CALCIUM-SEQUESTERING VESICLES AND CONTRACTILE FLAGELLAR ROOTS

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SUMMARY

The intracellular localization of calcium during contraction of striated flagellar roots of the green alga *Tetraselmis subcordiformis* was investigated using calcium pyroantimonate and calcium oxalate cytochemistry and energy-dispersive X-ray microanalysis.

Vesicles (0.25-0.50 μm diameter) located in the anterior-most region of the cell are calcium-sequestering organelles. These vesicles exhibit a consistent and reproducible localization of electron-opaque pyroantimonate and oxalate salts of calcium. Striated contractile flagellar roots (CFR) show a periodic pattern of calcium antimonate (Ca–Sb) deposits following 'calcium shock' stimulation of contraction of this organelle. When CFRs are in a fully extended state they do not show Ca–Sb deposits. Deposits of Ca–Sb precipitate are present throughout the cytosol when CFRs are stimulated to contract.

The implications of these findings with respect to the regulation of intracellular levels of 'free' Ca**+** and to the mechanism of CFR contraction are discussed.

INTRODUCTION

Striated roots, also known as rhizoplasts, or fibrous system II roots (cf. Melkonian, 1980), occur in association with the basal apparatus of many flagellated or ciliated eukaryotic cells (Pitelka, 1974; Sleigh, 1979) and have been observed in association with centrioles and primary cilia in cultured fibroblasts (Tucker, Pardee & Fujiwara, 1979). Striated flagellar roots are contractile organelles, at least in certain organisms (Salisbury & Floyd, 1978). Calcium specifically triggers an extraordinary contraction of the contractile flagellar roots (CFRs) of the green alga *Tetraselmis subcordiformis*. Cyclic CFR contraction and extension occur in the presence of both calcium and ATP (Salisbury & Floyd, 1978). As we have previously suggested, force applied to the flagellar apparatus as a result of CFR contraction could be sufficient to alter the orientation of the anterior end of the cell and thus serve as an internal 'rudder' in order to bring about the frequent directional changes observed during *Tetraselmis* swimming (Manton & Parke, 1965; Salisbury *et al.* 1981a).

Cytochemical techniques are available for the intracellular localization of calcium (Komnick, 1962; Komnick & Komnick, 1963; Klein, Yen & Thureson-Klein, 1972). When these cytochemical methods are supplemented by electron-probe microanalysis (Russ, 1971; Cantino & Hutchinson, 1982) a reliable and sensitive localization of intracellular calcium can be obtained. This study was undertaken to determine the intracellular localization of calcium in relation to the contraction stage of the CFR of *T. subcordiformis*, a quadriflagellate green alga.
MATERIALS AND METHODS

Rapidly growing *T. subcordiformis* Butcher (Stein), Synonym: *Platymonas*, cultured in an enriched sea-water as described earlier (Salisbury & Floyd, 1978) were harvested and washed twice in an artificial sea-water (ASW) containing 400 mM-NaCl, 8.5 mM-KCl, 25 mM-MgCl₂, and 5 mM-NaSO₄ adjusted to pH 6.5. The cells were preincubated in either ASW alone, or ASW plus 2 mM-CaCl₂ ('calcium shock' conditions) for < 1 min prior to cytochemical incubation.

**Calcium cytochemistry**

(a) Pyroantimonate method: cells were resuspended in a solution containing 60 mM-KSb(OH)₆ (Fisher Scientific Co.), 20 mM-sucrose, 40 mM-glycine, adjusted to pH 7.8 and 1 % OsO₄ (Klein *et al.* 1972; Saetersdal, Myklebust, Berg Justesen & Olsen, 1974). (b) Sodium oxalate method: cells were incubated in ASW containing 20 mM-sodium oxalate for 30 min and then fixed in 2 % glutaraldehyde for 1 h and post-fixed in 1 % OsO₄ for 30 min.

Control samples were fixed as above without prior incubation in either cytochemical reaction mixture. All samples were dehydrated in an acetone series with 2 % uranyl acetate in the 75 % acetone step, and embedded in Spurr's resin. Thin sections, approximately 70-80 nm thick (silver interference colours) for the cytochemical investigations and thick sections, approximately 250 nm thick (green interference colours) for the electron-probe microanalysis were made on a Sorval MT1 ultramicrotome. Thin-sectioned material was either post-stained with uranyl acetate and lead citrate or not post-stained and observed with a Zeiss EM9 or Jeol 100CX electron microscope. Selected thin sections were floated on 1 mM-ethyleneglycol-bis-(β-aminoethyl ether), N,N'-tetraacetic acid (EGTA), 1 mM-ethylenediamine tetraacetic acid (EDTA) or distilled water at 35 °C for 1 h prior to electron microscopy.

**Electron-probe microanalysis**

Thick sections of cells prepared for calcium cytochemistry were picked up on Formvar-coated copper grids and carbon-coated (~10 nm) in a vacuum evaporator. Sections were observed with a Hitachi S500 scanning electron microscope operating in the STEM mode. The microscope was equipped with an Ortec 6230 energy-dispersive analysis system with a Si(Li) X-ray detector and a POP 11/04 computer. Analysis was performed at an accelerating potential of 20 keV, a total beam current of approximately 100 µA on an energy emission scale of 10 keV. Cell profiles and regions containing the flagellar apparatus (anterior) and chloroplast (posterior) were scanned for X-ray emission spectra.

**RESULTS**

The ultrastructure of a contractile flagellar root of *Tetraselmis* is illustrated in Fig. 1. This cell was fixed in ASW without any prior treatment. The section includes a portion of the anterior region of the cell and shows one of two CFRs present in this cell. A second CFR is partially visible passing out of the plane of section at the lower left. The upper CFR is in an extended configuration and is approximately 2.4 µm long. Eleven fibrous zones composed of 5-7 nm filaments are delineated by electron-dense cross-striations (Fig. 1, arrows). The CFR is associated proximally with the flagellar apparatus and distally with the plasma membrane (Robenek & Melkonian, 1979). Numerous vesicles (0.25-0.50 µm diameter) are located in the anterior-most region of the cell throughout the cell cycle. In rapidly growing cultures these vesicles appear to have electron-transparent contents. In aged cells, however, the vesicles sometimes contain granular material (not shown). General aspects of vegetative cell ultrastructure have previously been reviewed by Manton & Parke (1965) and McLachlan &
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Parke (1967), and structural details of the flagellar apparatus and associated CFRs have been presented by Melkonian (1979), and Salisbury et al. (1981a).

Calcium cytochemistry

Table 1 summarizes the pyroantimonate cytochemistry observations. Cells fixed in the presence of pyroantimonate for calcium cytochemistry retained good overall structural preservation. Fig. 2 illustrates the anterior region of another Tetraselmis cell oriented as in Fig. 1. In this cell the CFR is also in an extended configuration. Dense deposits of calcium pyroantimonate (Ca-Sb) precipitation product are present inside the 0.25-0.50 μm diameter vesicles located near the CFR in the anterior-most region of the cell (Fig. 2, arrows). Only sparse deposits are observed in the cytoplasm in general and on the CFR.

Table 1. Survey of calcium localization by the pyroantimonate method during striated flagellar root contraction

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Ca-Sb deposits</th>
<th>SCR contracted</th>
<th>SCR extended</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior vesicles</td>
<td>Dense precipitation</td>
<td>Dense precipitation</td>
<td></td>
</tr>
<tr>
<td>Flagellar root</td>
<td>13-30 discrete zones</td>
<td>Few scattered deposits</td>
<td></td>
</tr>
<tr>
<td>General cytoplasm</td>
<td>(311 ± 55/μm³)</td>
<td>(33 ± 13/μm³)</td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>(475 ± 43/μm³)</td>
<td>(15 ± 5/μm³)</td>
<td></td>
</tr>
<tr>
<td>Chloroplast</td>
<td>(24 ± 6/μm³)</td>
<td>(26 ± 3/μm³)</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Frequent deposits</td>
<td>Frequent deposits</td>
<td></td>
</tr>
<tr>
<td>Nucleolus</td>
<td>Dense deposits</td>
<td>Dense deposits</td>
<td></td>
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</tbody>
</table>

* Estimates for Ca-Sb particle density were made by counting the number of deposits in a known surface area of section and assuming an average section thickness of 75 nm. Density = number of Ca-Sb particles/μm³ section × 13.3/μm, ± standard error.

Upon calcium shock, CFRs are induced to contract (Salisbury & Floyd, 1978). Cells fixed in the pyroantimonate medium following a calcium shock pretreatment also show a vesicular localization of Ca-Sb deposits (Figs. 3-5). Furthermore, deposits of Ca-Sb precipitate occur as 25-30 discrete zones of electron-dense particles along the length of the CFR (Fig. 3, arrows) fixed in intermediate stages of contraction. The diameter of these zones increases and zones appear to fuse as CFRs continue to contract (Fig. 4) until finally 13-15 zones are formed in the completely contracted organelle. Ca-Sb deposits were also observed within cross-sectional profiles of CFRs (Fig. 5) and thus these deposits are not simply adsorbed onto the surface of this organelle. In addition, during CFR contraction, numerous individual Ca-Sb deposits are scattered throughout the cytoplasm (Figs 3-5).

Ca-Sb deposits were also observed in mitochondria, nuclei and nucleoli during all stages of CFR extension and contraction. The number of Ca-Sb deposits within the nuclear compartment and in the cytoplasm increased during calcium shock. The
number of deposits observed within chloroplasts was small and remained constant following various pretreatments.

Deposits of Ca–Sb precipitate could be completely removed by floating thin sections on solutions of EGTA or EDTA, and removal of these deposits produced holes in the sections where the deposits had once been (Fig. 6). EGTA has been shown to chelate several divalent cations, and in particular calcium with high affinity, and has been previously used to dissolve Ca–Sb precipitates (Legato & Langer, 1969; Klein, Yen & Thureson Klein, 1972; Saetersdal, Myklebust, Berg Justesen & Olsen, 1974). In contrast, this treatment does not significantly chelate and dissolve other cation–antimonate precipitates (Legato & Langer, 1969; Klein et al. 1972; Saetersdal et al. 1974).

Cells fixed after preincubation in sodium oxalate for 30 min showed electron-dense crystals of calcium oxalate (see below) inside 0.25–0.50 μm diameter vesicles, which were located in the anterior region of the cell near the CFRs (Fig. 7). Calcium oxalate crystals were not observed in other regions of the cell.

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**Fig. 1.** Electron micrograph showing a portion of the anterior region of a *Tetraselmis* cell illustrating a contractile flagellar root in an extended configuration. The CFR (cfr) is associated proximally with the flagellar apparatus and distally with the plasma membrane. Eleven fibrous zones composed of 5–7 nm filaments are delineated by electron-dense cross-atriations (arrows). Numerous vesicles (v) (0.25–0.50 μm diameter) are located in the anterior-most region of the cell in the vicinity of the CFRs. b, basal body. Bars in Figs. 1–7, 0.5 μm.

**Fig. 2.** Electron micrograph of a portion of the anterior region of a *Tetraselmis* cell, which had been fixed in the presence of pyroantimonate and OsO₄. The CFR visible in this section is in an extended configuration. Vesicles (v) located in the anterior region of the cell in the vicinity of the CFR show dense deposits of Ca–Sb precipitation product. Few deposits occur in the cytosolic compartment. s, starch.

**Fig. 3.** Electron micrograph of a portion of the anterior region of a *Tetraselmis* cell, which had been fixed in the presence of pyroantimonate and OsO₄ after calcium-shock stimulation of CFR contraction. Note the increased level of cytosolic deposits of Ca–Sb precipitation and the discrete zones of precipitation along the contracting CFR. n, nucleus; m, nucleolus.

**Fig. 4.** Electron micrograph of a portion of a *Tetraselmis* cell, which had been fixed as in Fig. 3. As contraction continues the dense zones of Ca–Sb precipitation along the CFR appear to broaden and fuse (arrows). b, basal body.

**Fig. 5.** Calcium antimonate deposits occur throughout the CFR when the organelle is fixed in the presence of pyroantimonate and OsO₄ after calcium-shock stimulation of CFR contraction. Note the increased level of cytosolic deposits of Ca–Sb precipitation and the discrete zones of precipitation along the contracting CFR. s, starch.

**Fig. 6.** Deposits of Ca–Sb precipitate can be completely removed by floating the thin sections on solutions of EGTA or EDTA, but not on water alone. This micrograph shows holes (arrows) in vesicles located in the anterior region of a *Tetraselmis* cell that are produced after EGTA treatment. The section has not been post-stained. s, starch.

**Fig. 7.** Electron micrograph of a portion of the anterior region of a *Tetraselmis* cell, which had been fixed after incubation for 30 min in the presence of sodium oxalate. Calcium oxalate crystals (arrow) form inside vesicles (v) located near CFRs in the anterior region of the cell.
Fig. 8 depicts X-ray microprobe evidence, which directly confirms the vesicular localization of calcium by the pyroantimonate and oxalate methods. The inset in the upper portion of the figure shows an STEM image of a thick-sectioned *Tetraselmis* cell from the same block as the TEM image shown in Fig. 3. This cell was fixed in the pyroantimonate medium after calcium shock. Electron-dense vesicles (0.25–0.50 μm diameter) are clearly evident in the anterior region of the cell (Fig. 8, upper inset, box a). The posterior region of this cell is occupied by the chloroplast and is free of these vesicles and major accumulations of precipitation (Fig. 8, upper inset, box b). Ultrastructural resolution is limited due to the thickness of the sections used (250 nm) and operational features of the instrumentation. The X-ray emission spectra for the anterior region (Fig. 8, upper inset, box a) and the posterior region of the same cell (Fig. 8, upper inset, box b) are shown in Fig. 8 spectra A and B, respectively. A number of peaks appear in both spectra and represent stray signal from the copper grid (Cu), aluminium hardware (Al), and vapour of the silicon-based diffusion pump oil (Si), or result from the particular techniques used in preparing the specimen, i.e. osmium (Os). The distinctive difference between the two spectra is the prominent calcium and antimonate emissions from the anterior region of the cell (Fig. 8, spectrum A) and the diminution of these peaks from the posterior vesicle-free region of the cell (Fig. 8, spectrum B). The emission peak at 3.69 keV represents the Ka line of calcium. The spectral regions 0.09 keV below and 0.15 keV above the calcium line represent the Lα and Lβ emissions, respectively, for antimonate. The proximity of the calcium Ka line and the antimonate Lα line is below the practical resolution of the detector. Under the conditions of analysis and with the low sample-mass the instrumentation is operating near its detection limits and the signal-to-background ratio for calcium: antimonate is predictably low. Nevertheless, a concentration of energy emission at 3.69 keV is clearly correlated with the cellular region containing electron-dense vesicles. CFR profiles could not be distinguished in these STEM images of thick sections.

Thick sections of sodium-oxalate-treated cells were similarly analysed by electron-probe microanalysis. The inset in the lower portion of Fig. 8 shows an STEM image of a thick-sectioned *Tetraselmis* cell from the same block as the TEM image shown in Fig. 7. The anterior region of this cell contains a number of vesicles with electron-opaque contents (Fig. 8, lower inset, box c), while the posterior region of the same cell (Fig. 8, lower inset, box d) is free of these vesicles. These two regions were analysed separately for their X-ray emission spectra and the results are illustrated in Fig. 8, spectra C and D, respectively. The spectrum for the anterior vesicle-containing region of the cell (Fig. 8, spectrum C) shows a distinct peak for calcium at 3.69 keV unobstructed by emissions of other elements. The spectrum for the posterior vesicle-free region of the same cell does not show a calcium emission peak significantly above background (Fig. 8, spectrum D). This electron-probe microanalysis of oxalate-treated cells demonstrates a direct localization of calcium in the vesicle-containing anterior region of *Tetraselmis* cells.
Fig. 8. Electron-probe analysis of calcium cytochemistry in *Tetraselmis* cells. Upper inset: STEM image of a thick-sectioned *Tetraselmis* cell after fixation in pyroantimonate and OsO₄. Boxes *a* and *b* indicate the regions scanned for the X-ray emission spectra *A* and *B*, respectively. Note the numerous electron-dense vesicles in the anterior region of this cell (box *a*) and the distinct X-ray peak at 3.69 keV in the spectrum for this region. Also note the absence of such vesicles in the posterior region of the cell (box *b*) and diminution of signal around 3.69 keV in the corresponding X-ray emission spectrum (*B*). Lower inset: STEM image of a thick-sectioned *Tetraselmis* cell after incubation in sodium oxalate for 30 min prior to fixation. Electron-dense vesicles are evident in the anterior region of this cell (box *c*) and the X-ray spectrum (*C*) for this region shows a distinct calcium emission peak at 3.69 keV. The posterior region of this cell does not contain dense deposits (box *d*) and the corresponding X-ray spectrum (*D*) for this region shows only a small emission peak at 3.69 keV. Background X-ray emissions for copper, aluminium, silicon and osmium are indicated below in spectrum *D*. 
Calcium cytochemistry and X-ray microanalysis demonstrate that the anterior region of *Tetraselmis* contains calcium-sequestering vesicles (0.25–0.50 μm diameter). When striated flagellar roots are stimulated to contract by calcium shock, additional deposits of Ca–Sb localization appear in the cytoplasm, suggesting that a transient rise in 'free' Ca²⁺ levels may be occurring. This transient rise in free Ca²⁺ appears to be confined to the cytosolic and nuclear compartments since the relative amounts of Ca–Sb deposits in mitochondria and chloroplasts appear unchanged. Concurrent with the apparent rise in cytoplasmic Ca²⁺ new Ca–Sb deposits appear along contracting CFRs and are organized into a distinct banding pattern. Thus, it appears that CFRs bind Ca²⁺ during contraction. We have shown earlier (Salisbury & Floyd, 1978) that during contraction the striated pattern of CFRs changes in an ordered and complex fashion. During contraction two amorphous zones form on either side of each shortening fibrous band. These amorphous zones broaden and appear to fuse as contraction continues, until finally there are 10–13 of these zones in the fully contracted organelle (Salisbury & Floyd, 1978). The pattern of Ca–Sb deposits along contracting CFRs observed in this study corresponds to the amorphous zones. I suggest that calcium

Fig. 9. Diagram illustrating the anterior region of a *Tetraselmis* cell and the hypothetical role for the regulation of contractile flagellar root contraction by the calcium-sequestering vesicles.
binding to the filaments of CFRs induces a conformational change in their structure, converting them into the amorphous pattern. This conversion probably occurs through a supercoiling of the individual filaments (Salisbury, unpublished observations).

The level of free Ca$^{2+}$ in the eukaryotic cytoplasm is generally maintained under powerful homeostatic controls at concentrations below 10^{-7} M in resting cells (see Barritt, 1981, for a review). Transitory fluxes of calcium across membrane barriers are involved in regulation of contraction in muscle cells (Weber, 1966; Ebashi & Endo, 1968; Saetersdal, Myklebust, Berg Justesen & Engedal, 1977), in the protozoon Spirostomum (Etienne, 1976), the vorticellid spasmoneme (Carasse & Favard, 1966; Routledge, Amos, Yew & Weis-Fogh, 1976), plant and animal spindle apparatus (Hepler, 1977) and flagella (Hofwill & McGregor, 1975; Naitoh, 1969; Satir, 1976). Technical limitations of the methods for calcium localization used in this study make quantitation of the amount of Ca-Sb precipitation within the calcium-sequestering vesicles difficult. However, on the basis of the observations presented in this study I postulate that the calcium-sequestering vesicles in the anterior region of Tetraselmis play a role in regulating calcium-induced contraction of CFRs (Fig. 9) in a manner perhaps similar to that of the sarcoplasmic reticulum of striated muscle cells. Our cytochemical observations suggest that these vesicles possess the molecular machinery necessary for calcium sequestration. In addition, we have recently observed a cytochemically demonstrable ATPase associated with the delimiting membranes of these vesicles (Salisbury, unpublished observations). Transient release of Ca$^{2+}$ from these vesicles into the cytoplasm and its subsequent re-sequestration (cf. Rose & Loewenstein, 1975) could induce cyclic contraction and extension of CFRs. We cannot, however, eliminate a possible role for smooth endoplasmic reticulum, mitochondria or the plasma membrane in regulation of CFR contraction by influencing the 'free' Ca$^{2+}$ in the vicinity of CFRs (Brinley, 1973; Lehninger, 1970).

Finally, calmodulin, the ubiquitous calcium-binding protein of eukaryotes, is known to be a major cytoplasmic receptor for Ca$^{2+}$. Calmodulin mediates a variety of cellular activities involving transient fluxes of Ca$^{2+}$ as signals (Cheung, 1980a; Wang & Waismann, 1979; Salisbury, Condels, Maihle & Satir, 1981b), including the transport of calcium across membranes (cf. Cheung, 1980b; Watterson & Vincenzi, 1980). Calmodulin is present in the cytoplasm in high concentrations and has four high-affinity calcium-binding sites ($K_d = 10^{-8}$ to $10^{-5}$ M; Klee, Crouch & Richman, 1980). We expect most 'free' Ca$^{2+}$ in the cytoplasm to be rapidly bound by calmodulin and possibly additional Ca$^{2+}$ binding proteins. Since the pyroantimonate method for calcium localization is sensitive to Ca$^{2+}$ concentrations in the neighbourhood of $> 10^{-4}$ M (Klein et al. 1972) I suggest further that cytoplasmic Ca-Sb deposits may represent potential sites of Ca$^{2+}$ bound to calmodulin or other Ca$^{2+}$ binding proteins.

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