Scanning Electron Microscopy

Volume 1985
Number 1 *1985*

Article 39

12-26-1984

X-Ray Microanalysis in Cryosections of Natively Frozen Paramecium Caudatum with Regard to Ion Distribution in Ciliates

M. Schmitz
University of Bonn, Ulrich-Haberland-Str.

R. Meyer
University of Bonn, Wilhelmstr.

K. Zierold
Max Planck Institute

SCANNING ELECTRON MICROSCOPY/1985/I (Pages 433-445)
SEM Inc., AMF O'Hare (Chicago), IL 60666 USA

0586-5581/85$1.00+.05

X-RAY MICROANALYSIS IN CRYOSECTIONS OF NATIVELY FROZEN *PARAMECIUM CAUDATUM* WITH REGARD TO ION DISTRIBUTION IN CILIATES

M. Schmitz*, R. Meyer**, K. Zierold***

* Inst. Cytol., Univ. Bonn, Ulrich-Haberland-Str. 61 a, 5300 Bonn, FRG
** Inst. Physiol. II, Univ. Bonn, Wilhelmstr. 31, 5300 Bonn, FRG
*** Max-Planck-Inst. f. Systemphysiol., Rheinlanddamm 201, 4600 Dortmund 1, FRG

(Paper received January 24 1984, Completed manuscript received December 26 1984)

Abstract

Cells of Paramecium caudatum were shock-frozen without pretreatment for cryoultramicrotomy and freeze-dried for subsequent X-ray microanalysis. Na, Mg, P, S, Cl, K, and Ca were detected in different amounts in several subcellular compartments. In particular, calcium was localized below the cell surface (pellicle). Trichocysts were found to contain significant amounts of Na in their base but not in the tip. Na, Mg, P, S, Cl, K, Ca were found in electron dense deposits within the lumen of the contractile vacuole. A small K concentration was found in the cytoplasm and in the mitochondria. X-ray microanalysis of the element distribution in different subcellular compartments provides information for the understanding of cellular functions such as exocytosis, locomotion, and ion regulation.

KEY WORDS: Paramecium caudatum, ciliates, ion distribution, X-ray microanalysis, cryofixation, cryosection, trichocyst, calcium localization.

*Address for correspondence:
M. Schmitz
Institut für Cytologie, Universität Bonn,
Ulrich-Haberland-Str. 61a, 5300 Bonn 1, FRG
Phone No: (0228) 735301

Introduction

Ions, their transport and storage have become more and more important for the understanding of biological processes. Because of the relatively small size of ions and their ability of fast dislocation, they play an essential role in regulation at the cellular level. Just these characteristics, however, make the experimental approaches and the interpretation of the results difficult. Since energy dispersive X-ray microanalysis (EDS) in the electron microscope provides a lateral resolution on a subcellular level in combination with a satisfactory sensitivity for the explanation of biological phenomena, this technique is increasingly applied (reviewed by Moreton, 1981; Sumner, 1983). Unfortunately, chemical fixations even in combination with histochemical preparations cannot avoid artificial translocation of the highly mobile ions (Morgan, 1979). In order to maintain elemental distributions resembling the in vivo state, biological tissue is fixed physically by shock-freezing without pretreatment (reviewed by Plattner and Bachmann, 1982). We used the ciliate Paramecium caudatum, since its relatively small volume and thickness (volume below 10^{-6} ml and smaller than 50/um in diameter) is advantageous for cryofixation. We studied the ion-gradients between different subcellular compartments by means of EDS of cryosections. These results are discussed with regard to the involvement of ions in cellular processes in ciliates.

Materials and Methods

Cell material

Paramecium caudatum was raised in hay infusion at room temperature. The cells were enriched by light or centrifugation, and collected with a pipette before fixation (reviewed by Wichtermann, 1953).

Chemical fixation

Cells of Paramecium caudatum were fixed in a solution of 2% OsO_4 and 1% $K_2Cr_2O_7$ (Wohlfarth-Bottermann, 1957), dehydrated in ethanol and embedded in a modified Spurr's medium (Spurr, 1969; modification: Mascorro et al., 1976).

Physical fixation

Shock-freezing of the cells was performed by

either spraying droplets of suspensions on a polished metal surface cooled by liquid nitrogen below 83 K or by shooting small droplets containing two to five cells and as little medium as possible into liquid propane below 83 K. The cells were frozen on concave platelets of polymerized embedding medium or Balzer's gold planchettes of about 1 mm in diameter, as necessary for cryosectioning. Jet-freezing (Müller et al., 1980) was given up, since the cells were shot from the holders.

The shock-frozen cells on polymerized plastic platelets as well as the ones frozen on a polished metal surface were transferred into a Leybold freeze-dryer GT 1^R (Klein and Stockem, 1976) and dehydrated for 80 hrs in vacuum better than 10^{-4} Torr. The sample container was slowly warmed up from 83 K to a controlled temperature of 160 K. This corresponded to a sample temperature of 190 K. While maintaining the low pressure permanently, the temperature of the freeze-dried cells was slowly raised to 263 K within 36 hrs. The specimens were then infiltrated with modified Spurr's medium for 48 hrs and polymerized at 343 K for 10 hrs (Mascorro et al., 1976). Sections were cut with glass knives and picked up after floating on water or from dry glass knives (Masters et al., 1979; Ingram and Ingram, 1980; Barckhaus et al., 1980; Meyer et al., 1982).

The sections were transferred to 50 mesh copper grids coated with Pioloform F^R (Stockem, 1970). The dry cut sections were at least 300 nm thick. Since they cannot be flattened completely on the grid and to obtain better contact to prevent charging, a second uncoated grid was glued onto the first.

Electron microscopy was carried out at 80 kV and 45° tilt in a conventional Philips EM 300 with scanning electron microscopy-"STEM"-unit and EDAX-microanalysis-unit (707 B).

Cryosectioning

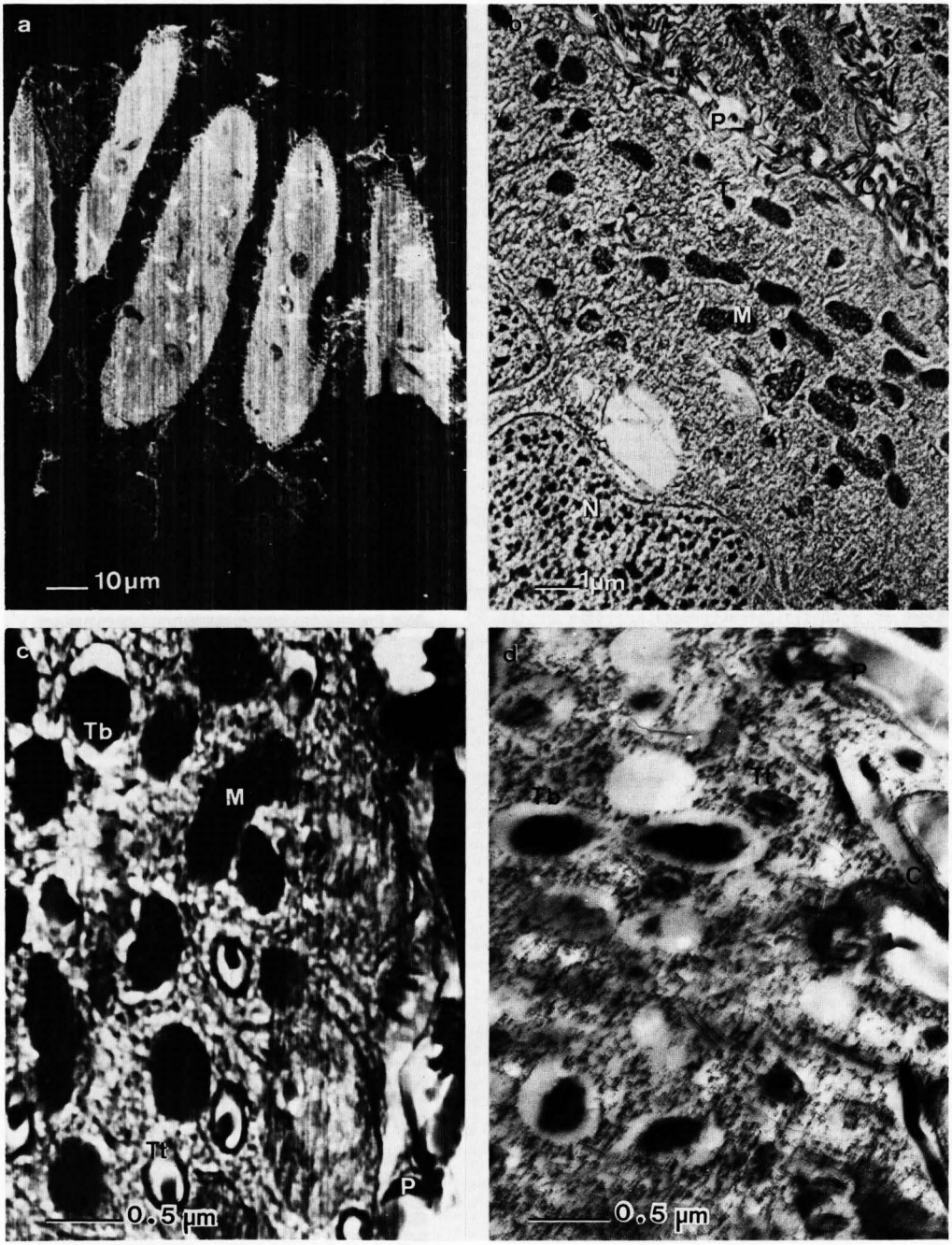
Just like sections of freeze-dried, vacuum embedded cells, cryosections were cut dry by glass knives (cryosectioning reviewed by Roomans et al., 1982; Sitte, 1982). At a temperature lower than 173 K to prevent drying and/or recrystallisation (Frederik, 1982), the sections were cut at high speed of about 20 mm/s, since this minimizes rolling up of cryosections and does not cause artefacts by melting, according to theoretical calculations (Hodson and Marshall, 1972) and to experimental evidence (Frederik and Busing, 1981; Karp et al., 1982).

The frozen-hydrated sections were transferred to a film-coated copper grid with an eyelash. To prevent charging, they were either pressed on the grid with a polished cold metal rod (Christensen, 1971; Wendt-Gallitelli et al., 1980; Zierold, 1982 a) putting up with the loss of sections or, alternatively, a second film coated grid was pressed on (Somlyo et al., 1977; Seveus, 1980) and disconnected again, thus, producing two grids with sections for the microscope (Zierold, 1982 a). Because of the extremely low contrast of frozen-hydrated sections in the electron microscope (Gupta et al., 1977; Gupta and Hall, 1981; Frederik, 1982; McDowall et al., 1983) and to obtain a better peak/background ratio (P/B) for EDS, the sections were freeze-dried in the transfer unit of a cryotransfer system or in the microscope (Zierold, 1982 a, b, 1983). Electron microscopy of the cryosections was performed by scanning transmission in a Siemens ST 100 F with a field emission gun at 100 kV and an energy dispersive X-ray system as described by Zierold (1982 b). For a more detailed discussion of the methods of cryopreparation see Meyer et al. in this volume.

Results

In ultrathin sections chemically fixed and plastic embedded cells of Paramecium caudatum exhibited the usual morphology and ultrastructural arrangement as reviewed by Vivier (1974). With EDS no significant signals of elements within the cells were detected except for those added during chemical fixation. Therefore, no pictures of morphology are presented and elemental distribution had to be studied in shock-frozen specimens. After shock-freezing without pretreatment, cells of Paramecium caudatum generally exhibit the same ultrastructural arrangement as those chemically fixed (Fig. 1). Although artifacts by ice crystal growth could not be avoided completely, they were negligible for cells used for EDS down to approximately 30 µm below the surface (for discussion of the influence of ice crystal damage on elemental distribution detected by EDS, see Zierold, 1984, and Meyer et al., this volume). There was no difference in the ultrastructure after spraying droplets of cell suspensions on liquid nitrogen cooled, polished metal surface, compared with specimens shot into liquid propane. Nevertheless, we preferred shooting into liquid propane, since after spraying the cells were difficult to find again. After cryosectioning and freeze-drying shock-frozen cells of Paramecium caudatum without pretreatment exhibit significant amounts of Na, Mg, S, Cl, K, and Ca in different subcellular compartments (Figs. 2a - f). Peaks of Cu, Si, and Au are artifacts caused by the method and are also present in control measurements outside the sections (Fig. 2). More than 2 µm under the

Fig. 1: Ultrastructure of shock-frozen Paramecium caudatum.
a) Dark field image in STEM of a cryosection with several paramecia. Vertical striations are caused by the noise of the field emission gun.
b) Cryosection in STEM (brightfield/darkfield). Subcellular compartments such as nucleus (N), mitochondria (M), trichocysts (T), cilia (C), and pellicle (P) can be identified.
c) Cryosection in higher magnification, same technique as b, shows a region below the cell surface. Cross-section of trichocyst body (Tb) and trichocyst tip (Tt), mitochondrion (M), pellicle (P).
d) Freeze-dried, embedded cell in ultrathin section, post-stained with lead citrate and uranyl acetate in TEM. Cross-sectioned trichocyst body (Tb) and trichocyst tip (Tt), pellicle (P), ciliary base (C).


a
10µm
b
P
C
M
N
1µm
c
Tb
M
Tf
P
0.5 µm
d
P
Tf
Tb
C
0.5 µm

pellicle significant amounts of phosphorus and potassium are demonstrated in the cytoplasm (Fig. 2a). Mitochondria contain additional amounts of sulphur varying from cell to cell, possibly representing different physiological conditions, but never calcium (Fig. 2b). In the cytoplasm adjacent to the pellicle and around the ciliary bases significant amounts of calcium and chlorine are obtained (Fig. 2c). Calcium has the highest peak in the spectrum besides the artifactual silicon. Therefore, its postulated function for the regulation of many cellular processes will be discussed later. The body of trichocysts always shows significant amounts of sodium, sulphur, and potassium, whereas sodium is missing in the tips of these organelles (compare Fig. 2d with 2e). Therefore, an artifactual influx of extracellular sodium can be excluded. However, it remains open to further investigations, how sodium is accumulated within the bases of trichocysts. It is also unclear, whether this accumulation is necessary for the storage and/or extrusion mechanism of organelles and/or whether it is involved in osmoregulation.

Contractile vacuoles are always in direct contact to the extracellular medium via pores. As an artifact of freeze-drying, sections of shock-frozen cells possess electron dense deposits, preferably along the inner side of the contractile vacuoles. These deposits contain very high concentrations of Na, Mg, P, S, Cl, K, and Ca. However, their concentration in the deposits varies from cell to cell. Since the cells were not fixed at a physiologically defined state of the contractile vacuoles it cannot be excluded that the different concentrations of elements within the deposits were caused by drying artifacts instead of different physiological conditions.

Discussion

In ciliates ion gradients are thought to be involved in different cellular functions such as contraction, locomotion, exo- and endocytosis, and osmoregulation. Most of the literature on element distribution is based on histochemical methods, biochemistry and electrophysiology. X-ray microanalysis of cryosections from natively frozen cells provides additional information on ion gradients between different subcellular compartments resembling the in vivo state. These results are discussed with regard to the present knowledge.

Contraction phenomena

Stirred up by speculations and findings of the role of calcium ions as an initiator of contraction in striated muscle (Heilbrunn, 1940; Heilbrunn and Wiercinski, 1947; reviewed by

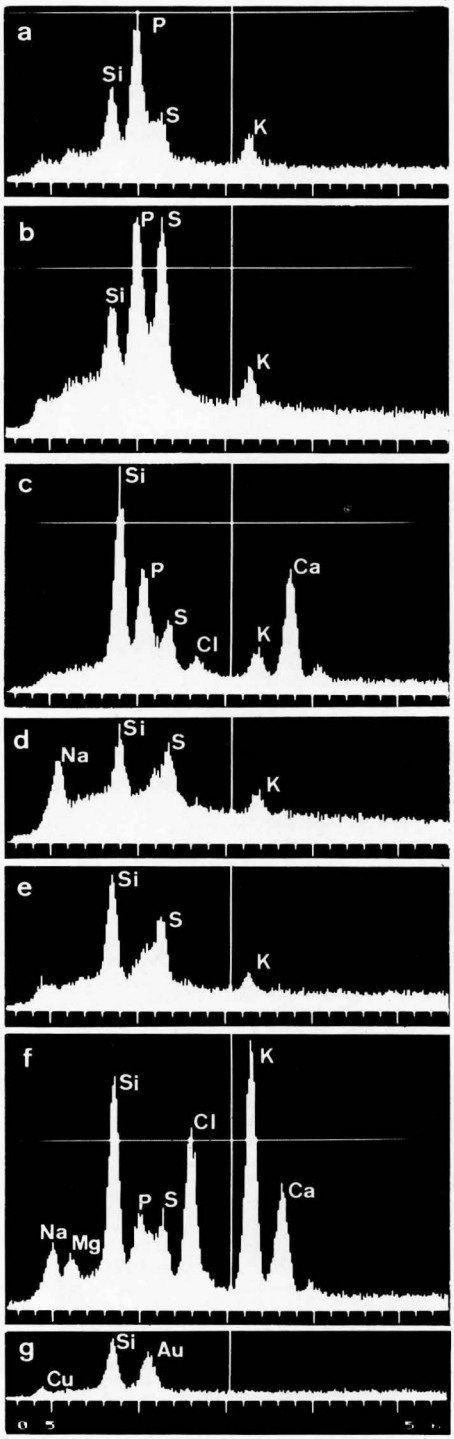


Fig. 2: X-ray spectra of different subcellular compartments in Paramecium caudatum.
a) Cytoplasm,
b) mitochondrion,
c) pellicle,
d) trichocyst body,
e) trichocyst tip,
f) deposit in contractile vacuole,
g) control outside the section.

Wilkie, 1968), attention was focussed on the contraction of the giant ciliate Spirostomum ambiguum. Organisms microinjected with the free-calcium-sensitive bioluminescent protein, aequorin, emitted light when electrically stimulated to contract. Furthermore, a filamentous peripheral network was demonstrated by electron microscopy and, after adding oxalate to the fixative, cytoplasmic vesicles contained crystalline, electron-dense precipitates morphologically defined as calcium oxalate. Ettienne (1970) proposed that an increase of the calcium concentration in cytoplasm, regulated by the vesicles with a role similar to the sarcoplasmic reticulum in striated muscle, initiated contraction of these cells.

Intracellular calcium pools within elongate dense bodies (EDB) were also found with EDS after glutaraldehyde fixation in embedded cells of Discophrya collini. This is a free living suctorian with tentacles able to contract. Increase of extracellular Ca and Mg, but not Ba, stimulated contraction. The application of external $CaCl_2$ to 10^{-1}M did not cause a detectable increase of Ca in the cytoplasm except for the EDB's, whereas after the same increase of extracellular Mg or Ba, these elements were not detected anywhere in the cells. It was suggested that EDB acted as calcium reservoir, controlling levels of cytoplasmic calcium (Hackney and Butler, 1981).

Peritrichous ciliates like Vorticella, Carchesium, and Zoothamnium possess contractile stalks, which coil helically or fold at speeds up to 170 lengths per second (Weis-Fogh and Amos, 1972). In these contractile organelles, called spasmonemes, precipitates of calcium were found in membranous tubules analogous to the sarcoplasmic reticulum (Favard and Carasso, 1965; Carasso and Favard, 1966). Whole spasmonemes were isolated from Zoothamnium and dried in the presence of solutions with different calcium concentrations. Electron microprobe analysis demonstrated 1.7 g more calcium per kg dry mass in the contracted compared to the extended organelles (Routledge et al., 1975). A spasmonemal calcium-binding protein, called spasmin, was extracted and characterized by sodium dodecyl sulphate-"SDS"-polyacrylamide gel electrophoresis and differentiated from actomyosin and tubulin (Routledge and Amos, 1977; Routledge, 1978).

Ciliary movement

Indications of calcium involvement in ciliary movement of the free living Paramecium aurelia were made by Plattner (1975) and Plattner and Fuchs (1975); using EDS they demonstrated calcium-binding sites by adding high concentrations of calcium to the fixative (Oschman and Wall, 1972) or after precipitation with oxalate according to Costantin et al. (1965). The 'ciliary granule plaques' at the ciliary base and the membranes of cilia contained electron dense deposits of Ca, P, and S. In Paramecium caudatum calcium binding sites in cilia were demonstrated accordingly (Tsuchiya, 1976). Basal bodies and membranes around ciliary axonemes also accumulated other divalent cations like Mg, Mn, Sr, Ni, Ba, and Zn after substitution for Ca (Fisher et al., 1976).

In shock-frozen cells of Paramecium caudatum without pretreatment high concentrations of calcium are present under the pellicle around the bases of cilia, since calcium is the most significant peak in the spectrum (Fig. 2c). Significant amounts of phosphorus, sulphur, chlorine, and potassium are also present, but except for chlorine they are comparable to the elemental distribution in the cytoplasm not adjacent to the pellicle (Fig. 2a). By our preparation technique a physiologically relevant presence of chlorine, probably as $CaCl_2$, is demonstrated. Earlier findings of this element were caused by high concentrations within the embedding media used, e.g. Epon.

Experiments described by Silvester et al. (1982) about shock-freezing of protozoa were performed at cooling rates up to 660 K/s, followed by freeze-substitution. Vitrification of cells requires cooling rates higher than 10^4K/s (Moor, 1964). For unambiguous morphological identification of subcellular structures and for the detection of ion gradients by EDS a mean ice crystal diameter smaller than 50 nm was accepted as satisfactory. This requirement was met in our analyzed cryosections of Paramecia. Methods including freeze-substitution were not used for EDS, in order to avoid the risk of ion translocation. Our results support the hypothesis that calcium is involved in the regulation of ciliary movement. Calmodulin, a calcium receptor protein, was localized by immunofluorescent studies in the microscope within the cilia and in a rim along the oral groove (Satir et al., 1980) and partially purified from Paramecium and Tetrahymena (Maihle and Satir, 1980). By SDS-polyacrylamide gel electrophoresis a molecular weight of about 17,000 was determined for calmodulin isolated from cilia and cells of Paramecium tetraurelia (Walter and Schultz, 1981). Calmodulins from ciliates seemed to share some antigenic determinants missing in higher organisms, because antiserum for calmodulin of Tetrahymena crossreacted with calmodulin of Paramecium but not with all of the higher organisms. By immunofluorescence microscopy the results of Satir et al. (1980) were confirmed. Additional fluorescence was localized at the anterior end of the cell and at the pores of the contractile vacuoles and its possible role for the function of the cells was discussed (Suzuki et al., 1982). An ultrastructural localization of calmodulins isolated from cilia, cell bodies, and whole cells of Tetrahymena was made by immunoelectron microscopy and alkali gel electrophoresis. Calmodulin was localized morphologically along the longitudinal axis of outer-doublet microtubules at regular intervals of about 90 nm and in the ciliary axoneme. Biochemically, calmodulin was located in the membrane plus matrix fraction and outer-doublet microtubule fraction, and its Ca^{2+}-dependent counterpart existed exclusively in the latter fraction. But neither calmodulin nor its counterparts were found in the crude dynein fraction (Ohnishi et al., 1982). This is controversial to earlier reports of Jamieson et al. (1980) and Blum et al. (1980), who found a highly purified Ca-calmodulin-sensitive dynein ATPase, isolated by affinity chromatography in Tetrahymena cilia, and detected calmodulin in the dynein fraction. Although progress was made by the characterization of Ca-binding proteins in ciliates and their localization within the cells,

their precise function for the regulation of intracellular Ca levels remained open to discussion.

Since a controlled influx of extracellular calcium or a release from intracellular calcium pools was essential for the understanding of the regulation of ciliary movements, electrophysiological studies using voltage clamp methods were performed by several groups. The experiments led to the characterization of a voltage-sensitive 'fast' Ca channel with millisecond kinetics, responsible for the brief calcium influx and cell depolarization that precede ciliary reversal (Naitoh and Eckert, 1968, 1969; Eckert, 1972; Machemer and Eckert, 1973; Oertel et al., 1977; Brehm and Eckert, 1978a, b; Satow and Kung, 1979). Nevertheless, this channel also seemed to be ion-dependent, because it showed different rates of activation and deactivation for Ca, Sr, and Ba currents (Saimi and Kung, 1982). Measurements of intracellular pH with recessed tip ion selective microelectrodes were also correlated to Ca currents through Ca channels monitored by voltage clamp in membranes of Paramecium caudatum. The normal internal pH was about 6.8. Internal acidification depressed the Ca current, while alkalization enhanced it (Umbach, 1982). Since the electrophysiology of ciliated, deciliated, and reciliated cells was compared (Ogura and Takahashi, 1976; Dunlap, 1977; Machemer and Ogura, 1979), there is general agreement, that this Ca channel is located within the ciliary membrane. Furthermore, 'slow' Ca-sensitive channels with 0.1 s kinetics were observed. The first was a K channel activated by Ca^{2+} entering through the 'fast' Ca channels (Brehm et al., 1978; Satow and Kung, 1980) and the second a Ca-sensitive Na channel triggered by internal Ca^{2} (Saimi and Kung, 1980). The gating of the Na channel seems to be affected by Zn^{2+} and Ni^{2+} and possibly Ca^{2+} or Mg^{2+} (cited from Saimi and Kung, 1982). Voltage-sensitive 'fast' channels were also reported for K after hyperpolarization (Oertel et al., 1978) and depolarization, responsible for delayed rectification (Oertel et al., 1977; Brehm et al., 1978; Satow, 1978; Satow and Kung, 1976). Antibodies against ciliary membrane proteins of Paramecium were able to immobilize the cells (Eisenbach et al., 1983). Their distribution over the entire cell surface was monitored by immunoelectron microscopy and after antibody binding, voltage-clamp experiments exhibited a reduction of the Ca-induced K current as well as a reduction of Ca current into the cells of Paramecium (Ramanathan et al., 1983). A more detailed review of ionic channels of Paramecium was presented by Adoutte et al. (1981).

Electron microscopical studies of ultrathin sections in combination with freeze-fracture replicas indicated that the alveolar sacs within the pellicle of ciliates act as calcium reservoirs for ciliary motility (Satir and Wissing, 1982). Our data are in agreement with that, since we obtained high concentrations of calcium in the region where the alveolar sacs are located (Fig. 2c). Because of their small size, however, it is uncertain, whether the calcium actually measured is exclusively located within the alveolar sacs. Additionally, alveolar sacs possessed a unique population of unit membrane particles and also frequently in their lumen lammelated inclusion bodies probably containing phospholipids (Satir and Wissing, 1982).

Exocytosis

First observations of the ultrastructural organization of cytoplasmic components in Paramecium after fixation with OsO_4 and $K_2Cr_2O_7$ were obtained by Wohlfarth-Bottermann (1958). The ultrastructure of their extrusive organelles, the trichocysts, was studied in detail by Hausmann et al. (1972a, b). First indications of an involvement of calcium in the exocytotic mechanisms of trichocysts in Paramecium aurelia were obtained by Plattner and Fuchs (1975), since the ionophores X-537 A or A 23187 stimulated exocytosis. Furthermore, Ca-binding sites were demonstrated in ultrathin sections of chemically fixed and embedded cells by EDS, after adding high concentrations of calcium to the fixative (Plattner and Fuchs, 1975; Fisher et al., 1976) or using pyroantimonate or oxalate for precipitation (Plattner and Fuchs, 1975; for methods see: Costantin et al., 1965; Oschman and Wall, 1972). Electron dense deposits containing calcium and phosphorus were observed on the inner side of the plasma membrane, the alveolar membrane, along the trichocyst membrane, and within trichocysts. Most trichocysts being in a 'resting' position were devoid of electron dense deposits. A few 'resting' trichocysts as well as most of those trichocysts just undergoing exocytosis contained deposits at the membrane of the trichocyst tip and of the trichocyst body as well as on the inner lamellar sheath structures. Cytoplasmic vacuoles containing high amounts of calcium were not found after glutaraldehyde fixation with high calcium, but after precipitation with oxalate. From these observations it was concluded that the deposits of the membrane around trichocysts might demonstrate an ATPase system for the triggering mechanism and that an influx of calcium into the trichocysts occurred just before exocytosis.

To test this hypothesis attention was focused on the membrane state before and during exocytosis. Several methods were developed to study still functional but undischarged trichocysts isolated from Paramecium cells (Steers et al., 1969; Anderer and Hausmann, 1977; Matt et al., 1978). A synchronized discharge of trichocysts was achieved by increasing intracellular, free calcium using ATP-blockers, Ca-ionophores, and lipid solvents (Matt et al., 1978), as applied to strains of Paramecium having different exocytotic capability (Matt et al., 1980). Freeze-fracture studies (Bachmann et al., 1972; Janisch, 1972; Plattner et al., 1973) exhibited at the docking sites of trichocyst rosettes of membrane-intercalated particles (MIP) missing in mutants without trichocysts (Beisson et al., 1976). Furthermore, a Ca^{2+}-ATPase was demonstrated cytochemically in these rosettes by electron microscopy in combination with EDS (Plattner et al., 1980). From the trigger experiments combined with the freeze-fracture studies and the histochemical experiments with EDS it was concluded that an increase of free, intracellular calcium estimated to be somewhere between 10^{-6} to 10^{-4}M (Matt et al., 1978) was necessary for exocytosis to occur. Furthermore, it was suggested that the

rosettes acted as Ca pumps or Ca channels and were involved in membrane fusion (Matt et al., 1980). The results of our experiments dealing also with the intracellular localization of calcium within Paramecium are often in agreement with the data presented by Plattner and Fuchs (1975) as well as Fisher et al. (1976), who added high concentrations of calcium to the fixative according to Oschman and Wall (1972). Both methods demonstrate that calcium is present under the pellicle, whereas 'resting' trichocysts also sometimes contain calcium after chemical fixation but never in our experiments (Figs. 2d, e). After application of precipitating agents such as pyroantimonate or oxalate, however, data were obtained by frequently finding Ca-containing deposits within 'resting' trichocysts or in so-called 'Ca-storing vesicles', both never observed in cryosections. Since shock-frozen cells represent a better model for the distribution of ions compared with chemically fixed cells, the risk of unspecific precipitations seems to be higher after additional application of precipitating agents to the fixative compared with the method of Oschman and Wall (1972). The danger of unspecific staining was also demonstrated for (OsFeCN) in a variety of biological objects including Paramecium caudatum by Schnepf et al. (1982). Our data support the hypothesis that calcium is involved in exocytosis by entering the trichocyst in high concentrations just before exocytosis. But by the demonstration of high concentrations of sodium within the bodies of resting trichocysts (Fig. 2d) a hitherto unknown factor for a possible function of trichocysts might have been added.

A possible role of calmodulin as a stimulator of Ca pumps in Paramecium cell membrane was discussed (Satir et al., 1980) and calmodulin was demonstrated to be a major component in purified, extruded trichocysts (Rauh and Nelson, 1981). In isolated and purified surface membrane complexes of Paramecium a Ca^{2+}-stimulated ATPase was detected (Nogushi et al., 1979) differing from those in exocytosis-incompetent strains (Bilinski et al., 1981b). Comparing the ion content of isolated trichocysts in condensed and in in vitro decondensed form, only phosphorus and sulphur were significant by EDS. Adding $CaCl_2$ before preparation for EDS, both tip and base of trichocysts accumulated the ions. It was concluded that calcium entered the lumen of the trichocyst vesicle through the membrane from the external medium after an exocytotic opening has been formed and then triggered decondensation (Bilinski et al., 1981a).

The possible role of the membrane during exocytosis (Plattner, 1981a, b), of microtubules during the docking process (Plattner et al., 1982), and of the alveolar sacs was discussed (Satir and Wissing, 1982).

Endocytosis and ion accumulation

Calcium seems to be involved in endocytotic processes of ciliates like the formation of food vacuoles, since by light immunofluorescence microscopy calmodulin was localized in the oral apparatus of Tetrahymena. This suggestion was supported by the suppression of food vacuole formation by the calmodulin inhibitor trifluoperazine (Suzuki et al., 1982). This is consistent with observations of Fok and Allen (1981), who found a decline in food vacuole formation accompanied by a decrease in Ca^{2+}-ATPase activity during stationary phase of culture cycle.

Food vacuole formation in Tetrahymena was also inhibited dose-dependent by lead (Nilsson, 1978, 1979) and nickel (Larsen and Nilsson, 1983), which presumably entered the cells through the plasma membrane and were detected by atomic absorption (AAS). Copper, however, stimulated endocytosis (Nilsson, 1981). After adaptation to heavy metals, they seemed to be accumulated in granules and eliminated by defecation since a constant value of nickel monitored by AAS, too, was maintained after some time (Larsen and Nilsson, 1983).

Intracellular accumulation of heavy metals such as Cd, Mn, Pb, Cu, and Zn was also detected by AAS in the marine ciliate Eufolliculina. Additionally, Na, Mg, P, S, Cl, K, and Ca were found by EDS and laser microprobe mass analysis (LAMMA^R) in air dried specimens of these protozoa (Mulisch et al., 1982).

Accumulation of Ca and P in the form of crystals and globular lithosomes was detected in air dried specimens of the marine ciliate Euplotes vannus by SEM and EDS (Hausmann and Walz, 1979).

In the ciliate Homalozoon vermiculare certain membrane-bounded organelles belong to the so-called parapharyngeal mass, which was believed to be involved in food vacuole formation. In isolated and air-dried material of the parapharyngeal mass an enrichment of Mg, P, K, and Ca was demonstrated by SEM and EDS. But the function of these accumulations was not yet explained (Kuhlmann et al., 1983).

Osmoregulation

The cytoplasm of fresh-water ciliates had an osmolarity from 120 to 210 mOsm/l (Kitching, 1938, 1951, Stoner and Dunham, 1970). Therefore, they were thought to be hyperosmotic to their environment. Since a passive influx of water and a passive outflux of ions resulted, the contractile vacuole was thought to maintain the osmotic gradient by actively pumping water out of the cells. Actually, contractile vacuole complexes appeared to be extremely sensitive to external osmotic changes. They increased their output if external medium became more diluted and decreased their output if the osmotic pressure in the medium was raised (Kitching, 1936, 1938, 1951; Gaw, 1936; Osanai, 1961a, b; Stoner and Dunham, 1970; inter alia). The frequency of contraction seemed to reflect the ionic balance in the medium. This was thought to be controlled by calcium and could be influenced separately from the mechanism of fluid production. Fluid production of the contractile vacuole was suggested to be taken over by the so-called spongiome, but little is known about its function or ion content except the fact that Paramecium did not seem to possess a Na/K-ATPase (Andrivon et al., 1977; for a detailed review on contractile vacuoles see Patterson, 1980). In freeze-dried cryosections of shock-frozen Paramecium cells electron dense deposits within contractile vacuoles are always present containing very high concentrations of Na, Mg, P, Cl, K, and Ca (Fig. 2f). Since variations of elemental distribution are observed at different yet undefined states of contraction in

cells surrounded by the same extracellular medium, the hypothesis is supported that the contractile vacuole is involved in ion regulation of ciliates. However, it cannot be excluded that different ion concentrations within the deposits are caused by freeze-drying. Therefore, freezing at physiologically defined states with subsequent analysis of frozen-hydrated sections would be desirable.

Conclusions

Earlier reports about the ion content in subcellular compartments of ciliates rely mostly on indirect methods, such as electrophysiology and biochemistry as well as precipitation techniques for the demonstration of ion binding sites. From experiments using these methods hypotheses were formulated involving ion gradients in various cellular functions. X-ray microanalysis of freeze-dried cryosections of shock-frozen Paramecia provides direct evidence for ion gradients between different subcellular compartments, thus, resembling the in vivo state. The EDS results generally agree with the leading hypotheses on the role of ions for cellular functions. Additionally, unreported ion gradients were detected, such as chlorine in cytoplasmic regions adjacent to the pellicle as well as high amounts of sodium in the trichocysts. The interpretation of these results, however, requires further EDS-data of frozen-hydrated cryosections from cells in better defined physiological conditions.

Acknowledgment

We thank Miss S. Dongard for skillful technical assistance.

References

Adoutte A, Ling KY, Forte M, Ramanathan R, Nelson D, Kung C. (1981). Ionic channels of Paramecium: From genetics and electrophysiology to biochemistry. J. Physiol. (Paris) 77, 1145-1159.

Anderer R, Hausmann K. (1977) Properties and structure of isolated extrusive organelles. J. Ultrastruct. Res. 60, 21-26.

Andrivon C, Wyroba E, Patterson DJ. (1977). Récherches préliminaires sur les ATPases de Paramecium aurelia. J. Protozool 24, 55A.

Bachmann L, Schmitt WW, Plattner H. (1972). Improved cryofixation: Demonstrated on freeze-etched solutions, cell fractions, and micellar organisms, in: Proceedings 5th European Regional Congress Electron Microscopy, Cosslett VE (ed.), The Institute of Physics, London, Bristol, 244-245.

Barckhaus RH, Höhling HJ, Krefting ER. (1980). Elektronenstrahlmikroanalyse an mineralisierendem Gewebe in der konventionellen Mikrosonde und im Elektronenmikroskop: Arten der Präparation und Darstellung einiger Ergebnisse. Acta Histochemica, Suppl. XXI, 167-180.

Beisson J, Lefort-Tran M, Pouphile M, Rossignol M, Satir B. (1976). Genetic analysis of membrane differentiation in Paramecium. Freeze-fracture study of the trichocyst cycle in wild-type and mutant strains. J. Cell Biol. 69, 126-143.

Bilinski M, Plattner H, Matt H. (1981a). Secretory protein decondensation as distinct, Ca^{2+}-mediated event during the final steps of exocytosis in Paramecium cells. J. Cell Biol. 86, 179-188.

Bilinski M, Plattner H, Tiggemann R. (1981b). Isolation of surface membranes from normal and exocytotic mutant strains of Paramecium tetraurelia. Ultrastructural and biochemical characterization. Europ. J. Cell Biol. 24, 108-115.

Blum JJ, Haynes A, Jamieson Jr GA, Vanaman TC. (1980). Calmodulin confers calcium sensitivity on ciliary dynein ATPase. Europ. J. Cell Biol. 87, 386-397.

Brehm P, Dunlap K, Eckert R. (1978). Ca-dependent repolarization in Paramecium. J. Physiol. (Lond.) 274, 639-654.

Brehm P, Eckert R. (1978a). Calcium entry leads to inactivation of calcium channel in Paramecium. Science 202, 1203-1206.

Brehm P, Eckert R. (1978b). An electrophysiological study of the regulation of ciliary beating frequency in Paramecium. J. Physiol. (Lond.) 283, 557-568.

Carasso N, Favard P. (1966). Mise en évidence du calcium dans les myonèmes pédonculaires de ciliés péritriches. J. Microsc. (Paris) 5, 559-570.

Christensen AK. (1971). Frozen thin sections of fresh tissue for electron microscopy, with a description of pancreas and liver. J. Cell Biol. 51, 772-804.

Costantin LL, Franzini-Armstrong C, Podolski RJ. (1965). Localization of calcium-accumulating structures in striated muscle fibres. Science 147, 158-160.

Dunlap K. (1977). Localization of calcium channels in Paramecium caudatum. J. Physiol. (Lond.) 271, 119-133.

Eckert R. (1972). Bioelectric control of ciliary activity. Science 176, 473-481.

Eisenbach L, Ramanathan R, Nelson DL. (1983). Biochemical studies of the excitable membrane of Paramecium tetraurelia. IX Antibodies against ciliary membrane proteins. J. Cell Biol. 97, 1412-1420.

Ettienne EM. (1970). Control of contractility in Spirostomum by dissociated calcium ions. J. Gen. Physiol. 56, 168-179.

Favard P, Carasso N. (1965). Mise en évidence d'un reticulum endoplasmatique dans le spasmonème de ciliés péritriches. J. Microsc. (Paris) 4, 567-575.

Fisher G, Kaneshiro ES, Peters PD. (1976). Divalent cation affinity sites in Paramecium aurelia. J. Cell Biol. 69, 429-442.

Fok AK, Allen RD. (1981). Axenic Paramecium caudatum. III. Biochemical and physiological changes with culture age. Europ. J. Cell Biol. 25, 193-201.

Frederik PM. (1982). Cryoultramicrotomy - recognition of artifacts, Scanning Electron Microsc. 1982; II: 709-721.

Frederik PM, Busing WM. (1981). Strong evidence against section thawing whilst cutting on the ultramicrotome. J. Microsc. 122, 217-220.

Gaw HZ. (1936). Physiology of the contractile vacuole in ciliates. I. The effect of osmotic pressure. Arch. Protistenkunde 87, 185-193.

Gupta BL, Hall TA, Moreton RB. (1977). Electron probe X-ray microanalysis, in: Transport of Ions and Water in Animals, Gupta BL, Moreton RB, Oschmann JL, Wall BY (eds.), Academic Press, New York, London, San Francisco, 83-143.

Gupta BL, Hall TA. (1981). The X-ray microanalysis of frozen-hydrated sections in scanning electron microscopy: An evaluation. Tissue and Cell 13, 623-643.

Hackney CM, Butler RD. (1981). Distribution of calcium in the suctorian Discophrya collini: An X-ray microanalytical study. Tissue and Cell 13, 453-459.

Hausmann K, Stockem W, Wohlfarth-Bottermann KE. (1972a). Cytobiologische Studien an Trichocysten. I. Die Feinstruktur der gestreckten Spindeltrichocyste von Paramecium caudatum. Cytobiol. 5, 208-227.

Hausmann K, Stockem W, Wohlfarth-Bottermann KE. (1972b). Cytobiologische Studien and Trichocysten. II. Die Feinstruktur ruhender und gehemmter Spindeltrichocysten von Paramecium caudatum. Cytobiol. 5, 228-246.

Hausmann K, Walz B. (1979). Feinstrukturelle und mikroanalytische Untersuchungen an den Kristallen und Lithosomen des Ciliaten Euplotes vannus. Protoplasma 99, 67-77.

Heilbrunn LV. (1940). The action of calcium on muscle protoplasm. Physiol. Zool. 13, 88.

Heilbrunn LV, Wiercinski FJ. (1947). The action of various cations on muscle protoplasm. J. Cell Comp. Physiol. 29, 15.

Hodson S, Marshall J. (1972). Evidence against through-section thawing whilst cutting on the ultramicrotome. J. Microsc. 95, 459-465.

Ingram FD, Ingram MJ. (1980). Freeze-dried, plastic-embedded tissue preparation: A review, Scanning Electron Microsc. 1980; IV: 147-160.

Jamieson GA, Vanaman Jr GA, Hayes A, Blum JJ. (1980). Affinity chromatography isolation of highly purified Ca-CaM sensitive dynein ATPases from Tetrahymena cilia. Ann. NY Acad. Sci. 356, 391-392.

Janisch R. (1972). Pellicle of Paramecium caudatum as revealed by freeze-etching. J. Protozool. 19, 470-472.

Karp RD, Silcox JC, Somlyo AV. (1982). Cryoultramicrotomy: Evidence against melting and the use of low temperature cement for specimen orientation. J. Microsc. 125, 157-165.

Kitching JA. (1936). The physiology of contractile vacuoles. II. The control of body volume in marine peritricha. J. Exp. Biol. 13, 11-27.

Kitching JA. (1938). The physiology of contractile vacuoles. III. The water balance of freshwater peritricha. J. Exp. Biol. 15, 143-151.

Kitching JA. (1951). The physiology of contractile vacuoles. VII. Osmotic relations in a suctorian with special reference to the mechanism of control of vacuolar output. J. Exp. Biol. 28, 203-214.

Klein H-P, Stockem W. (1976). Preparation of biological specimens for the scanning electron microscope. Microsc. Acta 78, 388-406.

Kuhlmann S, Walz B, Hausmann K. (1983). Characterization of the parapharyngeal mass of the ciliate Homalozoon vermiculare. Protoplasma 116, 209-212.

Larsen J, Nilsson JR. (1983). Effects of nickel on the rates of endocytosis, motility, and proliferation in Tetrahymena and determination on the cell content of the metal. Protoplasma 118, 140-147.

Machemer H, Eckert R. (1973). Electrophysiological control of reversed ciliary beating in Paramecium. J. Gen. Physiol. 61, 572-587.

Machemer H, Ogura A. (1979). Ionic conductances of membranes in ciliated and deciliated Paramecium. J. Physiol. (Lond.) 296, 49-60.

Maihle N, Satir BH. (1980). Calmodulin in the ciliates Paramecium tetraurelia and Tetrahymena thermophila. Ann. NY Acad. Sci. 356, 408-409.

Mascorro JA, Ladd MW, Yates RD. (1976). Rapid infiltration of biological tissues utilizing n-hexenyl succinic anhydride (HXSA)/vinyl cyclohexene dioxide (VCD), an ultra-low viscosity embedding medium. 34th Ann. Proc. EMSA, Claitor's Publ. Div., Baton Rouge, LA, 346-347.

Masters SK, Bell SW, Ingram P, Shelburne JD. (1979). Preparative techniques for freezing and freeze-sectioning macrophages for energy dispersive X-ray microanalysis, Scanning Electron Microsc. 1979; III: 97-122.

Matt H, Bilinski M, Plattner H. (1978). Adenosinetriphosphate, calcium and temperature requirements for the final steps of exocytosis in Paramecium cells. J. Cell Sci. 32, 67-86.

Matt H, Plattner H, Reichel K, Lefort-Tran M, Beisson J. (1980). Genetic dissection of the final exocytosis steps in Paramecium tetraurelia cells: Trigger analyses. J. Cell Sci. 46, 41-60.

McDowall AW, Chang J-J, Freeman R, Lepault J, Walter CA, Dubochet J. (1983). Electron microscopy of frozen hydrated sections of vitreous ice and vitrified biological samples. J. Microsc. 131, 1-9.

Meyer R, Schmitz M, Stockem W, Haas HG. (1982). Localization of Ca^{2+} at the plasma membrane of bullfrog myocardial cells. Z. Naturforch. 37c, 1180-1190.

Moor H. (1964). Die Gefrierfixation lebender Zellen und ihre Anwendung in der Elektronenmikroskopie. Z. Naturforsch. 62, 546-580.

Moreton RB. (1981). Electron-probe X-ray microanalysis: Techniques and recent applications in biology. Biol. Rev. 56, 409-461.

Morgan AJ. (1979). Non-freezing techniques of preparing biological specimens for electron microprobe X-ray microanalysis, Scanning Electron Microsc. 1979; II: 635-648.

Mulisch M, Hausmann K, Prosi F, Back H, Walz B. (1982). Inorganic components in the organic lorica of the ciliate Eufolliculina. Naturwissenschaften 69, 448.

Müller M, Meister N, Moor H. (1980). Freezing in a propane jet and its application in freeze-fracturing. Mikroskopie 36, 129-140.

Naitoh Y, Eckert R. (1968). Electrical properties of Paramecium caudatum. All or none electrogenesis. Z. Vergl. Physiol. 61, 453-472.

Naitoh Y, Eckert R. (1969). Ionic mechanisms controlling behaviour responses of Paramecium to mechanical stimulation. Science 164, 963-965.

Nilsson JR. (1978). Retention of lead within the digestive vacuole in Tetrahymena. Protoplasma 95, 163-173.

Nilsson JR. (1979). Intracellular distribution of lead in Tetrahymena during continuous exposure to the metal. J. Cell Sci. 39, 383-396.

Nilsson JR. (1981). Effects of copper on phagocytosis in Tetrahymena. Protoplasma 109, 359-370.

Nogushi MH, Inoué H, Kubo K. (1979). Calcium-activated adenosine triphosphatase activity in pellicles of Paramecium caudatum. J. Biochem. 85, 367-373.

Oertel D, Schein SJ, Kung C. (1977). Separation of membrane currents using a Paramecium mutant. Nature, 268, 120-124.

Oertel D, Schein SJ, Kung C. (1978). A potassium conductance activated by hyperpolarization in Paramecium. J. Membr. Biol. 43, 169-185.

Ogura A, Takahashi K. (1976). Artificial deciliation causes loss of calcium-dependent response in Paramecium. Nature, 264, 170-172.

Ohnishi K, Suzuki S, Watanabe Y. (1982). Studies on calmodulin isolated from Tetrahymena cilia and its localization within the cilium. Exp. Cell Res. 137, 217-227.

Osanai N. (1961a). Effects of osmotic pressure on the contractile vacuole of the fresh-water protozoa Cothurnia spec. Zool. Mag. 70, 160-164.

Osanai N. (1961b). Effects of Na, K, Mg, and Ca ions on the contractile vacuole of fresh-water protozoa Cothurnia spec. Zool. Mag. 70, 367-372.

Oschman JL, Wall BJ. (1972). Calcium binding to intestinal membranes. J. Cell Biol. 55, 58-73.

Patterson DJ. (1980). Contractile vacuoles and the associated structures: Their organization and function. Biol. Rev. 55, 1-46.

Plattner H. (1975). Ciliary granule plaques: Membrane intercalated particle aggregates associated with Ca^{2+}-binding sites in Paramecium. J. Cell Sci. 18, 257-269.

Plattner H. (1981a). Biologische Ultrastruktur-Forschung: Wege, Ziele und Grenzen, in: Konstanzer Universitätsreden, G. Hess (ed.), Universitätsverlag Konstanz GmbH, Konstanz, 5-63.

Plattner H. (1981b). Membrane behaviour during exocytosis. Cell Biol. Int. Rpts. 5, 435-459.

Plattner H, Bachmann L. (1982). Cryofixation: A tool in biological ultrastructural research. Int. Rev. Cytol. 79, 237-304.

Plattner H, Fuchs S. (1975). X-ray microanalysis of calcium binding sites in Paramecium. Histochemistry, 45, 23-47.

Plattner H, Miller F, Bachmann L. (1973). Membrane specializations in the form of regular membrane-to-membrane attachment sites in Paramecium. A correlated freeze-etching and ultrathin-sectioning analysis. J. Cell Sci. 13, 687-719.

Plattner H, Reichel K, Matt H, Beisson J, Lefort-Tran M, Pouphile M. (1980). Genetic dissection of the final exocytosis steps in Paramecium tetraurelia cells: Cytochemical determination of Ca^{2+}-ATPase activity over performed exocytosis sites. J. Cell Sci. 46, 17-40.

Plattner H, Westphal C, Tiggemann R. (1982). Cytoskeleton-secretory vesicle interactions during the docking of secretory vesicles at the cell membrane in Paramecium tetraurelia cells. J. Cell Biol. 92, 368-377.

Ramanathan R, Saimi Y, Peterson JB, Nelson DL, Kung C. (1983). Antibodies to the ciliary membrane of Paramecium tetraurelia alter membrane excitability. J. Cell Biol. 97, 1421-1428.

Rauh JJ, Nelson DL. (1981). Calmodulin is a major component of extruded trichocysts from Paramecium tetraurelia. J. Cell Biol. 91, 860-865.

Roomans GM, Wei X, Sevéus L. (1982). Cryoultramicrotomy as a preparative method for X-ray microanalysis in pathology. Ultrastruct. Pathol. 3, 65-88.

Routledge LM. (1978). Calcium-binding proteins in the vorticellid spasmoneme. Extraction and characterization by gel electrophoresis. J. Cell Biol. 77, 358-370.

Routledge LM, Amos WB, Gupta BL, Hall TA, Weis-Fogh T. (1975). Microprobe measurements of calcium binding in the contractile spasmoneme of a Vorticellid. J. Cell Sci. 19, 195-201.

Routledge LM, Amos WB. (1977). Calcium-binding contractile proteins in protozoa, in: Calcium-binding proteins and calcium function, Wassermann RH, Corradino RA, Carafoli E, Kretsinger RH, Mc Lennan DH, Sregel FL (eds.) Elsevier, North Holland, 439-453.

Saimi Y, Kung C. (1980). A Ca-induced Na-current in Paramecium. J. Exp. Biol. 88, 305-325.

Saimi Y, Kung C. (1982). Are ions involved in the gating of calcium channels? Science, 218, 153-156.

Satir BH, Garofalo RS, Gilligan DM, Maihle NJ. (1980). Possible functions of calmodulin in protozoa. Ann. NY Acad. Sci. 356, 83-91.

Satir BH, Wissing SL. (1982). Alveolar sacs of Tetrahymena: Ultrastructural characteristics and similarities to subsurface cisterns of muscle and nerve. J. Cell Sci. 55, 13-33.

Satow Y. (1978). Internal calcium concentration and potassium permeability in Paramecium. J. Neurobiol. 9, 81-91.

Satow Y, Kung C. (1976). A "TEA-insensitive" mutant with increased potassium conductance in Paramecium aurelia. J. Exp. Biol. 65, 51-63.

Satow Y, Kung C. (1979). Voltage sensitive Ca-channels and the transient inward current in Paramecium tetraurelia. J. Exp. Biol. 78, 149-161.

Satow Y, Kung C. (1980). Ca-induced K^+-outward current in Paramecium tetraurelia. J. Exp. Biol. 88, 293-303.

Schnepf E, Hausmann K, Herth W. (1982). The osmium tetroxide-potassium ferrocyanide (OsFeCN)

staining technique for electron microscopy: A critical evaluation using ciliates, algae, mosses, and higher plants. Histochemistry, 76, 261-271.

Seveus L. (1980). Cryoultramicrotomy as a preparation method for X-ray microanalysis, Scanning Electron Microsc. 1980; IV: 161-170.

Silvester NR, Marchese-Rogana S, Johnston DN. (1982). The relative efficiency of various fluids in the rapid freezing of protozoa. J. Microsc. 128, 175-186.

Sitte H. (1982). Instrumentation for cryosectioning, in: Electron microscopy 1982, Vol 1, the 10th European congress on electron microscopy, the organizing committee (eds.), Deutsche Gesellschaft f. Elektronenmikroskopie, Frankfurt/Main, 9-18.

Somlyo AV, Shuman H, Somlyo AP. (1977). Elemental distribution in striated muscle and the effects of hypertonicity. J. Cell Biol. 74, 828-857.

Spurr AR. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31-43.

Steers E, Beisson J, Marchesi VT. (1969). A structural protein extracted from the trichocyst of Paramecium aurelia. Exp. Cell Res. 57, 392-396.

Stockem W. (1970). Die Eignung von Pioloform F für die Herstellung elektronenmikroskopischer Trägerfilme. Mikroskopie, 26, 185-189.

Stoner LC, Dunham PB. (1970). Regulation of cellular osmolarity and volume in Tetrahymena. J. Exp. Biol. 53, 391-399.

Sumner AT. (1983). X-ray microanalysis: A histochemical tool for elemental analysis. Histochem. J. 15, 501-541.

Suzuki Y, Ohnishi K, Hirabayashi T, Watanabe Y. (1982). Tetrahymena calmodulin. Characterization of an anti-tetrahymena calmodulin and the immunofluorescent localization in Tetrahymena. Exp. Cell Res. 137, 1-14.

Tsuchiya T. (1976). Electron microscopy and electron probe analysis of the Ca-binding sites in the cilia of Paramecium caudatum. Experientia, 32, 1176-1177.

Umbach JA. (1982). Changes in intracellular pH affect calcium currents in Paramecium caudatum. Proc. R. Soc. B. 216, 209-224.

Vivier E. (1974). Morphology, taxonomy and general biology of the genus Paramecium, in: Paramecium. A current survey, van Wagendonk WJ (ed.), Elsevier, North Holland, 1-89.

Walter MF, Schultz JE. (1981). Calcium receptor protein calmodulin isolated from cilia and cell of Paramecium tetraurelia. Europ. J. Cell Biol. 24, 97-100.

Weis-Fogh T, Amos WB. (1972). Evidence for a new mechanism of cell motility. Nature, 229 127-128.

Wendt-Gallitelli MF, Stöhr P., Wolburg H, Schlote W. (1980). Cryoultramicrotomy, electron probe microanalysis and STEM of myocardial tissue, Scanning Electron Microsc. 1980; II: 499-509.

Wichtermann R. (1953). The biology of Paramecium. Mc Graw Hill, NY, 93-106.

Wilkie DR. (1968). Muscle, St. Martin's Press, NY, 1-67.

Wohlfarth-Bottermann KE. (1957). Die Kontrastierung tierischer Zellen und Gewebe im Rahmen ihrer elektronenmikroskopischen Untersuchung an ultradünnen Schnitten. Naturwissenschaften, 44,287-288.

Wohlfarth-Bottermann KE. (1958). Cytologische Studien II, die Feinstruktur des Cytoplasmas von Paramecium. Protoplasma, 49, 231-247.

Zierold K. (1982a). Preparation and transfer of ultrathin frozen-hydrated and freeze-dried cryosections for microanalysis in scanning electron microscopy, Scanning Electron Microsc. 1982; III: 1205-1214.

Zierold K. (1982b). Cryopreparation of mammalian tissue for X-ray microanalysis in STEM. J. Microsc. 125, 149-156.

Zierold K. (1983). X-ray microanalysis of frozen-hydrated specimens, Scanning Electron Microsc. 1983; II: 809-826.

Zierold K. (1984). Experiences in x-ray microanalysis of freeze-dried cryosections, Scanning Electron Microsc. 1984; IV: 1867-1874.

Discussion with Reviewers

H. Plattner: You mentioned the alleged role of alveolar sacs as calcium stores. Is that just by analogy to similar structures in other cell types or did you pursue here a suggestion which was raised in the literature some time ago? In other words, do you have experimental evidence for the alleged possibility that alveolar sacs could store calcium?

Authors: As was already pointed out in the discussion of the exocytosis, the possible role of the alveolar sacs for the calcium regulation of the ciliates was discussed by Satir and Wissing (1982) without having obtained experimental evidence. In cytoplasm adjacent to the pellicle and around the ciliary base high concentrations of calcium and chlorine are detected (Fig. 2c) compared with cytoplasm more than 2 µm away from the pellicle (Fig. 2a). Alveolar sacs could so far not be clearly identified within cryosections and high calcium is measured in the region where the alveolar sacs are located. But it is improbable that these data represent measurements within alveolar sacs exclusively, since alveolar sacs occupy only a very small share of this region. Therefore, the presented data are not sufficient to prove the proposed function of the alveolar sacs as places of calcium concentration and storage.

H. Plattner: You did not find "calcium storing vacuoles". We used this name for relatively large vacuoles with branched elongations and electron dense, calcium and sulphur containing deposits (Plattner and Fuchs, 1975) after chemical fixation. Given the wide diversity of lysosomal vacuoles (and their complicated interactions with endocytotic vacuoles, etc), would it appear feasible to you to look for different types of vacuoles with possibly different elemental composition?

I wonder why you did not mention the crystal bodies, which occur particularly in older cells, and which are also reported to contain calcium. Did you by chance look after these structures, too?

Authors: Certain subcellular compartments that can be clearly identified after chemical fixation and ultrathin sectioning of plastic embedded material cannot be recognized in cryosections of natively frozen Paramecium cells. Freeze-substituted or freeze-dried and vacuum embedded cells might provide more information about other subcellular compartments. Thus, this study presents X-ray spectra of the limited number of subcellular structures, which could be clearly identified in cryosections.

H. Plattner: I liked your data on the absence of calcium from "resting" trichocysts. We had found calcium to act as a trigger for protein decondensation during exocytosis (Bilinski et al., 1981a); concomitantly we found also no calcium in the matrix of "resting" trichocysts. However, after glutaraldehyde fixation in the presence of exogenous calcium, we found calcium in trichocysts which evidently were triggered by the fixation, since membrane fusions were visible (Plattner and Fuchs, 1975). Did you by chance also analyze freshly discharged trichocysts?
Authors: Unlike chemical fixation, shock freezing of Paramecium caudatum is so fast that a discharge of trichocysts is prevented. Therefore, the elemental distribution of trichocysts just undergoing exocytosis has not been monitored so far. To detect the elemental condition for triggering of exocytosis, experiments are desirable, that lead to a synchronized discharge, but still preserve unreleased trichocysts by shock freezing.

H. Plattner: As far as the trichocyst bases are concerned, can you pinpoint the different elements (a) in the halo between the matrix and the membrane or (b) in the matrix ("basis" in your terminology) itself?
Authors: The presented data come from a typical spot analysis within the basis of a trichocyst. Presumably, the halos around the bases of trichocysts (Fig. 1c and d) are freezing artefacts.

H. Plattner: Could you tell something about the possible presence of phosphorus, which we found to keep in vitro the trichocyst matrix ("basis" in your terminology) in the condensed state at a concentration anywhere above 10^{-5} M (Bilinski et al., 1981a)? Magnesium would also be interesting with this regard (c.f. Matt H, Plattner H. (1983). Decoupling of exocytotic membrane fusion from protein discharge in Paramecium cells. Cell Biol. Int. Rep. 7, 1025-1031), since it exerts a similar effect. What would the minimal concentrations be for just being detectable in a single trichocyst in a section of the type you used?
Authors: The detection limits of the X-ray microanalysis are in the mM range for these elements. E.g. the detection limit for elements of an atomic number higher than 12 in tissue material with 80% water content is about 3 mMol/l. For Mg (Z = 12) the detection limit is about 5 mMol/l. Since the spectra were taken from freeze-dried sections, the water content of the analyzed area must be known to correct for the in vivo situation.

R.D. Allen: What is the spot of the beam you are using, i.e., what is the resolution of the X-ray microanalysis work?

D. Sigee: What experimental parameters were used in obtaining the X-ray emission spectra? In particular, what was the beam current, probe size, duration of count, and what count rates were obtained?
Authors: X-ray microanalysis was carried out with an electron beam of 1-10 nA at an accelerating voltage of 100 keV. The counting time was 100s for each spectrum. The count rates were about 1000 counts per second, however, they varied depending on the electron density of the analyzed structures. The spot size was 30-50 nm in diameter, as found by comparison with contamination spots obtained in freeze-dried warm sections.

R.D. Allen: You show no pictures of the contractile vacuole. Is your analysis only of the vacuole, only of the spongiome with the fluid segregating organelles, or of both? If the material in the contractile vacuole is from the outside, how and when would it enter the contractile vacuole?
Authors: The presented X-ray microanalysis data originate from electron dense particles within the contractile vacuole itself, formed as an artefact of freeze drying of the section. Whether the elements derive from the spongiome of the extracellular medium cannot be distinguished by this method.

R.D. Allen: Will it be possible for you to pinpoint the exact location of calcium stores in the pellicle with this technique, given the complexity of structures and their nearness to each other in the pellicle such as alveoli, the cisternae under basal bodies, basal bodies themselves, the parasomal sacs, trichocysts tips, etc.?

G.M. Roomans: The analysis of the cytoplasm close to the pellicle shows a very high Ca signal, suggesting a Ca-accumulating structure. A conventional TEM picture would certainly enliven the discussion on what this structure could be.
Authors: The optical resolution of freeze-dried cryosections is mainly limited by the following factors: a. Ice crystal diameter, b. thickness of the section, c. weak contrast of unstained biological material.

Besides the basal bodies themselves as well as the trichocyst tips, the various membranous systems close to the pellicle have not yet been distinguished. Further advances in cryosectioning as well as the different techniques such as freeze-substitution or freeze-drying and vacuum embedding, possibly in combination with dry heavy metal staining, might solve these problems.

D. Sigee: The authors state that in fixed cells no significant signals of elements inside cells were detected, except those added during chemical fixation. Would the authors not expect that insoluble cations should be detected within fixed cells, and if so, why were none found?
Authors: Compared with freeze-dried cryosections chemically fixed and embedded material generally exhibits a remarkably lower peak to background ratio. Cations in fixed cells can be detected exclusively as natural crystallites or after histochemical treatment. Therefore, it is not surprising that we do not find significant amounts

of elements, besides those added during chemical fixation.

D. Sigee: The authors frequently use the term "significant" in relation to element detectability. What criteria were used for the assessment of significance of detection of particular elements in a) individual spectra, and b) a number of spectra obtained from each cell site?
Authors: We use the term "significance" in the following meaning: a. Peaks are clearly visible above the background, b. the particular element is generally detected within the subcellular compartment. The results presented in this investigation derive from 181 spectra of cryosections of Paramecium cells. 78 analyses of those were recorded to distinguish in the elemental composition between trichocyst basis and tip.

D. Sigee: The cryosection shown in Fig. 1b has a clear nucleus containing condensed chromatin. Were any spectra obtained from this part of the cell, and if so, what were the results?
Authors: The nucleus is usually located in the center of the Paramecium cell, where ice crystal growth is increased, compared with peripheral regions analyzed in this investigation. Therefore, it is difficult to decide, whether the structures in the nucleus demonstrated in Fig. 1b represent condensed chromatin. Nuclei were not analyzed in this study.

G.M. Roomans: A point of much interest is, of course, whether the K concentration measured in the cytoplasm agrees with the mentioned value of 120-210 mOsm/l. The lack of quantitation prevents any meaningful conclusion about osmoregulation in Paramecium.
Authors: We do not know the water content of the cytoplasm of paramecium. Assuming a water content of about 80%, the potassium concentration in the cytoplasm is about 10-20 mMol/l.