Strongylocentrotus purpuratus Spindle Tubulin. II.
Characteristics of Its Sensitivity to Ca++ and the Effects of Calmodulin Isolated from Bovine Brain and S. purpuratus Eggs

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ABSTRACT Tubulin was extracted from spindles isolated from embryos of the sea urchin Strongylocentrotus purpuratus and purified through cycles of temperature-dependent assembly and disassembly. At 37°C, the majority of the cycle-purified spindle tubulin polymer is insensitive to free Ca++ at concentrations below 0.4 mM, requiring free Ca++ concentrations >1 mM for complete depolymerization. However, free Ca++ at concentrations above 1 μM inhibits initiation of polymer formation without significantly inhibiting the rate of elongation onto existing polymer. At 15°C and 18°C, temperatures that are physiological for S. purpuratus embryos, spindle tubulin polymer is sensitive to free Ca++ at micromolar concentrations such that 3-20 μM free Ca++ causes complete depolymerization. Calmodulin purified from either bovine brain or S. purpuratus eggs does not affect the Ca++ sensitivity of the spindle tubulin at 37°C, although both increase the Ca++ sensitivity of cycle-purified bovine brain tubulin. These results indicate that cycle-purified spindle tubulin and cycle-purified bovine brain tubulin differ significantly in their responses to calmodulin and in their Ca++ sensitivities at their physiological temperatures. They also suggest that, in vivo, spindle tubulin may be regulated by physiological levels of intracellular Ca++ in the absence of Ca++-sensitizing factors.

Many observations suggest that calcium ions (Ca++) are involved in the regulation of tubulin during mitosis. For example, Ca++ depolymerizes microtubules that are polymerized in vitro (5, 20, 21, 29, 35, 36) as well as those in spindles isolated from dividing embryos (25, 27, 30, 31). Furthermore, Ca++ injected into the spindles of living cells causes a localized loss of spindle fiber birefringence which presumably corresponds to a depolymerization of microtubules (10, 14). The spindle birefringence returns spontaneously within minutes, possibly as a result of uptake of the injected Ca++ into endoplasmic reticulum within the spindle (6-9, 15, 23, 28) which may sequester and release it in a manner similar to that of the sarcoplasmic reticulum in muscle (7-9, 22, 25, 26, 32, 33).

If Ca++ regulates tubulin polymerization and depolymerization during mitosis, spindle tubulin must be sensitive to free Ca++ at concentrations within the physiological range of 10^{-7} to 10^{-5} M. Although mammalian brain tubulin that has been purified by cycles of temperature-dependent assembly and disassembly is insensitive to free Ca++ concentrations within this range (21), its sensitivity can be increased by the calcium-binding protein calmodulin (3, 16, 19). Furthermore, calmodulin has been identified by indirect immunofluorescence in mammalian cells in culture within regions of mitotic spindles where microtubule disassembly is known to occur (1, 3, 17, 38, 39). Whether spindle tubulin is similar to cycle-purified brain tubulin in its sensitivity to Ca++ and in its response to calmodulin, though, has not been reported.

In the preceding paper (13) we have shown that spindle tubulin extracted and purified from embryos of the sea urchin Strongylocentrotus purpuratus differs significantly from mammalian brain tubulin in a number of properties, among which is its ability to polymerize with a low critical concentration in
the absence of associated proteins. Because brain tubulin depends on associated proteins for its ability to polymerize with a low critical concentration as well as its insensitivity to Ca\(^{++}\) and interactions with calmodulin (D. K. Jemiolo, manuscript in preparation), spindle tubulin without these proteins may differ from brain tubulin in its interactions with calmodulin. Therefore, it is important to determine directly the nature of the interaction of Ca\(^{++}\) with spindle tubulin to better understand the regulation of microtubule assembly and disassembly during mitosis.

In this study, we investigated the Ca\(^{++}\) sensitivity of tubulin extracted from isolated sea urchin spindles and purified by cycles of temperature-dependent assembly and disassembly. In particular, we have determined the effect of Ca\(^{++}\) on the initiation and elongation of the tubulin polymerization and on the steady state amount of polymer present at its physiological temperatures and above. Furthermore, we have examined the response of spindle tubulin to calmodulin isolated from both bovine brain and S. purpuratus eggs.

MATERIALS AND METHODS

**Isolation and Purification of S. purpuratus Spindle Tubulin**

The *S. purpuratus* spindle tubulin used in these experiments was extracted by cold depolymerization from spindles isolated without glycerol and was purified by cycles of temperature-dependent assembly and disassembly in 100 mM PIPES, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM GTP, pH 6.8, as described in the preceding paper (13). The bovine brain tubulin used in these experiments was purified through three cycles of temperature-dependent assembly and disassembly in 100 mM PIPES, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM GTP, pH 6.8.

**Isolation of Calmodulin from S. purpuratus Eggs and Bovine Brain**

Calmodulin was isolated and purified from unfertilized *S. purpuratus* eggs by ethanol precipitation and Ca\(^{++}\)-dependent affinity chromatography using chlorpromazine-Sepharose and Triton-Sepharose by a method to be published elsewhere (W. H. Burgess, manuscript in preparation). Bovine brain calmodulin was isolated and purified by a method previously described (4).

Both bovine brain calmodulin and *S. purpuratus* calmodulin were dialyzed against 100 mM PIPES, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM CaCl\(_2\), pH 6.8, before use to ensure that the Ca\(^{++}\) bound to the calmodulin would not affect the concentration of free Ca\(^{++}\) calculated to be present when the concentration of Ca\(^{++}\) added equals the concentration of EGTA in the solution.

**Determination of Protein Concentration**

Protein concentration was determined by the method of Bensadoun and Weinstein (2) using bovine serum albumin as the standard.

**Determination of the Calcium-sensitivity of Cycle-purified Spindle Tubulin**

The tubulin in all of the experiments described, except that used to obtain data in Fig. 1, was in a buffer consisting of 100 mM PIPES, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM GTP, pH 6.8. For the experiment described in Fig. 1, 5 mM EGTA was substituted for the 1 mM EGTA usually used. In all assays, the tubulin was diluted into a total volume of 300 µl in masked microcuvettes 2 × 45 mm with a 10-mm pathlength (Markson Science, Inc., Bliss & Laughlin Industries, Del Mar, CA). A Perkin-Elmer model 526 recording spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) equipped with a thermoelectric temperature-controlled five-cell sample holder was used to monitor absorbance of the tubulin at 350 nm. With this instrument, the temperature of the samples can be changed between 15°C and 37°C in either direction usually in 3 min or less.

Using Lang-Levy pipettes, Ca\(^{++}\) was added in aliquots of 1, 2, or 5 µl volumes from a stock solution of 50 mM CaCl\(_2\) made in distilled, deionized H\(_2\)O. With this protocol, adding a total of 6 µl of CaCl\(_2\) stock makes the final concentration of Ca\(^{++}\) added equal to the concentration of EGTA present in the buffer with 1 mM EGTA. When considered necessary, equivalent volumes of H\(_2\)O were added to control samples to determine the effect of dilution on the tubulin.

**Determination of Free Calcium Concentrations**

The concentration of free Ca\(^{++}\) in the Ca-EGTA buffers used in this study was determined by the method of Portzehl et al. (24) using 2 × 10\(^{-6}\) M as the apparent association constant of EGTA at pH 6.8 for calcium. At pH 6.8, the binding of Ca\(^{++}\) to EGTA releases two hydrogen ions for each calcium bound (24). However, with the hydrogen ion buffer used in this study, 100 mM PIPES at pH 6.8 (pK of 6.8), there is no significant change in pH even when all of the EGTA is saturated with calcium. At equal concentrations of Ca\(^{++}\) and EGTA (1 mM/1 mM), the concentration of free Ca\(^{++}\) is calculated to be 20 µM. The concentration of Ca\(^{++}\) added in excess of the EGTA present plus 20 µM is approximately the concentration of free Ca\(^{++}\).

**RESULTS**

**Ca\(^{++}\)-sensitivity of Cycle-purified Spindle Tubulin Polymer at 37°C**

For these experiments, tubulin was extracted from spindles isolated from embryos of the sea urchin *S. purpuratus* and was purified through at least two cycles of temperature-dependent assembly and disassembly. In addition to the tubulin, which polymerizes with a critical concentration of 0.15–0.2 mg/ml at 37°C, the preparations contain a protein of 80 kdaltons (0.5–5%) and in some cases actin (12). The Ca\(^{++}\) sensitivity of this cycle-purified spindle tubulin was determined in two different ways: by adding Ca\(^{++}\) to the polymer once formed and by adding Ca\(^{++}\) to the tubulin before the initiation of polymer formation.

The effects of adding Ca\(^{++}\) to spindle tubulin polymer at 37°C are shown in Fig. 1. In this experiment, the spindle tubulin was polymerized at 37°C and aliquots of Ca\(^{++}\) were added sequentially, allowing establishment of new steady state levels of polymer (usually requiring ~10 min) between the additions. The results of this experiment are plotted as the percentage of polymer remaining at steady state in the sample containing Ca\(^{++}\) as compared to that in an equivalent sample to which H\(_2\)O alone was added versus the concentration of free Ca\(^{++}\). In all samples tested in this way, the majority of polymerization occurred at concentrations of free Ca\(^{++}\) between 0.1 mM and 1.0 mM, with 50% of the polymer remaining at concentrations of free Ca\(^{++}\) as high as 0.4 mM. Thus, at 37°C the majority of spindle tubulin polymer is insensitive to physiological concentrations of free Ca\(^{++}\).

**Effect of Ca\(^{++}\) on the Polymerization of Spindle Tubulin — Initiation and Elongation**

In contrast to the preceding results, adding Ca\(^{++}\) to the spindle tubulin before the initiation of polymer formation inhibits the rate of polymer formation at concentrations of free Ca\(^{++}\) much lower than are necessary to depolymerize the polymer once formed. This can be seen in Fig. 2 which shows a series of polymerizations of the same two samples of tubulin: one in the presence of concentrations of free Ca\(^{++}\) increasing from <10^{-8} M to 2 × 10^{-5} M and the other in the absence of added Ca\(^{++}\). After each polymerization, both samples were placed on ice for at least 10 min to ensure complete depolymerization. Then, an additional aliquot of Ca\(^{++}\) was added to one sample and polymerization was reinitiated by raising the temperature to 37°C.

The results indicate that free Ca\(^{++}\) at concentrations >1 µM decreases the rate of spindle tubulin polymer formation. Although not shown in this figure, the tubulin in the presence of
The temperature of both samples was then lowered (shaded areas of Fig. 3 c, d, and e) until the sample with the added Ca" had resulted had the Ca" been added to existing spindle tubulin polymer formed from an equivalent protein concentration. Furthermore, adding excess EGTA to a Ca"-inhibited sample at any time after initiation reverses the inhibition of the rate of polymer formation (Fig. 3). The inhibition of the rate of polymer formation by Ca" is presumably the result of the inhibition of either or both of two different processes—initiation and elongation. To distinguish between the two, we determined the effects of Ca" on spindle tubulin polymer formation in the presence and absence of existing homologous polymer onto which elongation could occur. In this experiment (Fig. 3) two equivalent samples of tubulin were polymerized at 37°C (during the time period, a). After steady state was established, aliquots of Ca" were added to one sample (Fig. 3 b), resulting in a final concentration of free Ca" of 20 μM which depolymerized 20% of the polymer. The temperature of both samples was then lowered (shaded areas of Fig. 3 c, d, and e) until the sample with the added Ca" was partially depolymerized, at which point the temperature was raised again to 37°C to allow repolymerization. During three consecutive cycles of temperature change, the amount of depolymerization allowed in the Ca" sample was increased by decreasing the final temperature reached, from 22°C in Fig. 3 c to 15°C in Fig. 3 e, before raising the temperature again.

While little depolymerization occurred in the sample with no added Ca" during the short intervals of time at lower temperatures (Fig. 3 c, d, and e), more depolymerization did occur in this sample during a longer exposure to the lower temperature (Fig. 3 f). Finally, both samples were placed on ice (Fig. 3 g) for at least 10 min to depolymerize all of the microtubules. Polymerization was then reinitiated by raising the temperature to 37°C (Fig. 3 h) and, after 24 min, excess EGTA was added to the Ca" sample (Fig. 3 i).

A comparison of rates of polymer formation in the sample containing 20 μM free Ca" during the rise in temperature following partial depolymerization, 0.0040 optical density U/min (ODs/min) in Fig. 3 c, 0.0030 ODs/min in Fig. 3 d, and 0.0020 ODs/min in Fig. 3 e, with the rate of polymer formation after the sample is placed on ice, 0.0002 ODs/min in Fig. 3 h, indicates that the rate of polymer formation is dramatically greater in the presence of polymer than in its absence and that the greater the amount of polymer present the greater the rate of polymerization. The slower rate of polymer formation in the presence of smaller amounts of polymer is probably due to the presence of fewer ends in the sample when less polymer is present, which is consistent with the suggestion that cold depolymerization of microtubules proceeds by an all-or-none mechanism (36) or with a scheme in which shorter microtubules disappear during depolymerization before longer ones (11). Thus, loss of polymer corresponds to a loss of whole microtubules, leaving fewer ends onto which elongation can occur. A comparison of the rate of elongation after partial depolymerization of the polymer in the presence of added Ca"+, for example 0.0040 ODs/min in Fig. 3 c, with that in the sample containing 20 μM free Ca" during the rise in temperature, shows that the rate of polymer formation is greater in the presence of polymer than in its absence and that the greater the amount of polymer present the greater the rate of polymerization.
with no added Ca$^{++}$ after prolonged exposure to the lower temperatures. 0.0043 ODs/min in Fig. 3f, indicates that the rate of elongation in 20 $\mu$M free Ca$^{++}$ can be similar to that in the absence of added Ca$^{++}$ if similar amounts of polymer are present onto which elongation can occur.

The inhibition of the initiation of polymer formation by Ca$^{++}$ is reversible by the addition of excess EGTA which results in a rate of polymer formation, 0.032 ODs/min (Fig. 3i), similar to that in the sample with no added Ca$^{++}$, 0.032 ODs/min (Fig. 3h), although some protein denaturation probably does occur in the Ca$^{++}$ sample over the course of the experiment as indicated by the lower level of steady state polymer in the Ca$^{++}$ sample. These results demonstrate that, at 37°C, free Ca$^{++}$ at concentrations between 1 and 20 $\mu$M reversibly inhibits the initiation of spindle tubulin polymerization without greatly affecting its rate of elongation or the steady state level of polymer present (>75% of that with no added Ca$^{++}$).

Fig. 3 also demonstrates that a decrease in temperature causes a more rapid rate of spindle tubulin depolymerization in the presence of 20 $\mu$M free Ca$^{++}$, for example 0.017 ODs/min (Fig. 3e), than in the absence of added Ca$^{++}$, 0.004 ODs/min (Fig. 3f). Consequently, the same duration of exposure to lower temperatures that causes a large depolymerization in the sample with added Ca$^{++}$ has little effect on the sample with no added Ca$^{++}$ (shaded areas of Fig. 3c, d, and e). This suggests the possibility that the spindle tubulin polymer is more sensitive to Ca$^{++}$ at the lower temperatures than at 37°C.

Ca$^{++}$-sensitivity of Spindle Tubulin Polymer at Physiological Temperatures for S. purpuratus Embryos

To investigate further the effect of temperature on the Ca$^{++}$-sensitivity of spindle tubulin, the amounts of steady state polymer at a series of different temperatures were determined for tubulin in physiological concentrations of free Ca$^{++}$. Because spindle tubulin selfassembles only very slowly at temperatures that are physiological for S. purpuratus embryos (13) and at concentrations of free Ca$^{++}$ within the micromolar range, the tubulin used in these experiments was first polymerized at 37°C, then Ca$^{++}$ was added and the temperature of the tubulin sample was lowered in sequential steps, reestablishing steady state at each temperature.

Fig. 4 shows the results of four different temperature-drop experiments done without added Ca$^{++}$ at protein concentrations of 0.73 and 1.15 mg/ml (previously presented in reference 12), with 3 $\mu$M free Ca$^{++}$ at a protein concentration of 0.73 mg/ml and with 20 $\mu$M free Ca$^{++}$ at a protein concentration of 1.15 mg/ml. In each case, the amount of polymer present at 37°C in the different Ca$^{++}$ concentrations is defined as 100% and the percentage of this polymer remaining at steady state as the temperature is lowered is plotted as a function of temperature. In the presence of 20 $\mu$M free Ca$^{++}$, no polymer remains when the temperature is lowered to 18°C. In 3 $\mu$M free Ca$^{++}$, 10% of the polymer remains at 18°C, but all is lost at 15°C.

The Ca$^{++}$-sensitivity of the tubulin at 15°C and 18°C is independent of whether the Ca$^{++}$ is added before or subsequent to lowering the temperature. This is shown in Fig. 5 in which the results of experiments where Ca$^{++}$ was added to tubulin polymer at 37°C, at 18°C, and at 15°C are plotted as the percentage of the amount of original polymer remaining at the different Ca$^{++}$ concentrations versus the concentration of free Ca$^{++}$. The amount of polymer present at the specified temperatures before the addition of Ca$^{++}$ is considered to be 100%. At both 15°C and 18°C, temperatures at which S. purpuratus embryos develop normally, all of the spindle tubulin polymer in these two samples is depolymerized by a concentration of free Ca$^{++}$, 3 $\mu$M, that is well within the physiological range. The fact that the tubulin at 18°C appears to be slightly more sensitive than that at 15°C is probably due to variability between the experiments.
Effect of Calmodulin on the Ca$^{2+}$-sensitivity of Spindle Tubulin

Because cycle-purified spindle tubulin polymer at 37°C is not sensitive to physiological concentrations of Ca$^{2+}$ and has a Ca$^{2+}$-sensitivity similar to that reported for cycle-purified mammalian brain tubulin, calmodulin was tested to determine whether it can increase the Ca$^{2+}$-sensitivity of the spindle tubulin as it does for brain tubulin. In these experiments, spindle tubulin was polymerized at 37°C to steady state in the presence or absence of a 10-fold molar excess (calmodulin/tubulin dimer) of calmodulin purified from either bovine brain or S. purpuratus eggs. Ca$^{2+}$ was added in aliquots up to a final free concentration of 20 μM, and new steady state levels of polymerization were established. The results of these experiments are shown in Fig. 6. In 20 μM free Ca$^{2+}$, 78% of the spindle tubulin polymer remained with bovine brain calmodulin and 80% of the tubulin polymer remained with S. purpuratus calmodulin in a control sample run in parallel but without the calmodulin, 79% of the polymer remained in 20 μM free Ca$^{2+}$ and the curve is essentially superimposable (not shown).

FIGURE 6 The effect of bovine brain calmodulin (a) and S. purpuratus calmodulin (b) on the Ca$^{2+}$-sensitivity of spindle tubulin. Spindle tubulin (0.9 mg/ml in a and 0.85 mg/ml in b) was polymerized at 37°C in at least a 10-fold molar excess (calmodulin/tubulin dimer) of bovine brain calmodulin (a) and S. purpuratus egg calmodulin (b). Ca$^{2+}$ was added in aliquots resulting in the concentrations of free Ca$^{2+}$ indicated. In 20 μM free Ca$^{2+}$, 78% of the polymer remained in the bovine brain calmodulin and 80% of the polymer remained in the S. purpuratus calmodulin sample. In a control sample equivalent to that with S. purpuratus calmodulin run in parallel but without the calmodulin, 79% of the polymer remained in 20 μM free Ca$^{2+}$ and the curve is essentially superimposable (not shown).

DISCUSSION

Here we have determined the effect of Ca$^{2+}$ on tubulin isolated from S. purpuratus spindles and purified by cycles of temperature-dependent assembly and disassembly. Our initial observations with this spindle tubulin polymerized at 37°C indicated that like cycle-purified mammalian brain tubulin, the majority of the polymer is insensitive to concentrations of free Ca$^{2+}$ below 10$^{-4}$ M and requires concentrations of free Ca$^{2+}$ above 1 mM for complete depolymerization. In addition, we found that at 37°C initiation of spindle tubulin polymerization is much more sensitive to Ca$^{2+}$ than is either elongation onto existing polymer or maintenance of the polymer once formed. That is, the addition of 3–20 μM free Ca$^{2+}$ causes a significantly slower rate of polymerization while not significantly inhibiting elongation or depolymerizing >15–25% of the polymer. Furthermore, we found that in contrast to the results with cycle-purified mammalian brain tubulin, calmodulin isolated from either bovine brain or S. purpuratus eggs does not affect the Ca$^{2+}$ sensitivity of spindle tubulin.

The insensitivity of spindle tubulin polymer to Ca$^{2+}$ at 37°C would indicate that, in vivo, Ca$^{2+}$ is not involved in the depolymerization of spindle microtubules, because they are not sensitive to free Ca$^{2+}$ concentrations within the range generally considered to be physiological, and because calmodulin does not increase the sensitivity of cycle-purified spindle tubulin polymer to Ca$^{2+}$. However, S. purpuratus embryos do not normally develop at 37°C. Optimal development occurs between 14°C and 18°C, although the embryos will develop at temperatures up to 22°C. Previously, we have shown that cycle-purified spindle tubulin will polymerize and will maintain polymer at temperatures (15–18°C) that are physiological for S. purpuratus (13). Therefore, we have investigated the Ca$^{2+}$
sensitivity of the spindle tubulin polymer at a series of different temperatures, including some within the physiological range for the embryos.

At temperatures between 15°C and 18°C, cycle-purified spindle tubulin polymer can be completely depolymerized by free Ca" at concentrations as low as 3 μM. It appears that this increased sensitivity to Ca" at physiological temperatures is due to a synergism between the effects of lower temperature and Ca" on the steady state amount of polymer present. Consequently, for each degree of change in temperature, there is a greater change in the amount of polymer present in samples with micromolar concentrations of free Ca" than in samples with no added Ca". Because the rate of depolymerization as the temperature is lowered from 37°C is considerably greater in the presence of Ca" than in its absence, the Ca" appears to be affecting the microtubules directly rather than affecting just subunit addition.

Regardless of the mechanism of this effect, these results demonstrate that at all temperatures that are physiological for S. purpuratus embryos, sea urchin spindle tubulin polymer is sensitive to physiological concentrations of free Ca". These results are consistent with those of Salmon and Segall (31) and Kiehart and Inoué (10, 14) which indicate that microtubules in sea urchin spindles, both isolated and in vivo, can be depolymerized by physiological concentrations of free Ca". They also support the suggestion that Ca" may be a regulator of the state of tubulin during mitosis. However, whether Ca" actually regulates tubulin during mitosis, and whether spindle tubulin in all species including those whose physiological temperatures are as high as 37°C is sensitive to physiological concentrations of Ca" or calmodulin is necessary to mediate Ca"-depolymerization of that tubulin has yet to be determined.

Recently, observations qualitatively similar to some of those presented above for cycle-purified spindle tubulin were reported for tubulin isolated from unfertilized eggs by ion-exchange chromatography. In their report, Nishida and Kumagai (18) demonstrated that, like spindle tubulin, at 35°C both the polymer of egg tubulin and elongation of egg tubulin onto existing polymer (in the case of isolated Tetrahymena cilia outer doublets) are both much less sensitive to Ca" than is initiation of egg tubulin polymerization. They also report that calmodulin isolated from both mammalian brain and sea urchin eggs does not increase the Ca"-sensitivity of egg tubulin polymer. This is similar to the lack of effect of calmodulin from these two sources on the Ca"-sensitivity of spindle tubulin polymer. In addition, these authors demonstrate that calmodulin from either source does not increase the Ca"-sensitivity of the initiation of egg tubulin polymerization. Presumably, calmodulin also does not increase the Ca"-sensitivity of the initiation of spindle tubulin polymerization because even at 37°C it is already inhibited by physiological concentrations of free Ca". Although the sensitivities of egg tubulin to Ca" reported by Nishida and Kumagai (18) are qualitatively similar to those presented here for spindle tubulin, there are significant quantitative differences in the Ca" sensitivities reported for the two types of tubulin. The concentrations of Ca" that reportedly affect whole egg tubulin at 35°C (18) are ~10- to 100-fold lower than those that we have found to have similar effects on spindle tubulin at 37°C. In addition, the unfertilized egg tubulin reportedly does not polymerize at concentrations 10 times greater than the critical concentration for spindle tubulin (13).

These quantitative differences may indicate that there are significant differences between unfertilized egg tubulin and spindle tubulin. However, in their study, Nishida and Kumagai (18) determined the Ca"-sensitivity of the unfertilized egg tubulin by adding Ca" to solutions of tubulin containing 1-1.5 mM EGTA and a hydrogen ion-buffering capacity (10 mM MES, whose pK of 6.1 is considerably lower than the pH 6.8 used in the assay) that is insufficient to prevent a considerable drop in pH, possibly as much as 0.6 U, due to the two hydrogen ions released from the EGTA for each Ca" bound. The change in pH that would occur in adding Ca" to those solutions would affect not only the steady state level of tubulin polymerization but also the apparent binding constant of the EGTA for the Ca", making impossible an accurate calculation of the concentration of free Ca" without knowing the new pH. Further, Suprenant and Rebhun (34), working with S. purpuratus egg tubulin isolated by column chromatography, find no differences in Ca"-sensitivity of polymer or nucleation at either 37°C or 18°C when compared to S. purpuratus spindle tubulin. Therefore, in light of these considerations, the results reported for the Ca" sensitivity of unfertilized egg tubulin should be interpreted with caution and the Ca"-sensitivities reported here for spindle tubulin and by Nishida and Kumagai (18) for unfertilized egg tubulin should not be compared directly.

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Note Added in Proof: While this paper was in review, a report by Stephen A. Berkowitz and J. Wolff (1981. J. Biol. Chem. 256:11216-11223) appeared. In that report, the authors demonstrate that micromolar concentrations of Ca" inhibit the nucleation but not the elongation of pure bovine brain tubulin, and that the Ca"-sensitivity of pure bovine brain tubulin polymer depends on both tubulin concentration and temperature of assay. In these respects, it appears that pure bovine brain tubulin and S. purpuratus spindle tubulin have similar sensitivities to Ca".

REFERENCES

1. Anderson, B., M. Osborn, and K. Weber. 1978. Specific visualization of the distribution of the calcium-dependent regulatory protein of cyclic nucleotide phosphodiesterase (modulator protein) in tissue culture cells by immunofluorescence microscopy: mitosis and intracellular bridges. Cytobiologie. 17:354-364.
2. Brandt, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. 70:241-250.
3. Brinkley, B. R., J. M. Marcom, M. J. Welsh, J. R. Dedman, and A. R. Means. 1978. Regulation of spindle microtubule assembly-disassembly: localization and possible functional role of calcium-dependent regulator protein. In Cell Reproduction: In Honor of Daniel Mazia. E. R. Dirksen, D. M. Prescott, and C. F. Fox, editors. Academic Press, Inc., New York. 299-314.
4. Burgers, W. H., D. K. Jemiolo, and R. H. Kretsinger. 1980. Interaction of Ca" and calmodulin in the presence of sodium dodecyl sulfate. Biochem. Biophys. Acta. 621:257-270.
5. Haga, T., T. Abe, and M. Kurokawa. 1974. Polymerization and depolymerization of microtubules in vitro as studied by flow birefringence. FEBS (Fed. Eur. Biochem. Soc.) Lett. 5:291-295.
6. Harris, P. 1975. The role of membranes in the organization of the mitotic apparatus. Exp. Cell Res. 96:409-421.
7. Harris, P. 1978. Triggers, trigger waves, and mitosis. A new model. In Cell Cycle Regulation. J. R. Riter, J. A. Cameron, G. P. Padilla, and A. M. Zimmerman, editors. Academic Press, Inc., New York. 73-104.
8. Hepler, P. 1977. Membranes in the spindle apparatus: their possible role in the control of microtubule assembly. In Mechanisms and Control of Cell Division. T. L. Rost and E. M. Griffof, editors. Dowden, Hutchinson, and Ross, Stroudberg, PA. 212-232.
9. Hepler, P. 1980. Membranes in the mitotic apparatus of barley cells. J. Cell Biol. 86:490-499.
10. Inoué, S., and D. P. Kiehart. 1978. In vivo analysis of mitotic spindle dynamics. In Cell...
Reproduction: In Honor of Daniel Mazia. E. R. Dirksen, D. M. Prescott, and C. F. Fox, editors. Academic Press, Inc., New York. 433-444.

11. Johnson, K. A., and G. G. Bonsy. 1977. Kinetic analysis of microtubule self-assembly in vitro. J. Mol. Biol. 117:1-31.

12. Keller, T. C. S., and L. I. Rebhun. 1978. Properties of isolated spindles and spindle tubulin. J. Cell Biol. 78(2): Pt. 2:304a (Abstr.).

13. Keller, T. C. S., and L. I. Rebhun. 1980. S. purpuratus spindle tubulin. Characteristics of its polymerization and depolymerization in vitro. J. Cell Biol. 93:788-796.

14. Kiehart, D. P., and S. Inoue. 1976. Local depolymerization of spindle microtubules by microinjection of Ca** ions. J. Cell Biol. 70(2, Pt. 2):230a (Abstr.).

15. Kinoshita, S., and I. Yazaki. 1967. The behavior and localization of intracellular relaxing system during cleavage in the sea urchin egg. Exp. Cell Res. 47:449-458.

16. Marcum, J. M., J. R. Dedman, B. R. Brinkley, and A. R. Means. 1978. Control of microtubule assembly-disassembly by calcium-dependent regulator protein. Proc. Natl. Acad. Sci. U. S. A. 75:7371-7375.

17. Means, A. R., and J. R. Dedman. 1980. Calmodulin an intracellular calcium receptor. Nature (Lond.). 285:73-77.

18. Nishida, E., and H. Kumagai. 1977. Calcium-sensitivity of the microtubule reassembly system. J. Biochem. (Tokyo). 82:303-306.

19. Omsted, J. B., and G. G. Borisy. 1975. Ionic and nucleotide requirements for microtubule polymerization in vitro. Biochemistry. 14:2996-3005.

20. Porter, K. R., and R. D. Machado. 1960. Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tip. J. Cell Biol. 40:678-691.

21. Rosenfeld, A., B. R. Brinkley, and R. C. Weisenberg. 1976. Magnesium stimulation of calcium binding to tubulin and calcium-induced depolymerization of microtubules. FEBS (Fed. Eur. Biochem. Soc.) Lett. 65:144-147.

22. Suprenan, K., and L. I. Rebhun. Assembly properties of unfertilized egg tubulins. J. Cell Biol. 87(2, Pt. 2):246a (Abstr.).

23. Welsh, M. J., J. R. Dedman, B. R. Brinkley, and A. R. Means. 1979. Tubulin and calmodulin. Effects of microtubule and microfilament inhibitors on localization in the mitotic apparatus. J. Cell Biol. 81:624-634.