at 0.25 mM Mg to 6.25 μM at 6 mM). Nevertheless, at all tubulin concentrations above 10 μM, the average rate of minus-end elongation was always greater at 6 mM Mg than at 0.25. Considered together, the consistent points about Mg and elongation are that increasing [Mg] increases the second-order association rate constant (k^e_2) and that a significant apparent dissociation rate is present at all Mg concentrations.

Rapid Shortening. Magnesium dramatically increased rates of rapid shortening after a catastrophe. Unexpectedly, the increases seen were partly quantal in nature. During a single rapid shortening event, microtubules often began to disassemble at one rate and then would abruptly switch to another rate, either faster or slower. This was apparent during the tracking of a rapid shortening event in real time, and confirmed by retracking replays of rapid shortening events when needed and then viewing the computer-generated graphs of length vs time on an expanded time scale. Rates appeared constant until the abrupt change took place.

Examples of life history data from three individual microtubules are presented in Figure 2. These illustrate the constant nature of most elongation phases, the abrupt transition to rapid shortening (catastrophe), and three different rapid shortening events. Panels B, D, and F of Figure 2 show the rapid shortening events of panels A, C, and E on an expanded time scale. The microtubules shown in panels A–D underwent breaks or changes in rate, while the microtubule in panels E and F exhibited a constant velocity for its entire length. Each tracking procedure was done manually with a mouse while running the video tape at real time. Since the manual tracking sometimes missed points, the disassemblies in Figure 2B,F were replayed 2 more times to confirm the accuracy of the first tracking.

Figure 2A,B shows the rapid shortening of a microtubule with plus-end free (called a "plus end" for convenience), that displayed three different regions of relatively constant disassembly. The [Mg] was 6 mM, [tubulin] 10 μM. The rates of disassembly, based on an average of the rates obtained for each region for the three tracking procedures, were 182, 55, and 174 μm/min. If a particular constant velocity region was missed during tracking, such as the third rate of the middle plot in Figure 2B, its rate was not included in the mean. From these data, it appears that the rate of disassembly during the first segment was essentially the same as the last, but the disassembly rate for the second segment was substantially different from the other two. Figure 2C,D shows a plus-end microtubule at 1 mM Mg and 10 μM tubulin. The rapid shortening event took place in two stages: the first 5 μm disassembled at 89 μm/min, and the last 12 μm disassembled at the markedly reduced rate of 22 μm/min. Figure 2E,F shows a plus microtubule end at 6 mM Mg and 13 μM tubulin that exhibited a single shortening velocity. This demonstrates that not all rapid shortening events were subdivided, and even long lengths of polymer, in this case about 30 μm, could be lost at a constant velocity. The rates determined from the repetitions shown in Figure 2E were 156, 154, and 159 μm/min.

We conclude that microtubules often abruptly change the rate at which they disassemble. Regions of constant velocity

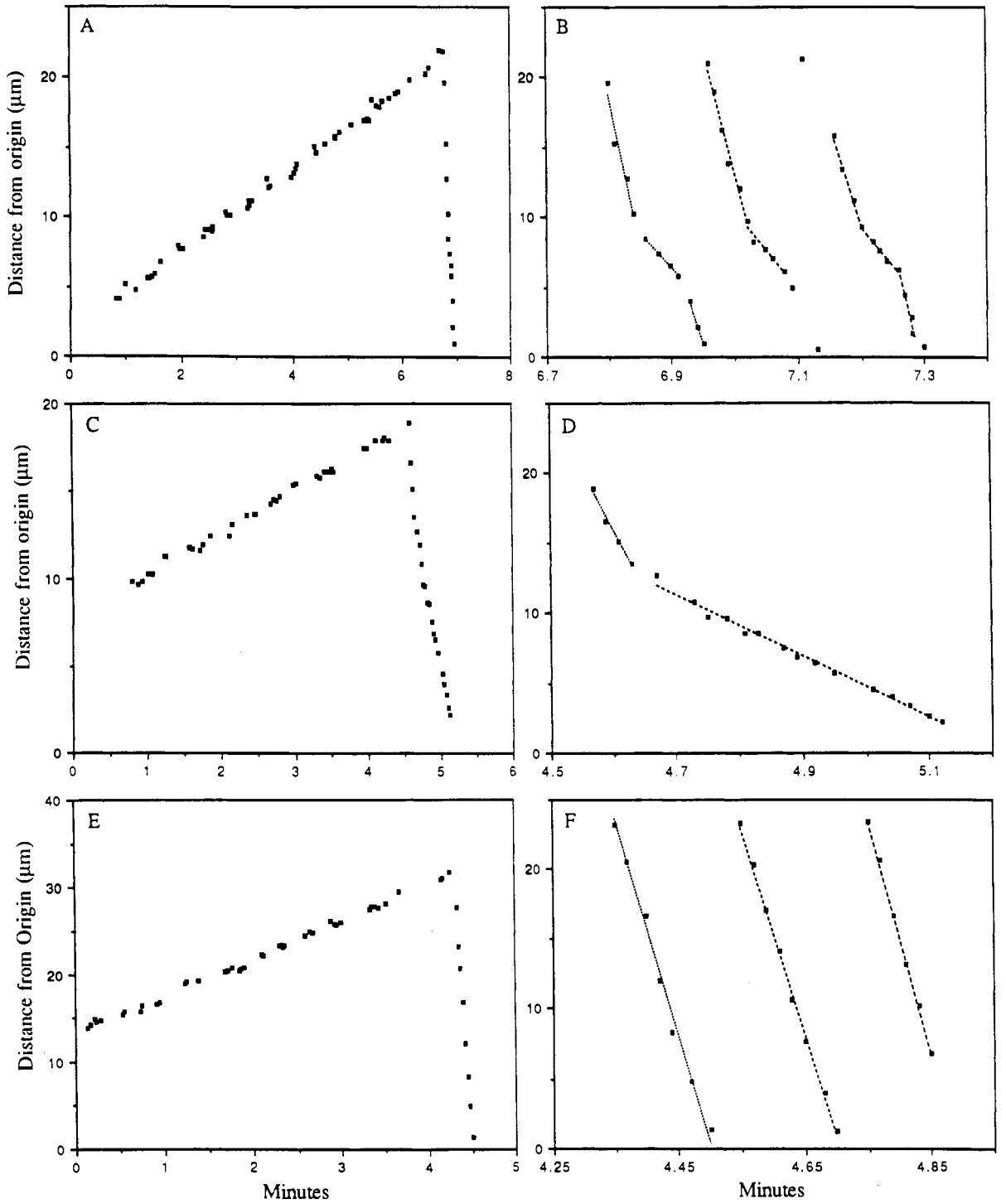

FIGURE 2: Life history plots of three microtubules. The lengths of microtubules over time were determined by following a videotaped image of growing and shrinking microtubules with a mouse-driven, computer-generated cursor in real time. Each horizontal pair (A, B, etc.) is one microtubule, with the right-hand figures (B, D, and F) showing the rapid shortening event on an expanded time scale (range: 0.5 min). The x and y axes of panels B, D, and F span equal regions of time and distance, so that the slopes of the disassembly plots can be easily compared. Panels B and F show the original shortening event shown in panels A and E, and two subsequent trackings of the same disassembly event, to compare with the first. Each closed square represents one click of the mouse. Dashed lines are least-squares regression lines.

varied stochastically from less than 1 μm to 10's of μm, and the frequency of their occurrence (or the average length of a constant velocity region) seemed to vary with [Mg]. Very high [Mg] (greater than 6 mM added Mg) seemed to exhibit long regions of high rates of disassembly (50 μm/min or greater), while zero added magnesium and 100 μM EDTA produced long regions with very low rates of disassembly (6–25 μm/min) and few changes in rate (data not shown). Multiple rates were observed primarily in the magnesium concentration range we report in this study, from 0.2 to 6 mM, and appeared to be independent of microtubule polarity.

Since rapid shortening appears to be zero order with respect to tubulin concentration [Walker et al. (1988) and the present study], data from all tubulin concentrations were used to generate Figure 3. In the top panel of Figure 3, disassembly rates are presented as an unweighted average of all the rapid shortening events seen at the Mg concentration indicated. For rapid shortening events with more than one rate, each segment or region that could be measured with confidence was included in calculating the mean.

Figure 3 (top) illustrates two main points; increased [Mg] resulted in increased rates of disassembly during a rapid

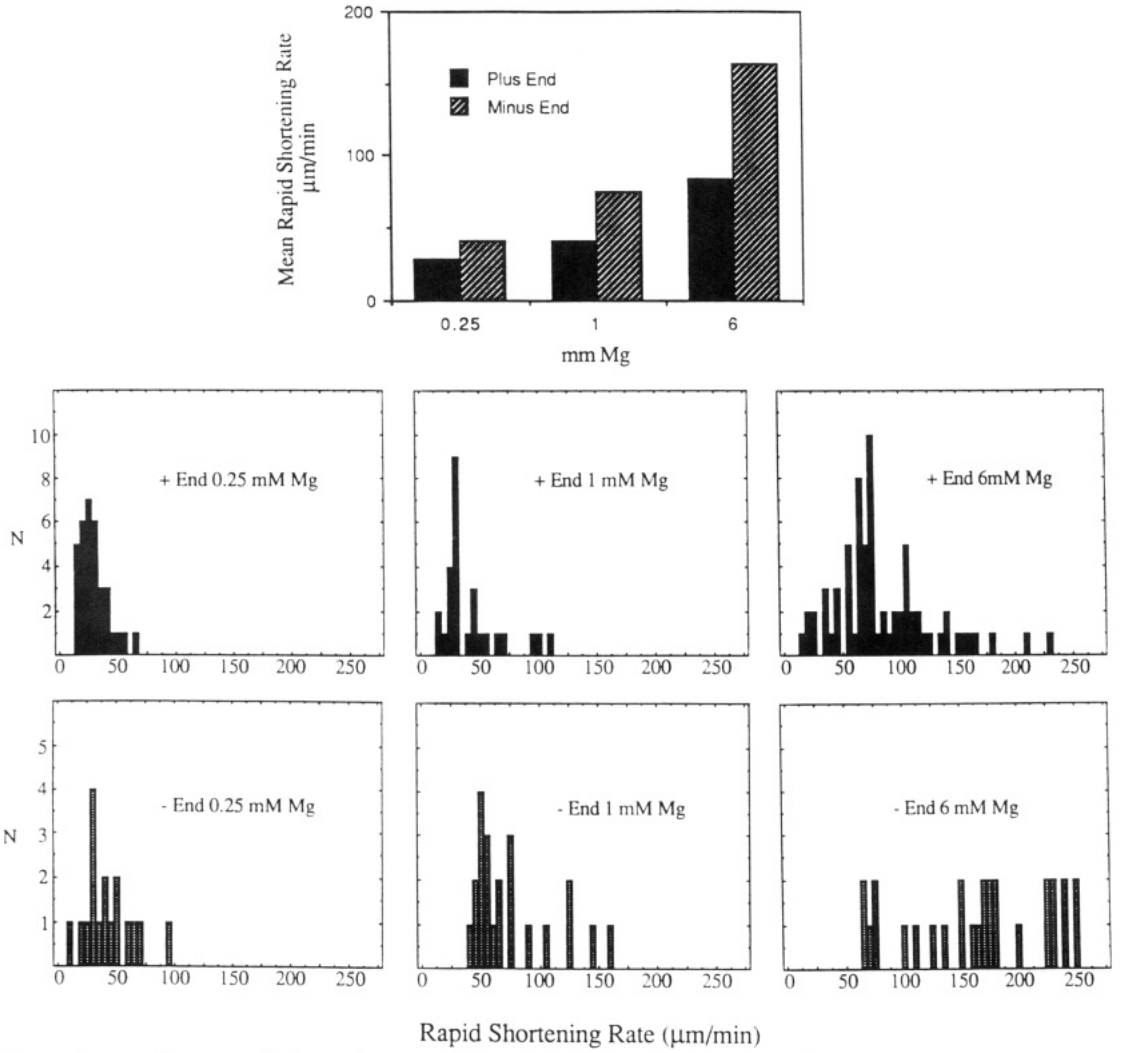

FIGURE 3: Effects of magnesium on rapid shortening rates. The top panel shows the mean rate of rapid shortening for all measurable episodes of rapid shortening, for plus and minus ends, and for all tubulin concentrations. Each constant velocity region was counted as 1 N. The bottom section is a frequency histogram for each of the data points in the top panel. All averages were calculated as unweighted, in that the length of polymer lost or the time spent at a particular rate was not factored in.

shortening event, and minus ends disassembled at about the same mean rate as plus ends at low [Mg], but became significantly faster than plus ends at high [Mg].

More detailed information is presented in the bottom panels of Figure 3. All individual episodes of rapid shortening are presented in a frequency histogram, with the number of times the rate occurred presented on the y axis. This shows the distribution of rates that were seen and illustrates how this distribution changed with [Mg]. Thus, although the average plus-end rate was about half that of the minus end, similar rates were often observed at both ends. Also, a wide range of rapid shortening rates was observed at any one [Mg]. While the average rate of rapid shortening increased about 3-fold at plus ends, and 4–5-fold at minus ends, the maximum range of rates observed was much greater: from minimum rates of 8–10 seen most often at 0.25 mM Mg, to a maximum of 230–250 μm/min at 6 mM. While low rates were sometimes seen even at high [Mg], high rates (>100) were not seen at low [Mg] (0.25 mM or less).

We wondered whether the rate of disassembly depended on the [Mg] present when the polymer was assembled, or depended only upon current buffer conditions. Using the flow cell, we could assemble microtubules at 0.25 mM magnesium and then dilute them into 6 mM magnesium buffer (with no tubulin present), and vice versa. The rapid shortening rates obtained were always determined by the immediate environment, and not the concentration of Mg that had been present during polymerization (data not shown).

It is not certain whether there is a maximum rate of rapid shortening. The 250 μm/min reported in Figure 3 for 6 mM Mg was not the maximum rate seen. At 7.5 mM Mg, instances of rapid shortening above 500 μm/min were observed, but these were quite infrequent. Increasing Mg above 6 mM to a maximum of 15 mM reduced the incidence of interspersed periods of slower disassembly, but the average minus-end rates remained 250–300 μm/min, and did not seem to increase. Frame by frame ($^1/_{30}$th s each) analysis of even the most rapid disassembly events shows that, within our limits of resolution, disassembly appeared to proceed only from the free end; e.g., the entire microtubule was of uniform contrast as disassembly took place.

Only some of the transitions between phases were significantly affected by [Mg]. The frequency of rescue (k_r) was decreased by Mg, particularly at minus ends. Figure 4 shows plus- and minus-end k_r vs free [tubulin] for 0.25, 1, and 6 mM Mg. Although k_r was previously reported to vary with tubulin concentration (Walker et al., 1988), we noticed little concentration dependence in our data over the tubulin concentrations we investigated (10–16 μM) (probably due to the fewer data points for any one [Mg] in the present study). This was especially apparent when compared with the differences seen between plus and minus ends, and high and low [Mg].

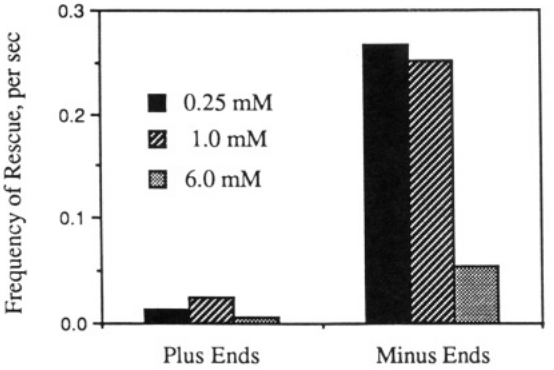

FIGURE 4: Frequency of rescue. Frequency of rescue was calculated by dividing the total number of rescues seen at a given concentration of tubulin and Mg by the total time all those microtubules spent in the rapid shortening phase, as described under Materials and Methods and in Walker et al. (1988). Data for all tubulin concentrations were pooled, as described in the text.

Also, there were about the same number of data points at each tubulin concentration for each Mg concentration investigated. We therefore pooled the data from all tubulin concentrations for Figure 4. The k_r at both ends became lower as [Mg] was increased, but at plus ends, this frequency was already so low that this difference was probably not significant. And, considering that k_r increased at 1 mM Mg at plus ends and then decreased again at 6 mM, the primary conclusion at plus ends is probably that there is little effect of [Mg]. However, minus ends exhibited a very high k_r at low [Mg], which was reduced toward the plus-end rate as [Mg] was increased.

At plus ends, the frequency of catastrophe (k_c) was not changed significantly over the Mg concentrations we explored, while the minus-end k_c did appear to change with [Mg], particularly at lower tubulin concentrations (Figure 5). The k_c of plus ends at 0.25 and 6 M Mg appeared identical over the range of tubulin concentrations examined (10–16 μM). In contrast, minus-end k_c for high and low [Mg] was similar at higher tubulin concentrations (13–16 μM), but at lower tubulin concentrations (8 and 10 μM), minus ends appeared to be stabilized at low [Mg]: the frequency of catastrophe remained at the 0.001–0.002 s^{-1} value observed at high tubulin concentrations, instead of rising to 0.006 s^{-1}.

Discussion

Mg ion affects, to a varying extent, each of the four parameters of dynamic instability. Increasing [Mg] enhanced both the rate of polymerization during the elongation phase and the rate of disassembly during the rapid shortening phase. The effects of [Mg] on the transitions between these phases, while more complex, can be summarized as follows: plus ends were relatively unstable at all concentrations of Mg, while minus-end transition frequencies were altered by changing [Mg]. Minus ends were about as unstable as plus ends at high [Mg], but were stabilized at low [Mg] (decreased k_c, increased k_r).

Walker et al. (1988) noted, in experiments done at 1 mM added Mg, that rapid shortening rates might be slightly higher at minus ends and the frequency of catastrophe slightly lower. Our present results indicate that the slight difference in rapid shortening rate was real and that this difference would have been seen more clearly at higher [Mg]. Similarly, the lower k_c at minus ends was also seen in the present work, but in this case, this effect would have been seen more clearly at lower [Mg] and low concentrations of tubulin. In both Walker et al. (1988) and Walker et al. (1989), minus ends were noted to be more stable than plus ends either in terms of transition frequencies during normal microtubule growth or after cutting

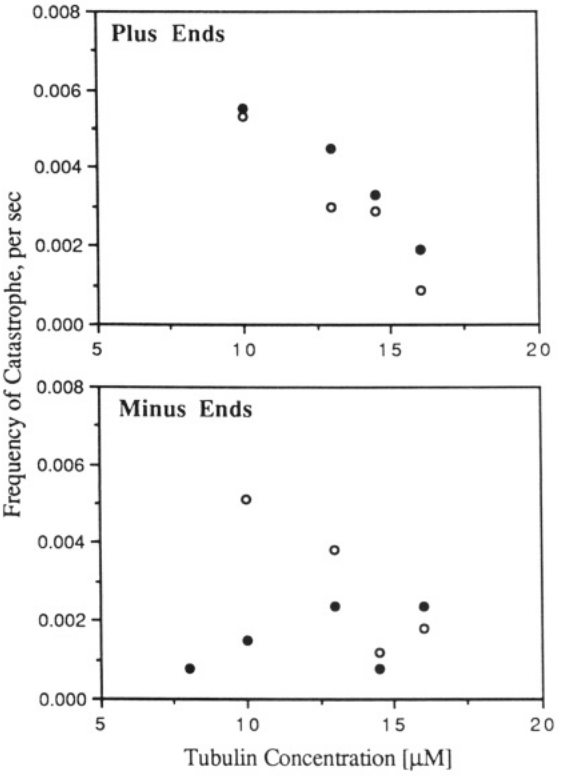

FIGURE 5: Frequency of catastrophe. Frequency of catastrophe was calculated in the same way as rescue: the total number of catastrophies seen was divided by the total time microtubules at a given tubulin concentration were observed in the elongation phase. Closed circles represent mean frequencies at 0.25 mM Mg; open circles, 6 mM Mg.

a microtubule. The present results are consistent with the earlier studies and suggest that the greater stability of minus ends would be diminished or abolished at high [Mg].

The effects of [Mg] on both elongation and rapid shortening rates took place over about the same concentration range at both ends. This implies that the Mg binding sites on tubulin, responsible for modulating elongation and rapid shortening rates, have similar affinities for Mg at both microtubule ends. It is therefore possible that the same binding site modulates both reactions at both ends. In contrast, while minus-end transition frequencies were also affected over this same range of Mg concentration, plus ends were not appreciably changed, implying that the destabilizing Mg site may be different from the site that affects rates. At this putative, transition-affecting site, either Mg does not bind to plus ends or the site is already saturated at the Mg concentrations used in this study (i.e., it has a significantly higher affinity for Mg at plus ends than at minus ends). This idea would suggest that stabilization of plus ends comparable to that seen at minus ends might occur, but only at concentrations of free Mg ion lower than 0.25 mM.

It may seem contradictory that the addition of one ligand can enhance the rate both of elongation and also of shortening. This can readily be understood, however, since two separate reactions are involved, each moving toward its separate equilibrium. During elongation, the balance of association and disassociation rates generates an effective critical concentration for elongation of 2–6 μM. This is comparable to the equilibrium constant (K_E) for binding of GTP–tubulin to an elongating microtubule end [see Walker et al. (1988) for discussion]. The second equilibrium, association and dissociation of GDP subunits onto a disassembling GDP–tubulin polymer "core" after a catastrophe, has a K_E that is probably in the range of 100 μM free GDP subunits or greater. An illustration of the dramatic difference in these two reactions

is that during elongation, tubulin subunits are calculated to dissociate at a rate of 25–30 subunits/s, while during rapid shortening GDP subunits dissociate from 250 to 8000/s, depending on the Mg concentration. Our experiments, at 8–24 μM GTP–tubulin and negligible amounts of free GDP–tubulin, are therefore above the K_E for elongation, favoring net assembly, and at the same time well below the K_E for GDP–tubulin addition, favoring disassembly after a catastrophe. The effect of Mg is to accelerate the movement of each of these reactions toward their individual equilibria.

Effect of Mg on the Rate of Elongation. We observed a clear increase in the elongation rate with increased Mg, and showed that this increase is primarily a result of an increase in the second-order rate constant for elongation (k^e_2) at both ends. This result is in contrast to the almost 2-fold reduction in this rate constant reported by Martin et al. (1987). In that study, the effects of microtubule number concentration and rate of nucleation were carefully separated from the effect of Mg on k^e_2 so these effects cannot explain the difference in our results. The difference may be due to the activity of the MAPs present in the study of Martin et al., or in their experimental procedure, which measured net assembly rates and could not distinguish between individual phases of microtubule growth. Our results are, however, in agreement with those of many early reports showing, both with and without MAPs, that Mg was either required for or promoted microtubule assembly (Weisenberg, 1972; Olmstead & Borisy, 1975; Frigon & Timasheff, 1975; Lee & Timasheff, 1977).

Mg Effect on Rapid Shortening. The slowest rates of rapid shortening were about 8–10 μm/min at each end. This rate was most common in buffers with no added Mg and 100 μM EDTA added (data not shown), but was also observed at Mg concentrations up to 6 mM, although less frequently. As [Mg] was increased, rates of rapid shortening increased also, in general confirmation of the results of Gal et al. (1988), Caplow et al. (1989), and O'Brien et al. (1989), but not in the manner expected of a simple ligand-modified system. Instead of rates that smoothly increased with increasing [Mg], microtubules often underwent quantal changes in rate during a single rapid shortening event, with a wide variety of rates evident at a single [Mg].

Since disassembly during rapid shortening appears to be zero order with respect to tubulin concentration, and, as mentioned above, the association of GDP–tubulin dimers is negligible under our conditions, enhancement of the rate of rapid shortening must reflect an enhancement of the first-order rate constant for dissociation of GDP subunits from the polymer end. This implies that Mg must be able to bind to GDP subunits while in the polymer and that this binding makes the dissociation reaction more facile. Recently, Melki et al. (1989) published a model of the conformations of GDP and GTP–tubulin that incorporated much of the known properties of both species. In this model, GDP–tubulin prefers a curved conformation in solution, when in a ring oligomer, or when disassembling at the end of a microtubule. When they are incorporated into the microtubule lattice, however, GDP–tubulin subunits are constrained by the bonds to neighboring subunits to stay in the straight conformation. In contrast, GTP subunits prefer a straight conformation in solution or in the stabilizing tip of a microtubule. Within this framework, our Mg results might be understood as a stabilization of the predominant forms of each type of species: the assembly-competent, straight form of GTP tubulin and also the curved, non-assembly-competent form of GDP–tubulin. Elongation rates would be enhanced by increasing the faction of GTP–tubulin subunits in the straight conformation, and disassembly during rapid shortening promoted by Mg binding to terminal GDP subunits and enhancing their rate of change from straight to bent form. This model is compelling, and helps in both visualizing and explaining the effects of Mg on dynamic instability.

Mechanism of Quantal Changes in Disassembly Rate during a Single Rapid Shortening Phase. The quantal changes in the rate of disassembly can only be understood as structural differences that are consistent along a particular segment of the microtubule, but which can vary from segment to segment and from microtubule to microtubule. One possibility is that the seam necessarily formed in a B-lattice microtubule polymer [discussed in Mandelkow et al. (1986)] might open up or close abruptly, causing an abrupt change in the number of points simultaneously allowing subunit dissociation. Another possibility is the formation of regions with extra polymeric structures attached to the microtubule wall [e.g., hooks (Burton & Himes, 1978)]. A preliminary investigation of microtubules nucleated from axonemes at 0.25, 1, and 6 mM Mg, and then fixed, pelleted, stained, embedded, and sectioned for electron microscopy, showed the presence of hooks or C-shaped projections on some of the microtubules. The relative number of hooks seen did not vary with [Mg] over the range explored, and so cannot explain the overall increase in rates of rapid shortening seen with increasing [Mg]. However, a more thorough structural study will be necessary to determine whether the presence of hooks along certain regions of some polymers might explain the abrupt changes in rates observed.

Implications for the GTP Cap. Models explaining dynamic instability picture a region of GTP–tubulin at microtubule ends that prevents the onset of rapid disassembly. Most of these "GTP cap" models have pictured GTP hydrolysis as taking place subsequent to the incorporation of a subunit into a microtubule end. Catastrophe takes place when the GTP cap is lost via dissociation of the terminal T's or through hydrolysis of the GTP bound to these subunits. If hydrolysis is not constrained to take place at a particular depth into the microtubule end, then as assembly rates increase, the cap should grow larger and the frequency of catastrophe diminish. We call this type of GTP cap model a "build-up" model. In some general aspects, this was the relationship observed both in our previous work (Walker et al., 1988) and in the present study. Increasing tubulin concentrations produced greater elongation rates, and also did produce decreasing frequencies of catastrophe. The problem is that the frequency of catastrophe is virtually unaffected by [Mg] (or is actually increased at the minus end for high Mg and low tubulin concentrations), in spite of the fact that elongation rates, and thus the size of a "build-up cap", are increased at both ends by higher [Mg]. Thus, a build-up cap is inconsistent with results of Mg addition for each end taken separately. Further problems appear when plus and minus ends are considered together. Microtubules in 6 mM Mg have frequencies of catastrophe at the two ends that are indistinguishable from one another, yet plus ends are elongating almost twice as fast as minus ends. Conversely, at low Mg and low tubulin concentrations, minus ends undergo an appreciably lower frequency of catastrophe (than at high [Mg] or at plus ends at the same Mg concentration), even though they grow more slowly than either plus ends at any Mg concentration or minus ends at high [Mg]. We conclude that, remarkably, the frequency of catastrophe does not correlate with elongation rate.

In order to salvage a build-up cap mechanism for catastrophe, one would have to postulate that Mg always enhanced the rate of GTP hydrolysis just enough to compensate for the

increased rate of elongation, while at the same time hydrolysis would have to proceed at rates that were different at the two ends precisely to the degree necessary to give an equivalent k_c at each end. This is possible theoretically as long as hydrolysis is not stochastic, since the two microtubule ends are different. However, this scenario is cumbersome and seems unlikely. It seems much more likely that the GTP cap is confined to the tip of a growing microtubule in such a way as to not allow any accumulation of GTP–tubulin subunits no matter how fast the microtubule grows. To us, the most likely models remain a one-subunit or a one-subunit-deep cap model, with stochastic dissociation and coupled GTP hydrolysis (O'Brien et al., 1987; Walker et al., 1988). In fact, the stochastic dissociation model appears well adapted to our present results in that the dissociation rate during elongation, postulated to be a major determinant of catastrophe, stayed about the same (for a given tubulin concentration) as [Mg] was varied, and so did the frequency of catastrophe.

Microtubule Oscillations. Under certain conditions, high concentrations of tubulin will undergo isothermal cycles of assembly and disassembly (Carlier et al., 1987; Mandelkow et al., 1988). These oscillations in assembly appear to be an interesting manifestation of dynamic instability and are most readily produced at very high concentrations of Mg (up to 20 mM) as well as high GTP–tubulin concentrations. Yet, the importance of high Mg concentrations, used in both studies, was not explored in detail or discussed, except for a mention by Mandelkow et al. (1988) that high [Mg] was included to stabilize assembly, i.e., to promote polymerization. Presumably, high [Mg] was included in the buffers of Carlier et al. (1987) for the same reason. Knowing that Mg also greatly accelerates disassembly during rapid shortening may alter significantly the interpretation of oscillatory behavior. In particular, the dissociation rate constant of 400 s^{-1} assumed by Carlier et al. (1987) now appears to be too low by a factor of 20. Our observations suggest a rate of at least 8000 s^{-1} for each end, and further demonstrate that at high [Mg] the plus and minus ends are equally active. These new data should be valuable in future modeling of oscillatory behavior, as well as our eventual understanding of the fundamental mechanisms of dynamic instability.

Relevance to the Cell. In newt lung epithelial cells, microtubules that in interphase might extend 50–60 μm, in mitosis extend an average of only 10–15 μm (Cassimeris et al., 1988). Although absolute microtubule lengths can vary widely from cell to cell, this is the typical pattern in most eucaryotic cells between interphase and mitosis. In addition, the shorter mitotic microtubules appear to be much more dynamic than those in interphase, as mentioned in the introduction. However, it is not known which specific parameters of dynamic instability are altered, or in what directions, as the cell enters mitosis.

In a pair of reviews, Walker (1986) and Walker and Duffus (1983) have proposed a central role for magnesium in control of the cell cycle. They point to evidence that magnesium is crucial for DNA synthesis and mitosis onset, and suggest mechanisms that might control free magnesium levels. More recently, Prescott et al. (1988) have described the reversible disassembly of both interphase microtubule arrays and stress fibers in PtK2 cells by microinjected magnesium. Although it has not been directly demonstrated that free magnesium levels fluctuate with the cell cycle, our results suggest that the rates of both elongation and rapid shortening would be increased by an increase in the Mg concentration. This by itself would increase microtubule dynamics, i.e., bringing free tubulin in and out of the polymeric form more often. High [Mg] increased elongation rates to a degree sufficient to achieve the 7.2 μm/min rates seen in interphase by Cassimeris et al. (1988) at tubulin concentrations expected in the cytoplasm. At the same time, however, high [Mg] produced rates of shortening much greater than those observed in that study. Comparison with the rates of elongation and shortening of spindle microtubules must await measurement of those rates, but our results demonstrate that rapid shortening rates could be very high. Interestingly, although one would expect a change in plus-end transition frequencies at the onset of mitosis [see Cassimeris et al. (1988) and Walker et al. (1988) for discussion], we did not see a significant change in either catastrophe or rescue frequency at plus ends over the Mg concentrations explored. It will be interesting to determine whether changes in transition frequencies do indeed take place in vivo, whether or not the Mg binding site is involved in the change, and whether the pattern of changes in the parameters of dynamic instability is the same or different from that seen with pure tubulin as Mg concentrations are altered.

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Phosphatidylserine Affects Specificity of Protein Kinase C Substrate Phosphorylation and Autophosphorylation†

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ABSTRACT: Protein kinase C substrate phosphorylation and autophosphorylation are differentially modulated by the phosphatidylserine concentration in model membranes. Both substrate phosphorylation and autophosphorylation display a cooperative dependence on phosphatidylserine in sonicated vesicles composed of diacylglycerol and either phosphatidylcholine or a mixture of cell lipids (cholesterol, sphingomyelin, phosphatidylethanolamine, and phosphatidylcholine). However, the concentration of phosphatidylserine required to support phosphorylation varies with individual substrates. In general, autophosphorylation is favored at intermediate phosphatidylserine concentrations, while substrate phosphorylation dominates at high phosphatidylserine concentrations. These different phosphatidylserine dependencies may reflect different affinities of particular substrates for negatively charged membranes. Increasing the negative surface charge of sonicated vesicles increases the rate of substrate phosphorylation. In contrast to the modulation exerted by phosphatidylserine, diacylglycerol activates protein kinase C equally toward substrate phosphorylation and autophosphorylation. These results indicate that both diacylglycerol and phosphatidylserine regulate protein kinase C activity in the membrane: diacylglycerol turns the enzyme on, while phosphatidylserine affects the specificity toward different substrates.

The Ca^{2+}/lipid-dependent protein kinase C plays a critical role in the transduction of extracellular signals that promote phospholipid turnover (Nishizuka, 1986, 1988). Receptor-mediated hydrolysis of phosphatidylinositol bisphosphate generates two important second messengers in the activation of protein kinase C: diacylglycerol and Ca^{2+}. Recent evidence suggests that one of these messengers, diacylglycerol, may also be formed by receptor-mediated hydrolysis of phosphatidylcholine (Besterman et al., 1986; Slivka et al., 1988). Protein kinase C becomes tightly associated with the plasma membrane in response to increased intracellular Ca^{2+} levels and is activated by diacylglycerol. The "translocation" of protein kinase C to the membrane in response to a variety of biological agonists, in many different cell types, is well documented (Kraft et al., 1982; Farrar & Anderson, 1985; Terbush & Holz, 1986). Nonetheless, the mechanism of membrane binding and activation of protein kinase C remains to be elucidated.

Protein kinase C binds to acidic membranes in a Ca^{2+}-dependent manner (Bazzi & Nelsestuen, 1987a). While this binding can usually be reversed by removal of Ca^{2+}, recent evidence indicates that, under the appropriate conditions, the binding may be irreversible (Bazzi & Nelsestuen, 1988b). Examination of binding to phospholipid monolayers and bilayers indicates that protein kinase C inserts into the hydrophobic core of the membrane (Bazzi & Nelsestuen, 1988a,b).

Activity of protein kinase C is dependent on phosphatidylserine (PS),[1] a lipid located on the cytoplasmic surface of the plasma membrane, and diacylglycerol (Kaibuchi et al., 1981; Boni & Rando, 1985; Bazzi & Nelsestuen, 1987a). The stoichiometry and specificity of the activation of protein kinase C by lipid has been determined by using a mixed micelle assay developed by Bell and co-workers (Hannun et al., 1985).

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[1] Abbreviations: DG, dioleoylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

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