Membrane Distribution in Dividing Endosperm Cells of *Haemanthus*

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**ABSTRACT** Membranes in cell-wall-free dividing endosperm cells of *Haemanthus* were examined after postfixation with osmium tetroxide-potassium ferrocyanide. We found that preservation and staining of membranes in metaphase cells was highly variable. Even adjacent cells often showed different degrees of preservation of membrane. However, this method does reveal a much more extensive membrane system in the mitotic spindle of *Haemanthus* than has been revealed previously using glutaraldehyde-osmium fixation. At prometaphase a system of membranes becomes associated with the kinetochore bundles. By metaphase, membranes constitute a prominent feature of kinetochore bundles, terminating near the kinetochores. Minipoles, identified by converging microtubules and associated membranes, are distributed in a zone extending laterally across the polar regions of the cell. The microtubules appear to terminate at the minipoles, whereas the membrane system becomes oriented generally perpendicular to the spindle axis and interfaces distally with a region of amorphous electron-dense material, helical polyribosomes, and cell organelles. The role of this extensive membrane system, if any, in chromosome movement is unknown. However, its distribution is coincident with the distribution of calcium-rich membranes and kinetochore fibers at metaphase in these cells (Wolniak, S. M., P. K. Hepler, and W. T. Jackson, 1981, *Eur. J. Cell Biol.*, 25:171-174). Thus, these membranes may function in creating calcium domains that, in turn, may play a regulatory role in chromosome movement.

Ultrastructural studies have shown a close association of membranous elements, such as endoplasmic reticulum (ER), Golgi apparatus, and vesicles, with the mitotic apparatus of many, but certainly not all, organisms (see references 10, 11, 13, 17 for reviews). A large number of spindles, particularly those of fungi, protozoa, and algae, are closed and contain no membranous components detectable by standard methods. In cells possessing membranes in the spindle, the membrane system may act as an anchor for spindle fibers and as a regulatory system controlling calcium concentrations (10, 13). Calcium ion concentration controls assembly-disassembly of microtubules in vitro, microtubule-dynein interaction, and force production in actomyosin systems. Calcium may act in the spindle through one or more of the systems to regulate the formation and disappearance of microtubules and the generation of the force required to move chromosomes (11, 17). Wolniak et al. (25) used the fluorescent chelate probe chlorotetracycline (CTC) to detect membrane-associated calcium in the dividing cells of *Haemanthus* endosperm. They distinguish two components of the fluorescence: one a punctate (discrete bright spots) component attributed to CTC-Ca$^{2+}$ bound to membranes of mitochondria and plastids; and the second a diffuse component attributed to CTC-Ca$^{2+}$ bound to the ER. Bright cones of diffuse fluorescence were found in the chromosome-to-pole region of the metaphase spindle. The fluorescence in these cones decreased during anaphase, resulting in uniform diffuse fluorescence in both half spindles. More recently, Wolniak et al. (26) have shown that these cones of CTC fluorescence coincide with the distribution of kinetochore fiber birefringence at metaphase. *Haemanthus* endosperm cells have been used for many years to study the dynamics of chromosome movement, particularly by Bajer et al. (e.g., 4). Though the ultrastructure of these cells has been studied intensively (e.g., 1, 2, 3, 16, 19, 20, 22), only occasional membranes have been observed to be associated with the spindle in glutaraldehyde-osmium fixed cells (1, 16, 22). That is, the number and distribution of membranes in the mitotic spindle are insufficient to account for the patterns of fluorescence found by Wolniak et al. (25). Their results suggested that an extensive membrane system may be present in the kinetochore fibers, especially at metaphase. Encouraged by the striking results obtained by Hepler (14) in dividing cells of barley (*Hordeum vulgare*) by postfixa-
tion of standard glutaraldehyde-fixed cells with osmium tetroxide-potassium ferricyanide (OsFeCN), a technique previously applied to the study of sarcoplasmic reticulum of muscle (5), we applied Hepler's (14) method to Haemanthus endosperm cells. In contrast to the earlier ultrastructural studies of Haemanthus, our results reveal that there is an extensive membrane system present in the spindle and that its extent and distribution are consistent with the membrane pattern inferred from the fluorescence studies of Wolniak et al. (25). However, it should be emphasized that the observed filling of lumina in the sarcoplasmic reticulum of muscle and the ER of barley cells with electron-dense material, permitting striking localization of the membrane system in these cells, does not occur in Haemanthus.

MATERIALS AND METHODS

Preparations of endosperm cells of Haemanthus katherinae Baker were made on glass cover slips coated with a 0.5% agar in 3.5% glucose containing 5 mM CaCl₂ according to the method of Jackson (18). After allowing the cells to flatten and adhere to the agar surface, the cover slips were fixed in vapor of 25% glutaraldehyde for 5 to 10 rain, rinsed in water vapor for 5 min and fixed in vapor of 2% OsO₄ for 5 to 10 min (15).

The potassium ferricyanide reagent was made by adding, just before use, freshly opened 4% aqueous OsO₄ to a buffered solution of potassium ferricyanide so that the final concentrations were 0.8% potassium ferricyanide and 2% OsO₄ in 50 mM cacodylate buffer, pH 7.4, with 5 mM CaCl₂. The cover slips were flooded with a drop of this solution and fixed for 5 to 7 min. The cover slips were rinsed once in 50 mM cacodylate buffer with 5 mM CaCl₂, once in water, and then fixed for 10 min in 2% aqueous uranyl acetate. After two rinses in water, the cells were dehydrated by passing the cover slips through an ethanol series.

After infiltration with Epon-araldite, the cells were flat-embedded between two cover slips by adding a cover glass lid using slivers of plastic along the edge. The cells were dehydrated by passing the cover slips through an ethanol series. After infiltration with Epon-araldite, the cells were flat-embedded between two cover slips by adding a cover glass lid using slivers of plastic along the edge as spacers. After polymerization, the cover slips were dissolved with hydrofluoric acid. Using a phase-contrast microscope, cells in the plastic wafer were selected, circled, cut out, and mounted on Epon blocks. Sections were cut with a diamond knife and mounted on formvar-coated slot grids. The sections were poststained with uranyl acetate and lead citrate and examined in a JEOL 100 CX electron microscope.

RESULTS

We found that both preservation and staining of membranes in metaphase cells were highly variable; even adjacent cells often showed different degrees of preservation of membrane. We did not see the filling of cisternal space with electron-dense material observed by Forbes et al. (5) in the sarcoplasmic reticulum and by Hepler (14) in the endoplasmic reticulum of dividing barley cells. Although Forbes et al. (5) were able to obtain staining of the sarcoplasmic reticulum by staining successively with osmium and ferricyanide, White et al. (24) found that the components had to be present simultaneously. In hopes of enhancing staining, we omitted postfixation with OsO₄ vapor before treatment with OsFeCN. However, we found that this caused conversion of tubular membranes into isolated vesicles.

The degree of preservation of the ground cytoplasm, as judged by the density of ribosomes and amorphous background material, was variable. The preservation of membranes appeared to be independent of the preservation of the ground cytoplasm, but in cells that appeared extracted the membranes were more apparent by contrast. This observation was also made by Moll and Paweleit (23) in comparing fixation with KMnO₄ to fixation with glutaraldehyde and OsO₄. Also, there were instances when the matrix was dense but the membranes were not preserved; they appeared to have melted into an amorphous background. Microtubules were poorly fixed by this procedure and were much reduced in number or even absent, compared to results obtained by Bajer (e.g., 1). We tried many variations on the duration of the various steps and on the concentrations of the fixatives used, but without finding a method that produced more consistent results. Nonetheless, preservation and visualization of membranes is much improved compared to that obtained previously in Haemanthus with conventional glutaraldehyde-osmium tetroxide fixation.

After breakdown of the nuclear membrane at prometaphase, microtubules quickly become associated with the kinetochores (1). In the prometaphase cell shown in Fig. 1, the kinetochore bundles of some of the chromosomes closest to the polar regions show long tubular membranes paralleling the microtubules. Mitochondria and plastids are redistributed during prophase and prometaphase. They begin to aggregate in the polar regions during prometaphase, and by metaphase they are clustered at the two extremities of the spindle. Dictyosomes and randomly arranged membranes are abundant among the organelles in the polar region of the spindle throughout mitosis. Membranes surround the entire spindle at the margin of the cell. In the kinetochore bundles, membranes form linear arrangements paralleling the kinetochore microtubules and terminate very close to the kinetochore, near the insertion of the kinetochore microtubules (Figs. 2 and 3). In sections of the spindle where nonkinetochore ("continuous") microtubules can be distinguished (1), they also appear to have membranes associated (Fig. 4). Membranes also appear within the spindle outside of kinetochore bundles and, in contrast to the linear membranes associated with microtubules, are randomly arranged (Fig. 2). By prometaphase, organization of the membranes of the polar regions has occurred. Within a wide undulating zone extending across the polar region, there are centers (minipoles; 4) where microtubules of the spindle and their associated parallel membranes converge and where the microtubules appear to terminate; at the point of convergence, the orientation of the membrane changes and becomes generally perpendicular to the axis of the spindle (Figs. 5 and 6). Microtubules at angles to the spindle axis are sometimes seen in this region. In some cells there appears to be a band of membranes connecting these minipoles. This is most obvious in flattened cells (Fig. 5). The margins of this band interface distally with a region characterized by helical ribosomes associated with electron-dense material that may be the surface of membranes in the plane of the section (Fig. 6).

Anaphase cells, in our experience, are more difficult to fix than cells at earlier stages. They are more likely to show gross artifacts, such as myelin figures or total extraction of the matrix, than cells at other stages. Sometimes the membranes are not preserved, but electron-transparent channels represent their presumed distribution before extraction. Membranes of the kinetochore bundle still retain a linear arrangement but appear to be much reduced. There are concentrations of membranes in the polar region and along the entire periphery of the spindle. By late anaphase the cell organelles are no longer found exclusively at the poles, but appear along the edge of the interzonal region. In the midzone, membranes from the periphery of the cell appear to be penetrating the edge of the spindle region, while only a few vesicles are found in the center of the midzone.

DISCUSSION

A comparison of Figs. 3 and 4 in this study with Figs. 11 and 12 in Bajer (1) illustrate the superiority of OsFeCN fixation over the standard glutaraldehyde-osmium tetroxide fixation for preservation of membranes. In Haemanthus, the polar region...
of the spindle is characterized by an organized membrane system throughout mitosis. Many workers (2, 6, 7, 20-22) have studied the distribution and arrangement of microtubules throughout mitosis in *Haemanthus*. They have found that by metaphase the microtubules of the kinetochore fibers form bundles that intermingle with bundles of nonkinetochore microtubules. By examination of thick sections by high voltage electron microscopy, Bajer and Mólé-Bajer (4) have shown that intermingling of bundles in the polar region results in local concentrations of converging microtubules in minipoles. We find that the minipoles are also the sites of convergence of the ER tubules that are arranged parallel to the microtubules. The minipoles appear to be connected by a band of membranes that run across the polar region perpendicular to the spindle. The microtubules appear to be anchored to membranes at the minipoles, but this is difficult to establish.

Franke et al. (8), using indirect immunofluorescent microscopy, have detected a “polar-endplate” of tubulin-containing material in the endosperm of *Leucojum aestivum* that appears to have a configuration very similar to the polar band that we find in *Haemanthus*. They perceive this as the terminus of the spindle fibers. In *Marsilea*, Hepler (12) found that the smooth endoplasmic reticulum of the spindle area ends at the poles in an interface with the adjacent helical polyribosome-rich non-spindle cytoplasm. In *Haemanthus*, the region distal to the polar band or end plate is characterized by polyribosomes, randomly arranged short segments of microtubules, and amorphous electron-dense material. In some micrographs, this amorphous material appears to be the surface of membranes arranged in the plane of the section. Beyond this is an accumulation of dictyosomes, mitochondria, plastids, and membranes, with the membranes appearing as randomly arranged tubules.

Our findings demonstrate that a membrane system is a prominent component of the kinetochore bundles in *Haemanthus*. Hepler (14) has shown that the membranes of the mitotic apparatus of *Hordeum* form a continuous system from pole to kinetochore. The deposition of electron dense material in *Hordeum* makes it easy to trace the continuity of membranes. Although we did not obtain the opacity depicted by Hepler (14), our membrane system was much more extensive. It is probable that in *Haemanthus*, also, the long fingers of membranes that penetrate the spindle area along the kinetochore fibers form a continuous system. By metaphase, membranes are found in every kinetochore bundle and close to the kinetochores. Nonkinetochore microtubules also appear to have aligned membranes associated with them. Within the spindle between kinetochore fibers, unaligned, randomly arranged membranes are found. Membranes surround the entire spindle. A concentric arrangement of membranes has been found around the spindle of mitotic cells of diverse species; for example, in HeLa (23), in *Plasmodiphora* (9), and in *Hordeum* (14). Moll and Paweletz (23) suggest that these membranes may serve to exclude cytoplasmic organelles from the spindle area and to compartmentalize transport of regulating molecules from or to the poles.

Wolniak et al. (25), in studying the distributions of membrane-associated calcium as detected by CTC fluorescence in dividing *Haemanthus* cells, find a punctate fluorescence that forms a perinuclear band during early prophase and a polar...
Figures 2-4 Fig. 2: Midregion of a metaphase cell. All kinetochore bundles have membranes aligned parallel to the microtubules. Outside the kinetochore bundles membranes are randomly arranged (RM). Bar, 1 μm. × 12,500. Fig. 3: Enlargement of the kinetochore (K) of a chromosome of the cell shown in Fig. 2. Note the apparent termination of a tubular membrane (arrow) very close to the insertion of a microtubule into the kinetochore. Bar, 1 μm. × 40,000. Fig. 4: A section from the equatorial region of a metaphase spindle, close to the surface of the spindle. Parallel nonkinetochore microtubules (arrows) can be distinguished from converging microtubules of the kinetochore bundles (arrowhead). Bar, 1 μm. × 25,000.
aggregation in late prophase continuing until telophase. This corresponds to the distribution of mitochondria, plastids, and dictyosomes that we have seen. The second type of fluorescence they observed was a diffuse fluorescence that was not at the cell surface. They conclude that this fluorescence is not associated primarily with the plasmalemma but originates largely from endomembranes. In the metaphase spindle, the diffuse type of fluorescence is found in bright cones that come to a point at the kinetochores and coincide with the position of kinetochore fibers. In a subsequent study, Wolniak et al. (26) demonstrated that these cones coincide with the distribution of kinetochore fiber birefringence. We find that the membranes have a linear arrangement in this region. Thus, we suggest that the randomly arranged membranes are not merely fixation artifacts but are distinguished from linearly arranged membranes by having lower concentrations of associated calcium.
That is, we suggest that the two membrane arrangements may constitute different functional domains. The discrete cones of fluorescence disperse during anaphase and the chromosome-pole region exhibits uniformly diffuse fluorescence. At the ultrastructural level, the membranes are also most prominent at metaphase and they seem to disperse during anaphase.

We have shown that in *Haemanthus* better preservation of membranes can be achieved with OsFeCN than had been possible previously with glutaraldehyde/osmium. The membrane system is even more extensive than that found by Hepler (14) in *Hordeum* using the OsFeCN fixation method. The role of this extensive membrane system, if any, in chromosome movement is unknown. However, its distribution is coincident with the distribution of calcium-rich membranes and kineto-chore fibers at metaphase in these cells (25, 26). Thus, these membranes may function in creating calcium domains that, in turn, may play a regulatory role in chromosome movement.

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