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# Calcium in Ciliated Protozoa: Sources, Regulation, and Calcium-Regulated Cell Functions

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In ciliates, a variety of processes are regulated by $Ca^{2+}$, e.g., exocytosis, endocytosis, ciliary beat, cell contraction, and nuclear migration. Differential microdomain regulation may occur by activation of specific channels in different cell regions (e.g., voltage-dependent $Ca^{2+}$ channels in cilia), by local, nonpropagated activation of subplasmalemmal Ca stores (alveolar sacs), by different sensitivity thresholds, and eventually by interplay with additional second messengers (cilia). During stimulus–secretion coupling, $Ca^{2+}$ as the only known second messenger operates at ~5 $\mu M$, whereby mobilization from alveolar sacs is superimposed by "store-operated $Ca^{2+}$ influx" (SOC), to drive exocytotic and endocytotic membrane fusion. (Content discharge requires binding of extracellular $Ca^{2+}$ to some secretory proteins.) $Ca^{2+}$ homeostasis is reestablished by binding to cytosolic $Ca^{2+}$-binding proteins (e.g., calmodulin), by sequestration into mitochondria (perhaps by $Ca^{2+}$ uniporter) and into endoplasmic reticulum and alveolar sacs (with a SERCA-type pump), and by extrusion via a plasmalemmal $Ca^{2+}$ pump and a $Na^+/Ca^{2+}$ exchanger. Comparison of free vs total concentration, $[Ca^{2+}]$ vs [Ca], during activation, using time-resolved fluorochrome analysis and X-ray microanalysis, respectively, reveals that altogether activation requires a calcium flux that is orders of magnitude larger than that expected from the $[Ca^{2+}]$ actually required for local activation.
**KEY WORDS:** Calcium, $Ca^{2+}$-ATPase, $Ca^{2+}$ pump, Cilia, Ciliates, Exocytosis, Protozoa, Secretion.

## I. Introduction

Publications on $Ca^{2+}$-regulated processes in cells are legion. Not all of the aspects established for "higher" eukaryotes, particularly mammalian cells,



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have been analyzed in ciliates or any other protozoan as yet. Nevertheless, some basic aspects were detected first in ciliates. The perception in the literature (usually referring to "lower" eukaryotes), except for pathogenic protozoa, varies from incredulous stupefaction, to neglection or admiration, as reflected by the following examples. "Just over 20 years ago, Paul Brehm and the late Roger Eckert reported a curious finding in a curious organism: voltage-dependent calcium channels in the ciliate *Paramecium* are not only opened by membrane depolarization, they are also inactivated during a sustained depolarization, by the very calcium that enters through the open calcium channels (Brehm and Eckert, 1978)", as noted by Levitan (1999) in an editorial comment in *Neuron*. Even worse, one may say, Ca channels were discovered in *Paramecium* long before they became known in neurons. Formerly, during stimulated exocytosis in *Paramecium*, the "decondensation" of secretory content (which in this cell is mediated by exogenous $Ca^{2+}$) was recognized as a distinct functional step (Section III.E.2). It took about 15 years before this was recognized as a separately regulated step in mammalian cells as well, unfortunately without mentioning previous work with *Paramecium*. As another example, the kinetics of "dense core vesicle" exocytosis is remarkably slower in any "higher eukaryotic" secretory cell, as compiled by Kasai (1999) when compared to trichocysts (Plattner *et al.*, 1992, 1993), but again protozoan cells are easily overlooked. The finding that massive exocytosis stimulation in *Tetrahymena* induces the transcription of a variety of genes (Haddad and Turkewitz, 1997) was fascinating, as was how well this was perceived by Hutton (1997). Hutton does not hesitate to identify the potential importance of this finding for an understanding of some pathogenic conditions, like some forms of diabetes mellitus. Another example is the discovery of a novel type of $Ca^{2+}$/phospholipid-binding proteins, the copines (Creutz *et al.*, 1998), before neuronal copines could be identified. All of these findings are pertinent to $Ca^{2+}$-dependent cell functions of general interest, beyond the field of ciliates where they were detected.

In general, however, the overwhelming number of people working with mammalian cells sets a baseline for the much smaller number of those working with protozoa, or even with ciliates. Despite this, special aspects of $Ca^{2+}$-regulated processes in some ciliates, like synchronous exocytosis, regular arrangement of well-defined subplasmalemmal Ca stores (alveolar sacs), and formation of concise functional microdomains (cilia vs exocytosis sites, each involving different channels), may overcompensate for some of the notorious negative aspects, like those we face in the pharmacology (aberrant or mostly lacking drug effects) and molecular biology (aberrant genetic code) of ciliates. Frequently, one also has to develop new cell fractionation procedures and test the value of key enzymes that are well-established in most other systems. Our work with $Ca^{2+}$ imaging in *Parame-*

*cium* was long hampered by cell mobility, lack of uptake of acetoxymethyl ester forms (therefore requiring microinjection), vacuolar sequestration, contraction, and recoil during massive trichocyst discharge.

Nevertheless, ciliates clearly are interesting systems, because they possess some special features. A close relationship to pathogenic members of the phylum Alveolata may be another reason to pursue analysis of $Ca^{2+}$-regulated processes in ciliates. In this review, we evaluate published work and identify important aspects for future research.

## II. General Overview of $Ca^{2+}$ Regulation in Eukaryotic Cells

### A. $Ca^{2+}$ as a Second Messenger

A $[Ca^{2+}]_i$ increase controls widely different cellular processes, such as activation of some cytosolic enzymes (Schulman, 1998), gene transcription (Bito *et al.,* 1997; Hardingham *et al.,* 1997; Chawla and Bading, 1998; Deisseroth *et al.,* 1998), fertilization (Galione *et al.,* 1993a; Lee *et al.,* 1993; Marchant and Parker, 1998), cell proliferation and differentiation (Berridge, 1995; Archer *et al.,* 1998), apoptosis (Jayaraman and Marks, 1998), cell contraction (Meissner, 1994; Liu *et al.,* 1997), photoreception (Berridge, 1997), intracellular membrane fusion (Peters and Mayer, 1998), exocytosis, including neurotransmitter release (Lindau *et al.,* 1992; Neher and Zucker, 1993; Regehr and Atluri, 1995; Henkel and Almers, 1996; Huang and Neher, 1996; Berridge, 1998; Kasai, 1999), endocytosis (Artalejo *et al.,* 1995; Henkel and Almers, 1996), phagocytosis (Kruskal and Maxfield, 1987; Randriamampita *et al.,* 1991; Ohmer-Schröck *et al.,* 1995), amoeboid movement (Gilbert *et al.,* 1994), and ciliary beat regulation (Lansley and Sanderson, 1999). For reviews, see Berridge (1997), Berridge *et al.* (1998), and Verkhratsky and Toescu (1998). Evidently most, but not all of these aspects, are applicable to ciliates.

### B. Sources of $Ca^{2+}$

For the different activation mechanisms and targets, $Ca^{2+}$ may originate from different sources, i.e., from the outside medium or from internal stores, and $Ca^{2+}$ of different origins may cooperate and/or exert mutual control. Variations to this intriguing cross-talk have been reviewed by Bootman and Berridge (1995), Clapham (1995), Berridge (1997, 1998), Barritt (1999), and Mackrill (1999). $Ca^{2+}$ influx from the extracellular medium can occur via receptor-activated or voltage-activated $Ca^{2+}$ channels,

for instance. In some systems, $Ca^{2+}$ influx can trigger $Ca^{2+}$-induced $Ca^{2+}$ release (CICR[1]), as in cardiac muscle *in vivo* [but in skeletal muscle sarcoplasmic reticulum (SR) only *in vitro*]. Alternatively, $Ca^{2+}$ depletion from stores may be the first step, which may cause store-operated $Ca^{2+}$ influx (SOC) by a coupling mechanism between stores and plasmalemma to be determined in detail. Eventually, the formation of second messengers, like inositol 1,4,5-trisphosphate ($InsP_3$), may precede and then cause store depletion. After store depletion, a chemical message, also unidentified so far, may activate $Ca^{2+}$ conductance in the cell membrane ($I_{CRAC}$, for $Ca^{2+}$-release-activated current). These mechanisms preclude the occurrence of $Ca^{2+}$-release channels activated by $InsP_3$ ($IP_3$) receptors. Yet some other stores possess $Ca^{2+}$-release channels sensitive to the plant toxin ryanodine (ryanodine receptors), just like the SR in muscle. Although in muscle cells, CICR and/or physical coupling to a voltage sensor (dihydropyridine receptor) serves as coupling between SR and plasmalemma, which are tightly, structurally coupled in "triads," such coupling is little understood in nonmuscle cells, both in structural and in functional terms. Also, the search for physiological equivalents of ryanodine goes on. Some ryanodine-type receptors, including those in SR, are sensitive to still other drugs, like caffeine (Ehrlich *et al.,* 1994) and 4-chloro-*meta*-cresol (4CmC) (Zorzato *et al.*, 1993; Herrmann-Frank *et al.,* 1996; Westerblad *et al.*, 1998; Kabbara and Allen, 1999), whereas some other stores may only respond to either ryanodine or caffeine (Giannini *et al.,* 1992).

To summarize, (i) $InsP_3$- and (ii) ryanodine- or caffeine-sensitive $Ca^{2+}$ stores (although these may only respond to one activator) generally exist in higher eukaryotes, and both types of stores may coexist within one cell (Meldolesi and Pozzan, 1998b; Golovina and Blaustein, 1997). As stated, their coupling to the extracellular "compartment" may also differ widely. All of this can yield widely different $Ca^{2+}$ activation patterns even within one cell type (Cheek *et al.,* 1993; Meldolesi and Pozzan, 1998b).

Quite a novel aspect is $Ca^{2+}$ signaling, from the outside to the inside, by a $Ca^{2-}$ sensor in the cell membrane (Quinn *et al.*, 1997; Vassilev *et al.*, 1997; Adebanjo *et al.,* 1998; Chattopadhyay *et al.,* 1998). Because it also senses tri- and polyvalent cations, like $Gd^{3+}$, $La^{3+}$, and polyamines, in the outside medium, it is now called a $Ca^{2+}$/(polyvalent cation)-sensing receptor

[1] Abbreviations: ABs, antibodies; AED, aminoethyldextran; $[Ca]_{i,o}$, total (dissolved and bound) intracellular or outer calcium concentration; $[Ca^{2+}]_{i,o}$, concentration of free (dissolved) calcium in/outside cell; CaBP, $Ca^{2+}$-binding protein; CaM, calmodulin; CaM-BP, CaM-binding protein; CaN, calcineurin; CaSR, $Ca^{2+}$/(polyvalent cation) sensing receptor; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release; CLSM, confocal laser scanning microscope; 4CmC, 4-chloro-*meta*-cresol; DAG, diacyl glycerol; EDX, energy-dispersive X-ray microanalysis; EM, electron microscope; ER, endoplasmic reticulum; G-protein, GTP-binding protein; $InsP_3$, inositol 1,4,5-triphosphate; $PInsP_2$, phosphatidylinositol 4,5-bisphosphate; PK, protein kinase; PK-A or -G, cAMP- or cGMP-activated PK; SOC, store-operated $Ca^{2+}$ influx; SR, sarcoplasmic reticulum.

(CaSR). Activation of CaSRs may cause $Ca^{2+}$ release from cortical stores (frequently, but not always, of the $InsP_3$-sensitive type) and $Ca^{2+}$ influx via unspecified cation channels. CaSRs may be of paramount importance for ciliates (Klauke *et al.,* 2000), as will be discussed in Section III.C.2.

## C. Regulation of $Ca^{2+}$ Dynamics

An increase in intracellular free (ionic) $Ca^{2+}$ concentration, $[Ca^{2+}]_i$, may occur by any of the mechanisms described previously. Upon stimulation, in some cells, phosphatidyl inositol 4,5-bisphosphate ($PInsP_2$) is hydrolyzed to diacylglycerol (DAG) and $InsP_3$. Whereas DAG activates protein kinase C (PK-C), $InsP_3$ releases $Ca^{2+}$ from pools endowed with a receptor. If one could establish the occurrence of one member of this functional "chain" in ciliates, the chances of finding some functionally related messengers and effectors would be high—quite a hot issue at this time. Also quite intriguing is the activation of the stores that are sensitive to drugs, ryanodine, and/or caffeine. Equivalent endogenous activators could be identified only in some systems, such as cyclic adenosine diphosphoribose (cADPR; Galione *et al.,* 1993b), $NAADP^+$ (Lee 1999), sphingosine 1-phosphate, cyclic guanosine 3′,5′-monophosphate (cGMP) in conjunction with nitric oxide (NO), and possibly some others (Verkhratsky and Petersen, 1998; Verkhratsky and Toescu, 1998). Aspects pertinent to ciliates will be addressed in Sections III.C.3, III.C.4, and III.E.1.

After interaction with the respective target molecules, and already on the way to the target, the $[Ca^{2+}]_i$ signal is downregulated by widely different mechanisms. Because activation, e.g., of exocytosis, depends on the fourth power of local $[Ca^{2+}]_i$ (Zucker, 1993; Chow *et al.,* 1994), precise values are important, although difficult to register. During activation, a range of $[Ca^{2+}]_i^{act}$ between ~300 n*M* and ~10 $\mu M$ generally will have to be considered (Verkhratsky and Toescu, 1998), and even greater in some neuronal systems (Chow *et al.,* 1994). Because dilution, also by diffusion, occurs by a square function of distance, actual $[Ca^{2+}]_i$ decreases greatly from a $Ca^{2+}$-influx or -release channel to the actual effector molecule. This requires careful "design" of a cell (also considering pleotropic effects of $Ca^{2+}$) during evolution and during planning of experimental protocols by those interested in its revelation (Section II.D).

On the way to a target molecule, $[Ca^{2+}]$ can be downregulated by $Ca^{2+}$-binding proteins (CaBPs). In the cytosol, these are mainly of the high-affinity/low-capacity type, like calmodulin (CaM), or effector proteins, like centrin (Sections III.A and III.B). Generally only 1 out of 100 (Neher and Augustine, 1992) or up to 2000 (Mogami *et al.,* 1999) $Ca^{2+}$ ions remains in free form, whereas the rest is bound rapidly to endogenous "buffers." As

the second rapid deactivation mechanism, one now may consider uptake into mitochondria (Xu *et al.,* 1997; Nguyen *et al.,* 1998; Csordás *et al.,* 1999; Duchen, 1999) by a $Ca^{2+}$ uniporter (depending on inner membrane potential), followed by the slower release of $Ca^{2+}$ via a $Na^{+}/Ca^{2+}$ exchanger (Duchen *et al.,* 1998; Rutter *et al.,* 1998). Some nonciliate protozoa (*Trypanosoma*) possess acidic $Ca^{2+}$ stores with a $Ca^{2+}/H^{+}$ antiporter (Xiong *et al.,* 1997), which may be assumed to work fast. Much more time is required for $Ca^{2+}$ sequestration into SR or ER (endoplasmic reticulum) or equivalent stores by a universal $Ca^{2+}$ pump (SERCA-type $Ca^{2+}$ pump, from SR/ER/$Ca^{2+}$-ATPase). This organellar $Ca^{2+}$ pump is ~105–110 kDa in size, has no CaM-binding domain, and, hence, operates without CaM activation (Martonosi, 1992). A universal occurrence is the $Ca^{2+}$/CaM-activated $Ca^{2+}$-ATPase in the plasmalemma, ~130 kDa in size, and endowed with a C-terminal CaM-binding domain (Carafoli, 1991, 1994). For some types of $Ca^{2+}$ pumps, $K_m$ values would be too low to handle $[Ca^{2+}]_i$ occurring during full activation of a cell, and their $v_{max}$ values would be too small to avoid long-term activation. To fully account for short- and long-term downregulation of $[Ca^{2+}]_i$ occurring after stimulation, all of the multiple regulation steps mentioned are required.

With ciliates, mere short- or long-term adaptation to variable $[Ca^{2+}]_o$ can entail striking electrophysiological and behavioral effects, because different $Ca^{2+}$-regulated processes are connected by feedback mechanisms (Machemer-Röhnisch and Machemer, 1989; Preston and Hammond, 1998). These effects may be much more pronounced than with most other cells.

## D. Methodologies Available

During activation, spatial and temporal resolution, as well as sensitivity required for any of the different $Ca^{2+}$-dependent processes, has to be considered. Some useful $Ca^{2+}$ chelators and fluorochromes are compiled in Table I. The total calcium concentration, [Ca], primarily represents bound Ca, whereas the concentration of ionically dissolved $Ca^{2+}$, $[Ca^{2+}]$, is much smaller. At rest, $[Ca^{2+}]$ in the cytosol generally is determined to be between ~30 and 100 n*M* ($[Ca^{2+}]_i^{rest}$) and may rise by between ~10 and 100 times upon activation ($[Ca^{2+}]_i^{act}$) (Verkhratsky and Toescu, 1998). [Ca] in the cytosol is ≥1 m*M,* i.e., ~$10^4$ times above $[Ca^{2+}]$. In stores like SR or ER, [Ca] is ≤50 m*M,* whereas lumenal $[Ca^{2+}]$ may be ≤50 $\mu M$ according to estimations (Meldolesi and Pozzan, 1998a). The estimations available have fluctuated by 4 orders of magnitude over the years (Bygrave and Benedetti, 1996). High [Ca] in stores is possible due to binding to high-capacity/low-affinity CaBPs (Pozzan *et al.,* 1994), as will be explained in Sections III.C.3 and III.C.4. Actual values for $[Ca^{2+}]$ and [Ca] in ciliates are summarized in Table II.

TABLE I
Properties of Some of the $Ca^{2+}$ Buffers ("Chelators") and $Ca^{2+}$-Sensitive Fluorochromes[a]

| Compound | $K_d$ ($\mu M$) | $\tau$ ($\mu$s) | Remarks |
|---|---|---|---|
| | Chelators | | |
| EGTA (ethyleneglycol tetraacetate) | 0.07–0.40 (pH 7.0–7.4) | 200 | ~10 times reduced reaction time *in vivo* |
| BAPTA [1,2-bis(*o*-aminophenoxy) ethanetetraacetate] | 0.1 | 0.5 | |
| $Br_2$-BAPTA | 3.6 | 0.5 | |
| Fluorochromes for Two $\lambda_{excit}$/One $\lambda_{emiss}$ Analysis (Conventional) | | | |
| Fura-2 | 0.22 | 0.5 | |
| Fura red | 0.13 | 1.5 | |
| Mag-fura red | 17.00 | 1.8 | |
| Fluorochromes for One $\lambda_{excit}$/One $\lambda_{emiss}$ Analysis (Fast Confocal Analysis) | | | |
| Fluo-3 | 0.32 | 0.5 | False signals may be generated if cells become locally distorted |
| Calcium green-2 | 0.57 | 0.5 | |
| Oregon green 488 BAPTA-5N | 20.00 | 0.5 | |
| Fluorochrome Mixture for One $\lambda_{excit}$/Two $\lambda_{emiss}$ Analysis (Fast Confocal) | | | |
| Fluo-3/Fura red | 0.32/0.13 | 0.5/1.5 | No false distortion signals, but nonlinear signal due to different $K_d$ |
| | Alternatives | | |
| Rapid (sub-millisecond times) $\lambda$ shift by special instrumental setup | | | Developments in progress |
| Multiphoton excitation, FRET (fluorescence–resonance energy transfer) | | | |

[a] Abbreviations: $K_d$, binding constant; $\tau$, time constant (reaction time for equilibrium *in vitro*); $\lambda_{excit}$, wavelength used for excitation; $\lambda_{emiss}$, emitted wavelength used for recording. Note: Ciliates, like *Paramecium,* do not easily take up and/or activate acetoxymethyl esters, which is opposite most mammalian cells. Fluorochrome signals have to be calibrated under conditions of intracellular pH and ionic milieu. For references to individual compounds, see cited original work and reviews, as well as Föhr *et al.* (1993), Haugland (1996), and Molecular Probes catalogue.

[Ca] can be determined by calibrated energy-dispersive X-ray microanalysis (EDX) in the electron microscope (EM), preferably operated in the scanning transmission mode (STEM). Depending on the primary electron spot size, e.g., 63 nm, 80 kV, at a section thickness of 500 nm, spatial resolution is calculated as 72 nm (Hardt and Plattner, 1999). This allows measurements even within flat cisternae. In conjunction with quenched-

TABLE II

Values Determined for Free and Total Ca Concentrations, $[Ca^{2+}]$ and [Ca], in the Cytosol of *Paramecium* and Some Other Ciliates in Nonactivated or Activated State[a]

| Cell type | Method | $[Ca^{2+}]$ | [Ca] | Notes, references |
|---|---|---|---|---|
| | | Nonactivated State | | |
| *Paramecium* | Electrophysiology | ≤100 n*M* | | Naitoh and Kaneko (1972) |
| | | 500 n*M* | | Nakaoka *et al.* (1984) |
| | | 600 n*M* | | Machemer (1989) |
| | Fluorochrome analysis | 36–75 n*M* | | Variable, depending on strain, Klauke and Plattner (1998) |
| | X-ray microanalysis | | ≤4 m*M* | Hardt and Plattner (1999a) |
| *Didinium* | Electrophysiology | 200 n*M* | | Pernberg and Machemer (1995b) |
| *Blepharisma* | Electrophysiology | ≤100 n*M* | | Matsuoka *et al.* (1991) |
| | | Ciliary Reversal Activation | | |
| *Paramecium* | Electrophysiology | >1 μ*M* | | Naitoh and Kaneko (1972), Nakaoka *et al.* (1984) |
| | | 2 μ*M* | | Machemer (1989) |
| | X-ray microanalysis | | 25 m*M* | At ciliary basis, Hardt and Plattner (1999) |
| *Blepharisma* | | >1 μ*M* | | Matsuoka *et al.* (1991) |
| | | Exocytosis Activation | | |
| *Paramecium*, cortex | Fluorochrome | 0.4–1.2 μ*M* | | Variable, depending on strain and stimulants Klauke and Plattner (1998) |
| | X-ray microanalysis | | 7.5 m*M* | Hardt and Plattner (1999) |
| | | Induction of Cell Elongation | | |
| *Blepharisma* | Electrophysiology | ≥300 n*M* | | Matsuoka *et al.* (1991) |

[a] Other intracellular ion concentrations, as compiled by Lumpert *et al.* (1990), are as follows: $[Na^+]$ = 3–4 m*M* (*Paramecium*), 2 m*M* (*Tetrahymena*), $[K^+]$ = 20–40 m*M*. $[Mg^{2+}]$ = 1 m*M* (*Stylonychia;* DePreyer and Deitmer, 1980) or 0.4 (to 1) m*M* for *Paramecium* (Preston, 1990a, 1998). Davis *et al.* (1998) determined $[K^+]$ = 18 m*M* in *Paramecium* and a cytosolic pH = 6.6. This is lower than the value of 6.8 in the compilation by Lumpert *et al.* (1990), which also summarizes values for [ATP] = 0.9–1.25 and [GTP] = 0.35 m*M,* all for *Paramecium.* Fluorochrome measurements have been calibrated within the framework of these data (Klauke and Plattner, 1998).

flow/freeze substitution, under conditions allowing for Ca retention (Knoll *et al.*, 1993; Hardt and Plattner, 1999), a time resolution in the range of milliseconds (ms), though only beyond a dead time of the apparatus of 30 ms, can be achieved (Knoll *et al.*, 1991a). Specific element identification

can also be provided by secondary ion mass spectroscopy (SIMS), although spatial resolution is ~30 times inferior to that with EDX, i.e., in the range of several micrometers ($\mu$m) (Stelly *et al.,* 1995). The highest spatial resolution for element localization ($\leq$10 nm) is provided by electron spectroscopic imaging (ESI), by the analysis of $\leq$50-nm thin sections on the EM level (Knoll *et al.,* 1993), although quantitation is still problematic. High time resolution may be achieved with any of these methods when combined with quenched-flow/freeze substitution. To achieve absolute quantitation, we have concentrated on EDX analyses (Hardt *et al.,* 1998; Hardt and Plattner, 1999, 2000). This allows for measurements of local [Ca] in stores and cytosol and, thus, for total $Ca^{2+}$ flux measurements within the cell in subsecond time analyses. The actual times required for different $Ca^{2+}$-dependent functions in *Paramecium* are presented in Table III.

Uptake of $Ca^{2+}$ during stimulation can be followed by $^{45}Ca^{2+}$ flux measurements, e.g., during cell stimulation (Kerboeuf and Cohen, 1990). By spraying cells into a 0°C solution, using a quenched-flow apparatus (Knoll *et al.,* 1991a), $^{45}Ca^{2+}$ flux analyses on a subsecond time scale are possible (Knoll *et al.,* 1992). By adding $Ca^{2+}$ buffers immediately before/during stimulation and spraying into a liquid cryogen, extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) can be adjusted to different values (Knoll *et al.,* 1991a, 1993). Quantitative analysis, e.g., of exo-endocytosis, by different follow-up procedures may ensue to determine $[Ca^{2+}]_o$ requirements of the different steps involved (Plattner *et al.,* 1997a).

Most important, of course, is the time- and space-resolved analysis of $[Ca^{2+}]_i$ during activation. For this purpose, fluorochromes of different $Ca^{2+}$ affinities ($K_d$ values) and different reaction times (to achieve saturation) are available (Table I). Flurochromes are modified $Ca^{2+}$ chelators that, upon $Ca^{2+}$ binding and irradiation with an appropriate $\lambda_{excitation}$, emit fluorescent light with a specific $\lambda_{emission}$. Together this implies that fluorochromes can indicate only the tendency of the genuine time course and of the real $[Ca^{2+}]_i^{act}$ value at a given spot, whose size may also surpass that of the actual "strategic" site (e.g., a membrane fusion site of <10 nm). Because activation frequently entails a shape change, e.g., by cell contraction, the signal recorded must be normalized. Data frequently were obtained by double-$\lambda$ recordings, requiring ~1 s per image to allow for filter changes. The alternatives were single-$\lambda$ analyses by rapid confocal laser scanning microscopy (CLSM) with an optoacoustic beam deflection system, allowing for a complete image within 33 ms or less, whereby apparent $[Ca^{2+}]_i^{act}$ is expressed as the relation between values in the activated vs the resting state, i.e., as $f/f_0$ ratio (Erxleben *et al.,* 1997; Klauke and Plattner, 1997, 1998). To follow the extremely rapid methodological progress in this field, consult, for example, data released by Molecular Probes and the producers of CLSM instrumentation.

TABLE III

Time course of Some $Ca^{2+}$-Mediated Processes/Effects in *Paramecium*[a]

| Process/effect | Time required | $t_{1/2}$ | Notes, references |
|---|---|---|---|
| | Technical Details | | |
| Fast freezing by quenched flow | | | 30-ms dead time, ~1-ms time resolution, Knoll *et al.* (1991) |
| 2λ fluorochrome recording | | | 1–2 s required per image pair |
| 1λ fluorochrome recording | | | 30 ms per image (fast CLSM) |
| EDX, transmission-EM, 80 kV | | | X-ray/structure coordination ~73 nm (cf. thickness of alveolar sacs lumen ~98 nm); Hardt and Plattner (1999, 2000) |
| | Biological Aspects | | |
| Ciliary beat | | | |
| Normal beat frequency | ~50 ms | | Required per beat cycle, ~20 Hz |
| Exo-endocytosis of trichocysts | | | |
| Individual membrane fusion | <1 ms | | Below limits of fast freezing |
| Individual $Ca^{2+}$-activated currents | | 21 ms | Currents accompanying exocytosis; Erxleben *et al.* (1997) |
| Individual contents discharge | <1 ms | | High-speed kinematography (Unpublished observations) |
| All exocytosis events, $[Ca^{2+}]_o = 500\ \mu M$ | 80 ms | 57 ms | Fast freezing (Knoll *et al.*, 1991a; Plattner *et al.*, 1992) |
| All endocytosis events, $[Ca^{2+}]_o = 500\ \mu M$ | 80–350 ms | 126 ms | Fast freezing (Knoll *et al.*, 1991a; Plattner, *et al.*, 1992) |
| $Ca^{2+}$ mobilization from alveolar sacs | <1 s | 30–80 ms | EDX (Hardt and Plattner, 2000) |
| Recognizable cortical $Ca^{2+}$ fluorochrome signal | 30 ms | | Fast CLSM analysis (Erxleben *et al.*, 1997) |
| $^{45}Ca^{2+}$ influx, onset | >30 ms | | Knoll *et al.* (1992) |
| peak | 1 s | | Kerboeuf and Cohen (1990) |
| All exo-endocytotic events $[Ca^{2+}]_o = 10\ mM$ tested | <80 ms | | Fast freezing (Plattner *et al.*, 1997a) |

[a] $t_{1/2}$ indicates the half-time required, in comparison to methodical possibilities. Data on exocytosis refer to AED stimulation. Abbreviations: CLSM, confocal laser scanning microscopy; EDX, energy-dispersive X-ray microanalysis.

More realistic values can be obtained by the injection of $Ca^{2+}$ chelators (buffers) of different $K_d$ values (Table I), in conjunction with analysis of their effect on the respective cell function (Klingauf and Neher, 1997; Neher, 1995, 1998). An example is trichocyst exocytosis in *Paramecium,* where partial or total inhibition is observed when $[Ca^{2+}]_i$ is quenched to 1 or 10 $\mu M$, respectively (Klauke and Plattner, 1997). Thus, real cortical $[Ca^{2+}]_i^{act}$ may be ~5 $\mu M$, whereas fluorochromes show maximum values of only ~0.7 $\mu M$. Some electrophysiological methods may yield rather precise values, but they are not always applicable to all cells. Large ciliates with a rigid surface structure are just one example. For instance, *Paramecium* can be patch-clamped only with cell membrane blisters (Saimi and Ling, 1990), whereas large-scale use remains to be established for ciliates. A broad spectrum of methods, including patch-clamp analysis and local photoactivation of caged compounds, has been reviewed by Neher (1998).

Another possibility is work with permeabilized models or with cortex fragments to analyze requirements for ciliary activity (Naitoh and Kaneko, 1972; Naitoh, 1995) or trichocyst exocytosis (Vilmart-Seuwen *et al.,* 1986; Lumpert *et al.,* 1990), respectively. Due to the multiple counterregulation processes, the injection of unbuffered $Ca^{2+}$ solutions is not very useful. The same may apply to the use of otherwise highly specific drugs. At least their effect on ciliates has to be tested in every case. An invaluable methodological advantage is the occurrence of a great variety of mutants to which ciliate biologists can turn for specific problems.

*Conclusion.* A wide variety of techniques are available to account for the widely different temporal and spatial resolution of $Ca^{2+}$-dependent processes in ciliates.

## III. $Ca^{2+}$ in Ciliated Protozoa

A great variety of $Ca^{2+}$-dependent processes are surveyed in Table IV. Figure 1 shows an example of two $Ca^{2+}$-dependent reactions in *Paramecium,* i.e., expulsion of trichocysts followed by the induction of backward swimming (ciliary reversal), occurring one after the other during a "dangerous encounter" with the predatory ciliate, *Dileptus,* the attacks of which *Paramecium* can thus escape. In the scheme of Fig. 2, we outline the potential sources and theoretically possible anatomical pathways of $Ca^{2+}$, as well as the microcompartments involved in $[Ca^{2+}]_i$ regulation. As we shall discuss throughout this review, not all of these anatomically possible pathways occur in reality, and $Ca^{2+}$ may take a different pathway depending on the type of stimulation.

TABLE IV

$Ca^{2+}$-Dependent Processes in *Paramecium* and [$Ca^{2+}$] Required[a]

| Process | Evidence of $Ca^{2+}$ requirement |
|---|---|
| Stimulated exocytosis | |
| Exocytotic membrane fusion | Block by $Ca^{2+}$ buffer injection, $[Ca^{2+}]_i^{threshold} \sim 5\ \mu M$ (Klauke and Plattner, 1998) |
| Secretory contents discharge | Block by reduced $[Ca^{2+}]_o \leq 0.1–1.0\ \mu M$ (Bilinski *et al.*, 1981a; Plattner *et al.*, 1985b) |
| Endocytotic membrane fusion | Acceleration with increasing $[Ca^{2+}]_o$ (Plattner *et al.*, 1997a), i.e., by $Ca^{2+}$ influx |
| Detachment of "ghosts" | Same observation (Plattner *et al.*, 1997a) |
| Constitutive exocytosis | |
| Parasomal sacs | No data available on $Ca^{2+}$ requirement; sites considered to be competent for constitutive exo- and endocytosis (Flötenmeyer *et al.*, 1999) |
| Cytoproct | $Ca^{2+}$ requirement to be expected from annexin positioning (Knochel *et al.*, 1996) |
| Contractile vacuole | No data available |
| Lysosomal contents release | Stimulation by $Ca^{2+}$ ionophore A23187, $[Ca^{2+}]_i$ shown by quin-2 (Tiedtke *et al.*, 1988) |
| Constitutive endocytosis | |
| Parasomal sacs | No data available |
| Phagocytosis | |
| Cytostome | $Ca^{2+}$ requirement to be expected from attached F-actin (see text) and CaM (Momayezi *et al.*, 1986) |
| Internal vesicle fusions | No data available; expectation from work with yeast (Peters and Mayer, 1998) |
| Cyclosis | To be expected from cortical localization from actin–myosin (see text) |
| Contractile vacuole function | $Ca^{2+}$ requirement to be expected from CaM binding (Momayezi *et al.*, 1986) |
| Nuclear functions | No data available |
| Cell shape change | $Ca^{2+}$ requirement inferred from local contraction at sites of $[Ca^{2+}]_i^{act}$ increase (Klauke and Plattner, 1997, 1998; Erxleben *et al.*, 1997) and from cortical localization of actin–myosin and centrins (see text) |
| Cell division | No data available |
| Conjugation | $Ca^{2+}$ requirement to be expected from attachment of CaBPs (Hanyu *et al.*, 1995) |
| Cell surface pattern formation | Indirect data only (see text) |

[a] $[Ca^{2+}]_i^{act}$ indicates values for which threshold or maximal activation is observed. Note that some stimulated processes are considerably accelerated by increasing $[Ca^{2+}]_o$, even when the primary event may be $Ca^{2+}$ mobilization from internal stores. (Usually $[Ca^{2+}]_o$ = 50 to 500 $\mu$M, as specified in references). Some processes may take place without any noticeable $[Ca^{2+}]_i$ increase.

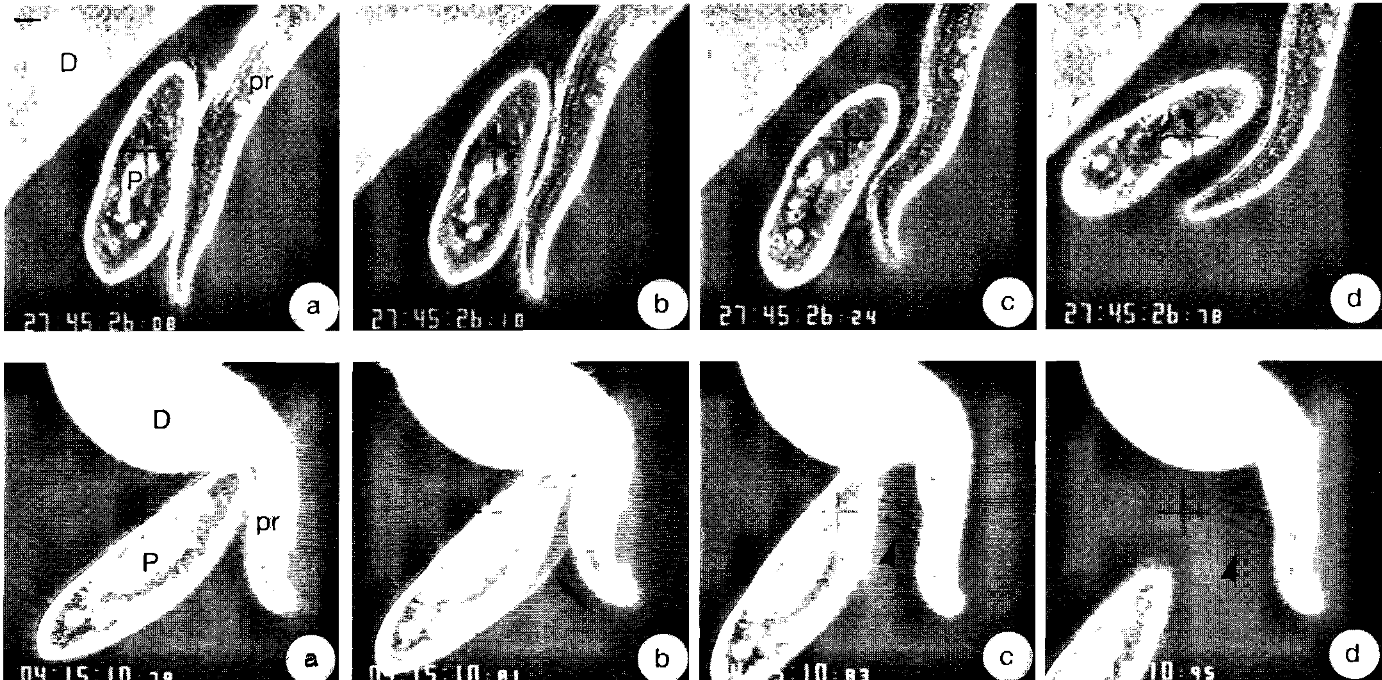



FIG. 1 $Ca^{2+}$ signaling not only is essential for basic cell functions but can be vital to escape a predator, as shown for a *Paramecium* (P) hitting the proboscis (pr) of *Dileptus* (D). In a wild-type cell (lower panels), this entails two $Ca^{2+}$-dependent processes: first, trichocyst exocytosis (arrowhead) and then ciliary reversal, with a very high escape rate. Note rapid backward movement at the arrow. A cell unable to release trichocysts (upper panels) cannot escape easily and will be ingested by the predator (fatal end not documented). Note time scale at bottom. From Knoll *et al.* (1991b).

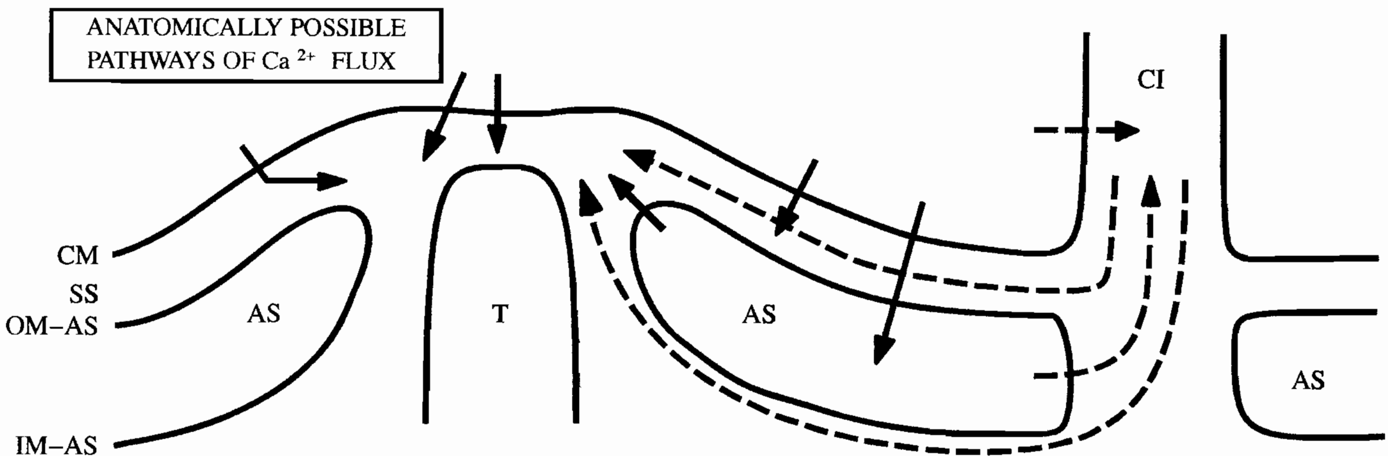



FIG. 2 Anatomically possible pathways of $Ca^{2+}$ flux in a ciliate. For realistic pathways, see text. Abbreviations: AS, alveolar sacs; CI, cilium; CM, cell membrane; IM-AS, inner alveolar sac membrane; OM-AS, outer alveolar sac membrane; SS, subplasmalemmal space; T, trichocyst (extrusome).

## A. Calmodulin (CaM) and CaM-Binding and CaM-Activated Proteins, Annexins, and Copines

Among CaBPs (Kretsinger, 1976), with an ever growing number of family members, CaM may be considered the best characterized and most widely distributed CaBP (Cheung, 1982) occuring in all eukaryotes. CaM regulates widely different cell functions (Klee *et al.*, 1986), like some protein kinases and phosphatases, as well as the plasmalemmal $Ca^{2+}$-ATPase (pump). For a summary of CaBPs in ciliates, see Table V.

TABLE V
$Ca^{2+}$-Binding Proteins (CaBPs) and $Ca^{2+}$-Activated Functions in *Paramecium* Cytoplasm and Plasmamembrane[a]

| Molecule | Characterstics | Possible functional implications, references |
|---|---|---|
| Actin–myosin system | See text | Cytoplasmic streaming (Sikora, 1981; Kersken *et al.*, 1986a). Phagosome formation (for refs., see text) |
| Annexins | $Ca^{2+}$/phospholipid BPs | Organelle docking (trichocysts, cytoproct; Knochel *et al.*, 1996) |
| Calmodulin (CaM) | Multiple functions | Multiple localization (Momayezi *et al.*, 1986) |
| $Ca^{2+}$/CaM-dependent protein phosphatase (calcineurin, CaN) | See Kissmehl *et al.* (1997b) | Exocytosis regulation (Momayezi *et al.*, 1987b) |
| $Ca^{2+}$/CaM-dependent protein kinases (CaM-kinases) | Not found | Sequence homologies in some $Ca^{2+}$-activated protein kinases (Kim *et al.*, 1998) |
| $Ca^{2+}$-activated protein kinase | Activated at ≥1 m*M* $Ca^{2+}$ | Function and physiological $Ca^{2+}$ requirement not yet established (Son *et al.*, 1993) |
| $Ca^{2+}$-inhibited protein kinase | Inhibited at ≥1 m*M* $Ca^{2+}$, casein kinase II type | Function and physiological $Ca^{2+}$ requirement not yet established (Kissmehl *et al.*, 1997a) |
| Copines | $Ca^{2+}$/phospholipid BPs | Function not yet established (Creutz *et al.*, 1998) |
| Ion channels | See Table VI | $Ca^{2+}$-conducting channels or $Ca^{2+}$ (CaM)-sensitive channels in plasmamembrane |
| Protein kinase C (PK-C) | Occurrence not established | Occurrence of signaling via trimeric G-proteins and PK-C not established |
| Synaptotagmin | $Ca^{2+}$ sensor at exocytosis sites | No equivalent known in *Paramecium* |

[a] For CaBPs in the secretory content of trichocysts and in calcium stores, see Section III.E.2.

CaM has been isolated and characterized from *Tetrahymena* (Suzuki *et al.,* 1981) and *Paramecium,* where it has been cloned by Kink *et al.* (1990). Molecular size ($M_r \sim 17$ kDa, $Ca^{2+}$-dependent $M_r$ shift), charge (p$I$ = 4.0), and $Ca^{2+}$-binding capacity (four $Ca^{2+}$-binding loops) are not very different from those of CaM from other sources. A usual $K_d$ for $Ca^{2+}$ reported in the range of 1–5 $\mu M$ (Watanabe and Nozawa, 1982) reflects conditions occurring in the cytosol during activation (Section II.D). More precisely, the $K_d$ of the different $Ca^{2+}$-binding loops of CaM depends on $[Mg^{2+}]$—an aspect of potential relevance for ciliary function (Section III.D).

Mutations in the CaM molecule from some *Paramecium* strains possess aberrant features. Because CaM regulates a variety of plasmalemmal cationic channels, including voltage-dependent $Ca^{2+}$ channels as well as $K^+$ and $Na^+$ channels in the somatic membrane (Section III.C.1), in some CaM mutants aberrant behavior could be cured by the injection of wild-type CaM (Section III.D). Another function is the assembly of exocytosis sites (Section III.E).

In *Tetrahymena* and *Paramecium,* EM localization studies and functional analyses fit together nicely. Originally, immunofluorescence revealed the enrichment of CaM on different vacuoles, ciliary basal bodies (Maihle *et al.,* 1981), as well as cilia, and the osmoregulatory system (Suzuki *et al.,* 1982). According to Watanabe and Nozawa (1982), functions attributable to CaM in *Tetrahymena* encompass phagocytosis, cell division, osmotic pressure regulation, exo-endocytosis, and ciliary reversal. They, as well as Schultz and Klumpp (1991), note as a salient biochemical feature strong activation by a $Ca^{2+}$/CaM complex of a membrane-bound form of guanylate cyclase in *Tetrahymena* and *Paramecium.* This enzyme is particularly enriched in pellicles, including significant activity contained in cilia. In fact, CaM had been detected as an inhibitor of cyclic nucleotide phosphodiesterase, and anti-CaM (*Paramecium*) antibodies (ABs) inhibit this enzyme in *Paramecium* (Klump *et al.,* 1984). With the same ABs, also in *Paramecium,* CaM was localized to cilia (membranes and peripheral microtubules), basal bodies, infraciliary lattice, trichocyst membranes (including docking sites), phagosomes, and the osmoregulatory system (Momayezi *et al.,* 1986). In *Paramecium,* guanylate cyclase was cloned and localized, by EM immunocytochemistry, to ciliary membranes and to the somatic cell membrane–alveolar sac membrane complex (Linder *et al.,* 1999). This may account for some effects of cGMP and $Ca^{2+}$ during ciliary activity (Section III.D). On the other hand, the restoration of exocytosis competence in cam$^-$ *Paramecium* strains by transfection with the wild-type CaM gene (Kerboeuf *et al.,* 1993) is compatible with the localization of CaM to exocytosis sites (Momayezi *et al.,* 1986; Plattner, 1987).

CaM-mediated functions may take place not only by the CaM-binding domains contained in some proteins but also by separate CaM-binding

proteins (CaM-BPs). Their cloning, biochemical characterization, and localization may yield clues to potential functions. In cilia from *Tetrahymena,* where >36 bands of CaM-BPs were found, a 55-kDa band dominated by far (Hirano and Watanabe, 1985). Some CaM-BPs bind to microtubules *in vitro* (Hirano-Ohnishi and Watanabe, 1988).

More details are available on *Paramecium*. In cilia, $Ca^{2+}$-dependent or -independent CaM-BPs occur (Evans and Nelson, 1989). At $[Ca^{2+}] = 0.5–1$ $\mu M$, CaM-BPs seen in [$^{125}$I]-(*Paramecium*)-CaM overlays from SDS gels primarily encompass a 63-kDa band and some additional bands of 126, 96, and 36 kDa. Sub-micromolar $[Ca^{2+}]$ is required for CaM binding to a 95-kDa protein. Upon cell fractionation, axonemes displayed CaM-BPs of 126, 96–105, and 63 kDa, but none were attributable to tubulin (55 kDa). Ciliary membranes showed 120-, 70-, and 63-kDa bands, whereas a 36-kDa band was shared by axonemes and membranes. In a figure shown for cell bodies, bands of about 120, 85, and 16–44 kDa were recognized. Generally, intensities were stronger with homologous CaM compared to bovine aliquots (Evans and Nelson, 1989).

By using [$^{125}$I]CaM of either origin for unpublished pilot studies in our lab [R. Kissmehl and M. Momayezi, unpublished data, compiled by Flötenmeyer (1999)], we detected a weak 63-kDa band in cortices only with bovine CaM, as reported by Evans and Nelson (1989) with *Paramecium*-CaM. (Unfortunately only bovine CaM was available to analyze bands of >100 kDa) We saw a strongest band of 126 kDa and weaker ones of 63 and 58 kDa, all enriched in cortex samples. A comparable CaM-binding band of ~120 kDa occurs in the cell cortex (Stelly *et al.,* 1991). Potential equivalents (on a speculative basis and allowing for some tolerance in $M_r$ values) would be the plasmalemmal $Ca^{2+}$/CaM-activated $Ca^{2+}$ pump (~130 kDa; Elwess and Van Houten, 1997) and calcineurin subunit A (Kissmehl *et al.,* 1997b), both with established CaM-binding capacity, and the nd7 gene product of 59 kDa (estimated from sequence; Skouri and Cohen, 1997) occuring at trichocyst docking sites, whose assembly depends on CaM (Kerboeuf *et al.,* 1993).

Unexpectedly, a casein kinase isolated from *Paramecium* clearly is inhibited by $[Ca^{2+}]$ (Kissmehl *et al.,* 1997a), whereas so far only the opposite was known from higher eukaryotes, as well as for some other kinases from *Paramecium* (Son *et al.,* 1993; Kim *et al.,* 1998). Because $[Ca^{2+}] \geq 1$ m$M$ is required for either inhibitory or stimulative effects with the *Paramecium* enzymes, additional factors may be required to account for physiological significance. So far, no $Ca^{2+}$/CaM-stimulated protein kinase (CaM-kinase) could be detected in ciliates. In *Paramecium* its role may be performed by a multigene family, of which two members have been cloned with cDNA-derived $M_r$ = 55.6 and 57.1 kDa, respectively (Kim *et al.,* 1998). They contain a CaM-like domain with four $Ca^{2+}$-binding sites, just as in CaM.

No conclusive information is available on any member of the PK-C family, some of which are $Ca^{2+}$-stimulated in metazoans. Injection into *Paramecium* of a synthetic CaM-binding peptide with a PK-C phosphorylation site increased periods of backward swimming (Hinrichsen and Blackshear, 1993). Yet more clear-cut evidence on the basis of molecular biology and biochemical work is required to prove or disprove more stringently the occurrence of PK-C, particularly as one frequently realizes in the literature that peptide injections may entail some effects unrelated to those of the intact protein and, hence, that results may be inconclusive.

Because in *Paramecium* conjugation is reported to depend on the relative proportion of $[K^+]_o$ vs $[Ca^{2+}]_o$ (Kitamura and Hiwatashi, 1984), one might expect $Ca^{2+}$-binding components at sites of prospective cell fusion. However, little is known on this aspect (see Section III.G).

CaM can make up $\leq 2\%$ of total cell protein (Momayezi *et al.,* 1986). A large fraction may be free and another fraction in the structure-bound form. The latter preferably will be seen in immunofluorescence and in cell fractionation studies. EM analyses using postembedding labeling should be less liable to redistribution and represent both CaM fractions. The most stringent results, however, may be provided by molecular studies. An example is transfection of *Paramecium* cam$^-$ mutants with the wild-type CaM gene, which can reestablish normally assembled exocytosis sites (with "rosettes" and "connecting material") and, consequently, exocytosis competence (Kerboeuf *et al.,* 1993), in agreement with immunolocalization of CaM in the cell surface complex, notably at trichocyst docking sites (see preceding discussion).

Some additional biochemical data fit these observations. The plasmalemmal $Ca^{2+}$-ATPase (pump) contains a CaM-binding domain (Elwess and Van Houten, 1997). The ATPase activity of axonemal dynein, notably of the 14S component, is enhanced greatly by CaM in *Tetrahymena* (Blum *et al.,* 1980). A $Ca^{2+}$/CaM-activated protein phosphatase type 2B (=calcineurin) may be involved in trichocyst exocytosis (Momayezi *et al.,* 1987b). Vacuole fusion in yeast has been shown to be a CaM-dependent process (Peters and Mayer, 1998). Finally, CaM has been localized to contractile vacuoles in *Dictyostelium* (Zhu *et al.,* 1993), where a plasmalemmal-type CaM-activated $Ca^{2+}$-ATPase occurs, as demonstrated in molecular genetic studies (Moniakis *et al.,* 1999).

Concomitantly, several "anti-CaM" drugs have been shown to impair a variety of cell functions in *Tetrahymena* (Watanabe and Nozawa, 1982) and *Paramecium.* [Note that these drugs are exceptional because they are equally reactive in ciliates and in mammals, in contrast to many other drugs (see Section III.D)]. This includes inhibition, by trifluoperazine (TFP) or W7, of normal (Otter *et al.,* 1984) or accelerated foreward swimming induced by chemical hyperpolarization (Yano *et al.,* 1996), as well as inhibition

of $Ca^{2+}$-activated currents accompanying exocytosis (Erxleben and Plattner, 1994). Quite remarkably, other members of the group Alveolata, like *Plasmodium* (Matsumoto *et al.*, 1987) or *Toxoplasma* (Pezzella *et al.*, 1997), also require CaM for exocytotic release of components of the "apicomplex," which parallels host cell infection.

Another type of $Ca^{2+}$-sensitive proteins, annexins, presumably also occur in ciliates, as we conclude from AB-binding studies (Knochel *et al.*, 1996). The role of these widespread $Ca^{2+}$-dependent phospholipid-binding proteins (Raynal and Pollard, 1994) is far from established in any system. In *Paramecium,* two types, each selectively recognizing ABs against common sequence (annexin) peptides, can be localized to trichocyst docking sites and to the cytoproct, respectively (Knochel *et al.*, 1996). A novel group of $Ca^{2+}$-dependent phospholipid-binding proteins, the copines, was detected in *Paramecium* (Creutz *et al.*, 1998). The $M_r$ of copines differs from that of annexins, and they possess C2 domains of about 120 amino acids, indicative of likely protein–membrane interaction, just as in protein kinase C (PK-C), phospholipase C, the $Ca^{2+}$ sensor synaptotagmin, and some other proteins occurring in the exocytotic docking/fusion complex. Any functional role or the precise localization of copines is not yet known.

*Conclusions.* CaM by far is the best analyzed CaBP in ciliates where it may exert multiple functions, including the activation of some pumps and ion channels. CaM localizes to exocytosis sites, for whose assembly it is mandatory. For ciliary activity, a potential key role of CaM is discussed in more detail in Section III.D. Some other CaBPs are poorly understood, whereas possible functions of contractile CaBPs will be discussed below.

## B. $Ca^{2+}$-Sensitive Contractile Cytoskeletal Elements

At least three different $Ca^{2+}$-dependent contractile filament systems are known from ciliates, i.e., those based on actin–myosin, spasmin, and centrin, respectively. In principle, $Ca^{2+}$-dependent cell contraction or shape change could be mediated by any of the three systems, but the third one is the focus of most interest. However, some other data also provide clues to important actomyosin function.

### 1. Actin and Myosin

The occurrence or localization of actin in ciliates has long been controversial. First, Tiggemann and Plattner (1981) showed cortical labeling in *Paramecium* by immuno- and affinity-fluorescence using DNaseI and heavy meromyosin (HMM) as rather specific tags. Then, on the EM level, Méténier (1984) decorated some cortical filaments with HMM, also in *Parame-*

*cium*. Because some groups, using HMM or S1 fragments as labels, obtained some divergent results (Cohen *et al.,* 1984), we injected *Paramecium* with rhodaminylated phalloidin (Kersken *et al.,* 1986a,b), an F-actin-stabilizing (and, thus, immobilizing) poison of the highest specificity known, with no side-effects being reported. Originally recognizable cortical labels gradually disappeared and were replaced by newly formed, heavily labeled transcellular filament bundles, which in the EM showed characteristic 6-nm filaments. If not for massive cell contraction, cortical F-actin may generate protoplasmic streaming, i.e., cyclosis, because this is sensitive to cytochalasin B (Sikora, 1981) and phalloidin (Kersken *et al.,* 1986a). This may account for the abolition of trichocyst docking by the actin-reacting agents, cytochalasin B (Beisson and Rossignol, 1975) or phalloidin (Kersken *et al.,* 1986a). The occurrence of actin in the cell cortex of *Paramecium* has been confirmed by Cohen and Beisson (1988).

Immunocytochemically, F-actin, together with the F-actin-binding protein $\alpha$-actinin, previously was localized to the cytopharyngeal basket of *Pseudomicrothorax* (Hauser *et al.,* 1980). In *Paramecium,* HMM and S1 labels were concentrated around the cytoproct and on nascent food vacuoles (Cohen *et al.,* 1984), as was injected rhodamin–phalloidin (Kersken *et al.,* 1986a). Concomittantly, cytochalasin B blocks food vacuole formation (Allen and Fok, 1985; Fok *et al.,* 1985; Fok and Allen, 1988; Allen *et al.,* 1995) and defecation (Allen and Fok, 1985). (The generally more specific form, cytochalasin D, was not easily available at that time.) Very intriguing were the absence of phalloidin labeling from the cleavage furrow (Kersken *et al.,* 1986a) and the lack of cytochalasin B effects on cytokinesis (Cohen *et al.,* 1984). Only later work with ABs against a N-terminal peptide, derived from homologous actin, allowed labeling of the cleavage furrow in *Tetrahymena* (Hirono *et al.,* 1987b).

No less intriguing was the occurrence of ciliary basal body labeling in *Paramecium* (Tiggemann and Plattner, 1981) and *Tetrahymena* (Hoey and Gavin, 1992). Without any detailed comment, this also shows up in work with *Pseudomicrothorax* (Hauser *et al.,* 1980) and *Tetrahymena* (Hirono *et al.,* 1987b). As will be discussed later, this now coincides with myosin localization.

Cloning of the actin gene in *Tetrahymena* (Cupples and Pearlman, 1986; Hirono *et al.,* 1987a) and *Paramecium* (Diáz-Ramos *et al.,* 1998) to a certain extent contributed to clarification of some aspects. The gene product encoded by the *Tetrahymena* actin gene analyzed is reported to possess some aberrant features, such as no DNaseI binding (Hirono *et al.,* 1989), whereas Fahrni (1992) could isolate actin of a typical 43 kDa from *Climacostomum* by using DNaseI affinity chromatography.

Considering $Ca^{2+}$-activated actomyosin functions, cloning of myosin in *Tetrahymena* (Kanzawa *et al.,* 1996; Garcés, 1998) was of high interest,

particularly because a double-headed, double-tailed form, similar to the myosin II type "classical" myosin, was found (Garcés *et al.,* 1995; Kanzawa *et al.,* 1996). Data on localization are scarce. So far, myosin has been localized to the "basal body–cage complex" (Garcés *et al.,* 1995), as F-actin had been previously (see earlier discussion).

*Conclusions.* We may expect a role for $Ca^{2+}$-stimulated actomyosin contraction in ciliates. This may account not only for food vacuole formation but also probably for the positioning of some organelles. Thereby cytoplasmic streaming (cyclosis) is involved at least indirectly, e.g., when "free" secretory organelles move around before they are "caught" by a microtubule for saltatory docking. Cytoplasmic streaming commonly is attributed to cortical actomyosin in a variety of cells. Localization of actin and myosin to ciliary basal bodies may entail a novel regulatory aspect (as unexpected as the discovery of kinesin in cilia). Because the sensitivity of ciliates to otherwise established drugs, like cytochalasins, may deviate greatly from that of mammalian cells (Zackroff and Hufnagel, 1998), it appears advisable in future work to take advantage of gene cloning and the production of more specific ABs.

### 2. Centrin- and Spasmin-Based Contractility

The other contractile system is based on the $Ca^{2+}$-mediated conformational change of a CaBP, i.e., intramolecular refolding upon $Ca^{2+}$ binding (Moriyama *et al.,* 1999). Originally, two isoforms of proteins, 18 and 20 kDa, both with quite similar amino acid sequences, were extracted from the contractile spasmoneme of the vorticellid *Zoothamnium* (Amos *et al.,* 1975). In *Vorticella,* whose stalk contracts in response to $Ca^{2+}$-store-mobilizing agents, like caffeine (Katoh and Naitoh, 1994), contraction is accompained by an all-or-none rise in $[Ca^{2+}]_i$ even in the absence of $[Ca^{2+}]_o$ (Katoh and Kikuyama, 1997). The spasmoneme of *Vorticella* also contains two "spasmins" of this size (Ohiai *et al.,* 1988). Nonstalked ciliates contain similar "spasmin-like" proteins, e.g., of 20, 18, 16, and 15 kDa in *Carchesium,* of 18, 17, and 16 kDa in *Epistylis* (Ohiai *et al.,* 1988), or of ~22 kDa in *Isotricha* and *Polyplastron* (Viguès and Grolière, 1985). On SDS–polyacrylamide gels these proteins show a $Ca^{2+}$-dependent mobility shift and, thus, can be assigned to EF-type CaBPs. Similar proteins extracted from the infraciliary lattice in *Paramecium* were of 23–24 kDa, and they cross-reacted with ABs against a 22-kDa protein from the ectoplasm boundary in *Isotricha* (Garreau De Loubresse *et al.,* 1991). These proteins form a large part of the "microfilament" system, not only in the cell cortex of *Paramecium* (Garreau De Loubresse *et al.,* 1991) but also at the cytopharynx, e.g., in *Nassula* and *Furgasonia* (Viguès *et al.,* 1999). *Tetrahymena* also possesses EF-type CaBPs of comparable size and cortical localization (Hanyu *et al.,* 1995, 1996). One

protein of 23kDa, as derived from cDNA (Takemasa *et al.,* 1990), would be within the size range of centrins, though it is considered different from centrin (Hanyu *et al.,* 1996).

Cloning and immunolocalization work with *Paramecium* (Garreau De Loubresse *et al.,* 1988; Madeddu *et al.,* 1996; Klotz *et al.,* 1997) allowed more detailed characterization of members of the "centrin" family, so called because they are universal components of centrosomes. Even though they may form the major component of the filament system of the cytopharynx (Viguès *et al.,* 1999) and the contractile infraciliary lattice (Klotz *et al.,* 1997), this does not preclude the occurrence of actomyosin in the cytopharynx region and cell cortex (see preceding discussion). Colocalization of F-actin and centrin-type filaments has not yet been performed, not even on the light microscope level.

By cloning and expression of cDNA encoding contractile CaBPs from *Vorticella,* Maciejeweski *et al.* (1999) demonstrated that two types of contractile CaBPs exist, one of the spasmin type (with two $Ca^{2+}$-binding domains) and one of the centrin type (with four $Ca^{2+}$-binding domains). The latter is typical of centrins (Moncrief *et al.,* 1990).

The $M_r$ of ~10 kDa of another CaBP (Kobayashi *et al.,* 1988) superficially reminds one of the long-known vertebrate CaBP, S100. Unfortunately no data are available on any other established CaBPs, like parvalbumin or troponins, in any ciliate. Analyses along these lines would be important considering the rapid reestablishment of $[Ca^{2+}]_i$ homeostasis, e.g., after secretion stimulation in *Paramecium* cells (Klauke and Plattner, 1997), which when calculated can hardly be mediated solely by $Ca^{2+}$ extrusion and sequestration (Plattner *et al.,* 1997a).

*Conclusion.* Centrins are widely distributed CaBPs in ciliates. They may serve not only for pattern formation and/or local contraction but also as a sink to bind a great deal of the $Ca^{2+}$ mobilized upon exocytosis stimulation (see Sections II.C and III.E).

## C. Potential Sources of $Ca^{2+}$

### 1. $Ca^{2+}$-Carrying and $Ca^{2+}$-Sensitive Ion Channels in the Cell Membrane

Which cell membrane channels (reviewed in Table VI) may be involved in the activation of different cell functions in ciliates, i.e., ciliary beat reversal and stimulated exocytosis?

Among ciliates, cation channels, including $Ca^{2+}$-carrying channels, are best analyzed in *Paramecium,* as reviewed by Kung and Saimi (1985) or Machemer (1988, 1989). Several channels are activated by $Ca^{2+}$, as reviewed

TABLE VI
$Ca^{2+}$ Pumps, $Na^+/Ca^{2+}$ Exchanger, $Ca^{2+}$ Channels, and Ion Channels Regulated by $Ca^{2+}$ or $Ca^{2+}$/CaM in *Paramecium*

| Molecule/function | Localization, characteristics | References |
|---|---|---|
| $Ca^{2+}$ pump | Plasmalemmal, 130 kDa, CaM-activated, absent from ciliary membrane | Wright *et al.* (1993), Elwess and Van Houten (1997), Van Houten (1998) |
| SERCA-type $Ca^{2+}$ pump | Alveolar sacs, 106 kDa, no CaM-binding domain; to be expected, in less copies, also in ER | Hauser *et al.* (1998), Plattner *et al.* (1999) |
| $Na^+/Ca^{2+}$ exchanger | To be expected in *Paramecium* [in cell membrane, trichocyst, or mitochondrial membrane? (see text)], occurring in cell membrane of *Euplotes* | Burlando *et al.,* (1999) |
| $Ca^{2+}$ uniporter | To be expected in mitochondria | Hardt and Plattner (2000) |
| $Ca^{2+}$ channels | | |
| Voltage-dependent | Cilia | Brehm and Eckert (1978), Eckert and Brehm (1979) |
| | Somatic plasma membrane? | Schultz *et al.* (1997) |
| Hyperpolarization-sensitive | Somatic plasma membrane? | Preston *et al.* (1992a,b) |
| Mechanosensitive | Enriched in anterior plasma membrane regions | Machemer (1986, 1988) |
| $Na^+$ channels | $Ca^{2+}$/CaM-activated, anteriorly enriched | Preston (1990b) |
| | Carries $Ca^{2+}$ (increases with decreasing $[Na^+]_o$) | |
| $K^+$ channels | $Ca^{2+}$/CaM-activated, posteriorly enriched | Preston (1990b) |
| $Mg^+$ channels | $Ca^{2+}$-activated | Preston (1990a) |

by Preston (1990a,b) or Preston and Saimi (1990), whereas a voltage-dependent $Ca^{2+}$ channel, first detected in *Paramecium,* is inhibited by increased $[Ca^{2+}]_i$ following activation (Brehm and Eckert, 1978; Eckert and Brehm, 1979). These channels have been localized to ciliary membranes because deciliation causes the loss of a $Ca^{2-}$ current induced by depolarization (electrically or by $[K^+]_o = 20$ m$M$) that normally accompanies a typical behavioral response, i.e., ciliary reversal (Eckert *et al.,* 1976; Ogura and Takahashi, 1976; Dunlap, 1977; Machemer and Ogura, 1979; Ogura and Machemer, 1980; Machemer 1986, 1988, 1989). This selective localization

has been challenged, because agents used for deciliation per se can inactivate voltage-dependent $Ca^{2+}$ channels (Schultz *et al.*, 1997). The state of the discussion is summarized in Fig. 3. Another complication is that voltage-dependent $Ca^{2+}$ channels are not the only type of $Ca^{2+}$ channels, because mechanosensitive (Machemer and Ogura, 1979; Ogura and Machemer, 1980) as well as hyperpolarization-sensitive $Ca^{2+}$ channels can be found on the somatic membrane (Preston *et al.*, 1992a,b). In addition, $Ca^{2+}$ can also be carried by anteriorly enriched $Na^+$ channels, particularly at low $[Na^+]_o$ (Saimi 1986). Some unexpected $Mg^{2+}$ channels (Preston, 1990a) and posteriorly enriched $K^+$ channels (Satow and Kung, 1980b; Machemer, 1988; Preston, 1990b) also are activated by $[Ca^{2+}]_i$. As will be discussed later, a selection of these channels, including $Na^+$ and $K^+$ channels, may be activated during AED-stimulated exocytosis (Erxleben and Plattner, 1994) due to their $Ca^{2+}$ sensitivity and, thus, can be used diagnostically to monitor subplasmalemmal $[Ca^{2+}]_i$ increase. However, $Ca^{2+}$ channels actually responsible for $Ca^{2+}$ influx, superimposing $Ca^{2+}$ mobilization from alveolar sacs, during stimulated exocytosis have not been identified in detail as yet. They seem to be unspecific cation channels (Klauke *et al.*, 2000).

Altogether it is well-established that ciliary reversal normally operates by $[Ca^{2+}]_o$ influx via voltage-dependent $Ca^{2+}$ channels in the ciliary membrane, even if similar channels would additionally occur in the somatic membrane. Most relevant evidence comes from work with *Paramecium* pawn mutants, which are devoid of any functional ciliary $Ca^{2+}$ channels and therefore cannot move backward (Satow and Kung, 1980a). $Ca^{2+}$-carrying depolarization-induced currents occur strictly in parallel to ciliary reversal, and both are absent from sufficiently "tight" pawn mutants, like d4-500r (Haga *et al.,* 1982). During AED-stimulated exocytosis, a cortical $[Ca^{2+}]_i$ increase can cause short ciliary reversal in wild-type as in pawn cells, probably by spillover into cilia (Plattner *et al.,* 1984; Erxleben and Plattner, 1994). In fact, both phenomena may occur consecutively when a predator is defeated, first by rapid local trichocyst exocytosis and then by ciliary reversal [Knoll *et al.* (1991b), Fig. 1].

From a more general point of view, we face the problem of how a cell can independently regulate two different $Ca^{2+}$-regulated processes, both occurring in its cortex. One may consider the selective arrangement of sites of $Ca^{2+}$ mobilization and/or influx relative to the different target structures (trichocysts and cilia being spaced in *Paramecium* at ~1–2 $\mu$m intervals) by different local levels of $[Ca^{2+}]_i^{act}$ achieved with the different stimuli, in addition to the different $Ca^{2+}$ sensitivity of targets and/or the involvement of some additional second messengers, i.e., cAMP and cGMP, in cilia (Section III.D).

A variety of mutant strains have been isolated over the years, primarily on the basis of behavioral observations. These include not only "pawn"

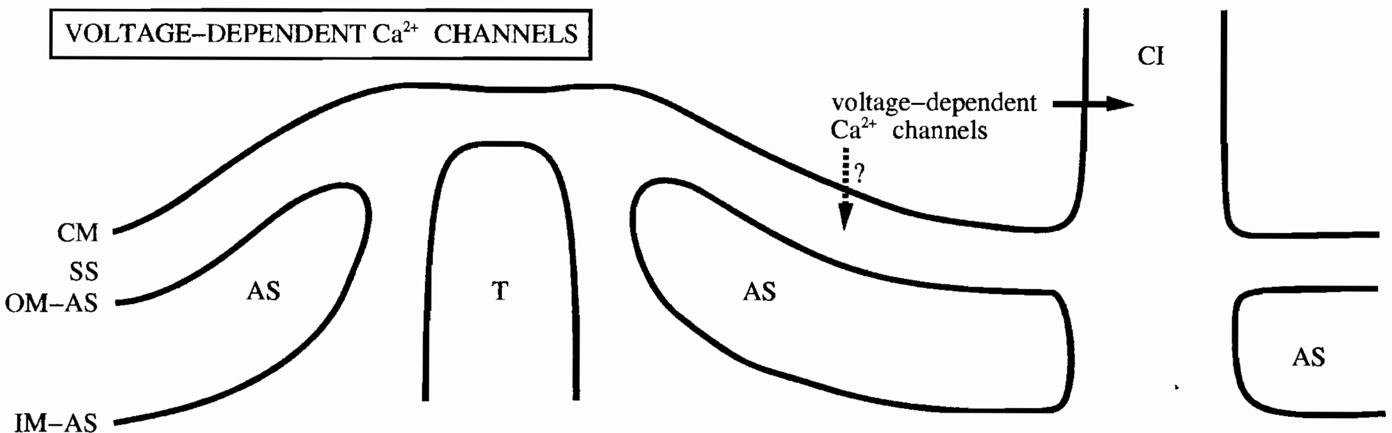



FIG. 3 Voltage-dependent $Ca^{2+}$-influx channels have been established to occur in ciliary membranes, but are postulated by some authors also to occur in somatic cell membrane regions. For abbreviations, see Fig. 2.

mutants, which cannot swim backward upon a depolarization stimulus, but also mutants designated "shy" or "pantophobiac." In pantophobiac cells, several amino acid exchanges and posttranslational modifications (lysine methylation) were found to parallel the behavioral defect (tendency for backward swimming at low $[Ca^{2+}]_o$) (Schaefer *et al.,* 1987a,b). Because widely different channels may be affected, a surprising multitude of mutants can be cured by the injection of wild-type CaM (Kung and Saimi 1985; Preston *et al.,* 1991; Kung *et al.,* 1992; Saimi and Kung, 1994; Saimi and Ling, 1995). Alternatively, anti-CaM drugs can affect behavior on the basis of the effects on a variety of currents, which are activated more or less by a $Ca^{2+}$/CaM complex. For instance, W7, i.e., *N*-(6-aminohexyl)-5-chloro-l-naphthalenesulfonamide, chlorpromazine, R24571 (calmidazolium), or TFP can block voltage-dependent $Ca^{2+}$ channels in cilia and, hence, block backward swimming (Haga *et al.,* 1982; Ehrlich *et al.,* 1988). Inhibitory effects have been recognized with somatic $Na^+$ currents and $K^+$ currents, as we could confirm (Erxleben and Plattner, 1994). Channel inhibition also is achieved by injection of an antisense oligonucleotide complex complementary to CaM, which depresses ciliary reversal (Hinrichsen *et al.,* 1992). In addition, non-CaM-type, low-$M_r$ proteins are assumed to be involved in the activation of the ciliary voltage-dependent $Ca^{2+}$ channel, as shown by microinjection into pawn cells that can thus be cured (Haga *et al.,* 1984).

The situation is much less clear for ion channels involved in the activation of exocytosis, whereby $Ca^{2+}$ comes in part from alveolar sacs and in part from the outside (Section III.E.1). The latter component may pass indirectly via alveolar sacs when they are refilled as they release $Ca^{2+}$ into the cytosol (Erxleben and Plattner, 1994; Erxleben *et al.,* 1997; Hardt and Plattner, 1999, 2000). With regard to exocytosis, we know only that ciliary voltage-dependent $Ca^{2+}$ channels are unlikely to be involved (see discussion to follow). Which plasmalemmal $Ca^{2+}$-carrying channels may be involved in AED-stimulated trichocyst exocytosis?

Some impermeable polyamines activate some of the currents under consideration, whereas other amines are inhibitory, so that questionable penetration properties have to be kept in mind, e.g., with the frequently used aminoglycoside, neomycin. Impermeable polyamines, like AED (40 kDa, 1 $N^+H_3$ group/kDa), or also some aliphatic polyamines with the appropriate spacing of amino groups (Plattner *et al.,* 1985b) induce synchronous exocytosis at $\geq 10^{-6}$ *M*. Lysozyme, highly positively charged at pH = 7 due to its p*I* ~ 10, can also induce exocytosis, though only at $10^3$ times higher concentration (Hennessey *et al.,* 1995). At low concentrations, both lysozyme or AED also produce ciliary reversal, recognizable by a depolarizing action potential. (Its designation as a "receptor potential" may be misleading because this term could be understood to imply binding to a receptor protein in the cell membrane.) Although the occurrence of a lysozyme

receptor has been assumed (Kuruvilla *et al.,* 1997), it is difficult to imagine that this would bind all of the different cationic trigger compounds (including cationic dyes used to stimulate mucocyst release in *Tetrahymena*) or that as many receptors would exist. This aspect, including the possibility of the involvement of a $Ca^{2+}$/(polycation)-sensing receptor, is addressed in more detail in Section III.C.2.

One potential explanation for the stimulation effect of polyamines comes from their effects on a variety of other cell types, involving unspecific activation of different ion channels. This includes $[Ca^{2+}]_o$-dependent exocytosis stimulation not only in mast cells, by "compound 48/80" (Lagunoff *et al.,* 1983), but in a variety of cell types (Williams, 1997a), including neuronal and pituitary cells (Shorte and Schofield, 1996), some of which also possess cortical Ca stores (Tse *et al.,* 1997) though in much less well-defined form. Polycationized latex beads stimulate not only trichocyst exocytosis in *Paramecium* (unpublished observations) but also differentiation of cultured muscle cells, caused by a $[Ca^{2+}]_i$ increase (Zhu and Peng, 1988). Work with reconstituted ion channels has shown that polyamines alter ion conductivity by interaction from the inside or from the outside (Weiger *et al.,* 1998).

Among polyamines, primarily endogenous low-$M_r$ compounds like spermi(di)ne or cadaverine are considered, yet large ones evidently can exert similar effects. Low-$M_r$-type polyamines may act from the inside or from the outside, because they are released by different cells and they are known to be synthesized in ciliates, e.g., in *Tetrahymena* (Yao *et al.,* 1984). Nevertheless, it is unlikely that AED mimics any effect of endogenously produced polyamines, because these low-$M_r$ species are not very efficient stimulators of trichocyst exocytosis in *Paramecium* (Plattner *et al.,* 1985b). Somehow a cell surface component of the predatory cells, to be warded off by trichocyst exocytosis, may contain a component similar to AED, whose local application can perfectly mimic the effects of a predator's attack (Knoll *et al.,* 1991b). As we assume, AED may cause the mobilization of $Ca^{2+}$ from alveolar sacs in a primary step and $Ca^{2+}$ influx through channels still to be identified in a superimposed second step. Yet which channel may be relevant for the second step?

An increase in $[Ca^{2+}]_i$ at the cytosolic side cell membrane upon AED stimulation has been documented (Knoll *et al.,* 1993) by the ESI method (see Section II.D) and measured indirectly by the registration of anteriorly enriched depolarizing $Na^+$ inward currents and posteriorly enriched hyperpolarizing $K^+$ outward currents (Erxleben and Plattner, 1994). Both of these currents are activated by $Ca^{2+}$/CaM (see preceding discussion), and they can be abolished by injecting EGTA or by adding permeable W7 or TFP (Erxleben and Plattner, 1994). The fact that only EGTA injection, but not always application of anti-CaM drugs, blocks AED-elicited exo-

cytosis could imply that anti-CaM drugs would be required at concentrations above those actually used, if one assumes that CaM plays any role in exocytosis regulation altogether (Momayezi *et al.,* 1987b). We definitely can exclude mechanosensitive $Ca^{2+}$ channels, and we largely exclude hyperpolarization-sensitive $Ca^{2+}$ channels (Erxleben and Plattner, 1994). We do not assume the implication of such channels in mediating $Ca^{2+}$ influx into the cytosol during AED stimulation (Cohen and Kerboeuf, 1993; Kerboeuf and Cohen, 1990, 1996), particularly because electrical (de- or) hyperpolarization does not cause exocytosis (Erxleben and Plattner, 1994). Depolarization also does not trigger extrusome release in *Didinium* (Hara *et al.,* 1985). At this time it admittedly is difficult to imagine how some other established CaM-sensitive channels would be involved, if our asumption is correct that $Ca^{2+}$ influx would more or less directly serve refilling alveolar sacs during their $Ca^{2+}$ depletion (Erxleben and Plattner, 1994; Erxleben *et al.,* 1997; Klauke *et al.,* 2000).

A variety of papers deal with the effects of CaM on ion channel conductivity and swimming behavior in *Paramecium.* The authors analyzed the effects of mutations, injection of wild-type CaM, CaM peptides, or antisense oligonucleotides, transfection with the wild-type gene, and CaM-antagonistic drugs (Hennessey and Kung, 1984; Hinrichsen *et al.,* 1986, 1990, 1992; Kanabrocki *et al.,* 1991). For reviews, including some pioneering work on genetic function repair, see Kung and Saimi (1985), Preston (1990b), Preston *et al.* (1991), Kung *et al.* (1992), Saimi and Kung (1994), or Hinrichsen *et al.* (1995).

Finally, we have to take into account that, for any more precise pharmacological and biochemical analyses, unfortunately there are not always sufficiently specific drugs available. Frequently, their specificity may be questionable or their $K_d$ values may vary widely. This is, in fact, a more general problem we face in work with ciliates. For instance, among a variety of channel blockers available for higher eukaryotes, only $TEA^+$ (tetraethylamine) is established as a (unfortunately not highly specific) blocker of $K^+$ channels in different ciliates (Krüppel *et al.,* 1991; Preston *et al.,* 1992a). No highly selective blockers are available for $Na^+$ channels or for most of the $Ca^{2+}$ channels (Eckert *et al.,* 1976; Kung and Saimi, 1985), except when some of the biogenic neuro-reactive $Ca^{2+}$ channel blockers are used at somewhat high concentrations, e.g., in *Euplotes* (Krüppel and Wissing, 1996). An exception is amiloride, which in millimolar concentrations inhibits hyperpolarization-sensitive $Ca^{2+}$ channels in *Paramecium* (Preston *et al.,* 1992a), but which, in our hands, does not inhibit exocytosis. In sum, one of the few details concerning channel activities during exocytosis that is sufficiently clear is the irrelevance of ciliary $Ca^{2+}$ channels. In fact, pawn mutants or deciliated wild-type cells respond equally well to AED by trichocyst release (Plattner *et al.,* 1984).

To summarize, we have to explain several observations pertinent to AED-induced trichocyst exocytosis in *Paramecium*. (i) We have evidence, obtained by widely different methods, that $Ca^{2+}$ mobilization from alveolar sacs is a first step that is rapidly superimposed by store refilling as a second step, while internal release occurs (Erxleben and Plattner, 1994; Erxleben *et al.,* 1997a; Plattner *et al.,* 1997a; Hardt and Plattner, 1999, 2000). (ii) Whereas this situation resembles that in skeletal muscle SR, in freeze–fracture replicas from cell and alveolar sac membranes, no tetrameric particle aggregates corresponding to a voltage sensor and/or a $Ca^{2+}$-release channel typical of sarcolemma/SR connections (Franzini-Armstrong and Jorgensen, 1994; Franzini-Armstrong and Protasi, 1997) could be recognized (Plattner *et al.,* 1999). (iii) A $Ca^{2+}$-induced $Ca^{2+}$-release (CICR) mechanism, as occurs with the SR *in vitro* (Meissner, 1994), could not be established with isolated alveolar sacs (Länge *et al.,* 1995) or reconstituted channels from surface membranes (Zhou *et al.,* 1995). Therefore, an important difference between alveolar sacs and SR must exist, including their coupling to any plasmalemmal $Ca^{2+}$-carrying channels, although otherwise both of these structures share important features in common (Länge *et al.,* 1995). The type of coupling of $Ca^{2+}$ influx to store depletion described herein would be of the SOC type (see Section II).

In this context, the effects of the low-$M_r$ aminoglycoside, neomycin, as described for *Paramecium* as well as for higher eukaryotic cells, remain to be explained. In mammalian cells, neomycin can block signal transduction via $PInsP_2$ turnover, when applied inside a cell or when allowed to penetrate (Malgaroli *et al.,* 1990; Phillippe, 1994). Considering that $PInsP_2$ turnover is assumed to be uncertain to occur in ciliates (Section III.C.3), it is interesting to note that neomycin can, nevertheless, inhibit some channel functions in *Paramecium*. So far this is strictly established only for voltage-dependent $Ca^{2+}$ channels (Gustin and Hennessey, 1988). Interestingly, in *Paramecium* neomycin inhibits trichocyst exocytosis (Plattner *et al.,* 1985b) and, as we found out much later, $Ca^{2+}$ influx (Klauke *et al.,* 1999). By virtue of its charge and small size, neomycin may inhibit some plasmalemmal channel function, just as it inhibits internal $InsP_3$-activated $Ca^{2+}$-release channels in mammalian cells. This assumption is compatible with the inhibition of $^{45}Ca^{2+}$ exchange by neomycin in hepatocytes (Hughes *et al.,* 1988) and some currents activated via NMDA receptors in pyramidal cells of the rat hippocampus (Lu *et al.,* 1998). If one considers the secretagogue effect of the broad spectrum of amines and polyamines in ciliates, whether an aminated compound can stimulate or inhibit one or the other of the different channels may depend on shape and charge distribution. Some other experiments in our laboratory, with $Mn^{2+}$-induced $Ca^{2+}$-signal quenching in fluorochrome analyses during AED stimulation, indicate that the plasmalemmal $Ca^{2+}$-carrying channels involved may be unspecific channels (Klauke *et al.,* 2000).

Their type still awaits further specification, particularly with regard to CaM sensitivity (see preceding discussion).

*Conclusions.* A detailed electrophysiological description is confronted with a poorly understood function of some ion channels in ciliates. The best understood channel type is the voltage-sensitive $Ca^{2+}$-influx channel in ciliary membranes, whereas $Ca^{2+}$-influx channels relevant for exocytosis remain to be identified.

### 2. A $Ca^{2+}$ Sensor in the Plasma Membrane?

Besides any possible direct effect of cationic compounds on ion channels (Section III.C.1), could a plasmalemmal $Ca^{2+}$ sensor protein be a potential candidate for AED-mediated activation? A $Ca^{2+}$ receptor has been described for some mammalian cell types that are in charge of regulating $[Ca^{2+}]$ homeostasis and/or uptake and calcification (Yamaguchi *et al.*, 1998). Such $Ca^{2+}$ sensors also are known to sense some other positively charged molecules in the medium (Quinn *et al.*, 1997; Vassilev *et al.*, 1997). In the parathyroid, the cDNA of the CaSR, which senses $Ca^{2+}$ and tri- to multivalent (poly)cations, is equivalent to a 120-kDa protein with the classical number of seven membrane-spanning domains (Brown *et al.*, 1993). Whereas such receptors frequently are acting via trimeric GTP-binding proteins (G-proteins) and $PInsP_2$ turnover (which both are not yet known to occur in ciliates; see Section III.C.3), some forms of $Ca^{2+}$ sensor proteins that possibly are devoid of these signal transmission characteristics have been described (Adebanjo *et al.*, 1998; Chattopadhyay *et al.*, 1998). In some gland cells, CaSRs are associated with SOC-type influx channels that carry $Ca^{2+}$, though they may be of little sensitivity (Warnat *et al.*, 1999), and some may be coupled to intracellular calcium stores (Shorte and Schoffield, 1996), just as we assume for *Paramecium* (Klauke *et al.*, 2000). Another variability is sensitivity to the aminoglycoside, neomycin, which activates CaSRs in some (Vassilev *et al.*, 1997; Yamaguchi *et al.*, 1998), but not all, mammalian cells (Bruce *et al.*, 1999). Any neomycin effects are particularly intriguing because this drug can exert widely different effects (Section III.C.1). Activation of SOC-type $Ca^{2+}$-influx channels, which we assume to participate in AED-stimulated trichocyst exocytosis (see preceding discussion), by $Ca^{2+}$/CaM also remains an open question.

We also do not know with certainty whether CaSR molecules really exist in ciliates and how they could mediate $Ca^{2+}$ influx. The identity of a "lysozyme receptor" of 42 kDa in *Tetrahymena* and 58 kDa in *Paramecium* described by Kuruvilla and Hennessey (1998) would have to be analyzed in this regard. A mere increase in $[Ca^{2+}]_o$ to 10 m*M* causes a rapid and intense cortical $[Ca^{2+}]_i$ increase, certainly by some other plasmalemmal $Ca^{2+}$ channels, yet no exocytosis ensues (Erxleben *et al.*, 1997; Klauke *et*

*al.,* 1999). This can be explained by the notorious stabilizing effect of high [$Ca^{2+}$] on lipid bilayers (Frankenhaeuser and Hodgkin, 1957), causing increasing rigidity (Papahadjopoulos, 1978) and, hence, increasing incapability of membrane fusion. Only brief exposure of *Paramecium* to $[Ca^{2+}]_o \leq 10$ m*M*, e.g., during AED stimulation in the subsecond time range using a quenched-flow apparatus, can greatly accelerate the exo-endocytotic cycle (Plattner *et al.,* 1997a).

*Conclusion.* As in other cells, the postulate of a CaSR still requires detailed analysis.

### 3. Cortical Ca Stores (Alveolar Sacs)

Alveolar sacs are attached tightly at the cell membrane, thus allowing the formation of a subplasmalemmal space of ~15 nm width maintained by visible membrane-to-membrane links (Plattner *et al.,* 1991). These are unidentified proteins that superficially recall the feetlike connections at terminal SR cisternae in striated muscle. However, in ciliates, no tetrameric substructures have ever been shown to occur, in contrast to SR where tetrameric dihydropyridine receptors (acting as voltage sensors) in the cell membrane match tetrameric ryanodine receptor-type $Ca^{2+}$-release channels in the SR membrane (Meissner, 1994). In *Paramecium* some drugs are inactive (Länge *et al.,* 1995), and de- or hyperpolarization of the cell membrane potential does not cause $Ca^{2+}$ release (Erxleben and Plattner, 1994).

The situation we postulate for *Paramecium* (Klauke *et al.,* 2000), is summarized in Figs. 4 and 5. A CaSR may be coupled to an unspecific cation-influx channel in the cell membrane. This may be associated with a $Ca^{2+}$-release channel in the nearby outer membrane of alveolar sacs. The precise entry pathway of $Ca^{2+}$ is not yet known.

The first evidence for alveolar sacs serving as Ca stores came from EDX analyses in *Coleps* (Fauré-Fermiet *et al.,* 1968), where Ca enrichment forms a conspicuously dense material in the lumen, particularly after precipitation in the insoluble form. Even the occurrence of a Ca pump has been precluded in these studies by enzyme cytochemistry. The isolation of alveolar sacs from *Paramecium* by Adoutte's group (Stelly *et al.,* 1991) allowed thorough analysis of $^{45}Ca^{2+}$-uptake and -release kinetics (Länge *et al.,* 1995, 1996). Structural analyses of $Ca^{2+}$ dynamics during exocytosis stimulation have been performed on a subsecond time scale using ESI (Knoll *et al.,* 1993) and EDX methods (Hardt *et al.,* 1998; Hardt and Plattner, 1999, 2000). Still, $Ca^{2+}$-release channels were identified only indirectly by their responsivity to SR activators, caffeine (Länge *et al.,* 1995, 1996; Klauke and Plattner, 1998) and 4-chloro-*meta*-cresol (4CmC; Klauke *et al.,* 1999). As with SR, to achieve maximal activation, caffeine has to be applied in concentrations up to 50 m*M,* whereas only $\leq 1$ m*M* 4CmC is sufficient. 4CmC activates

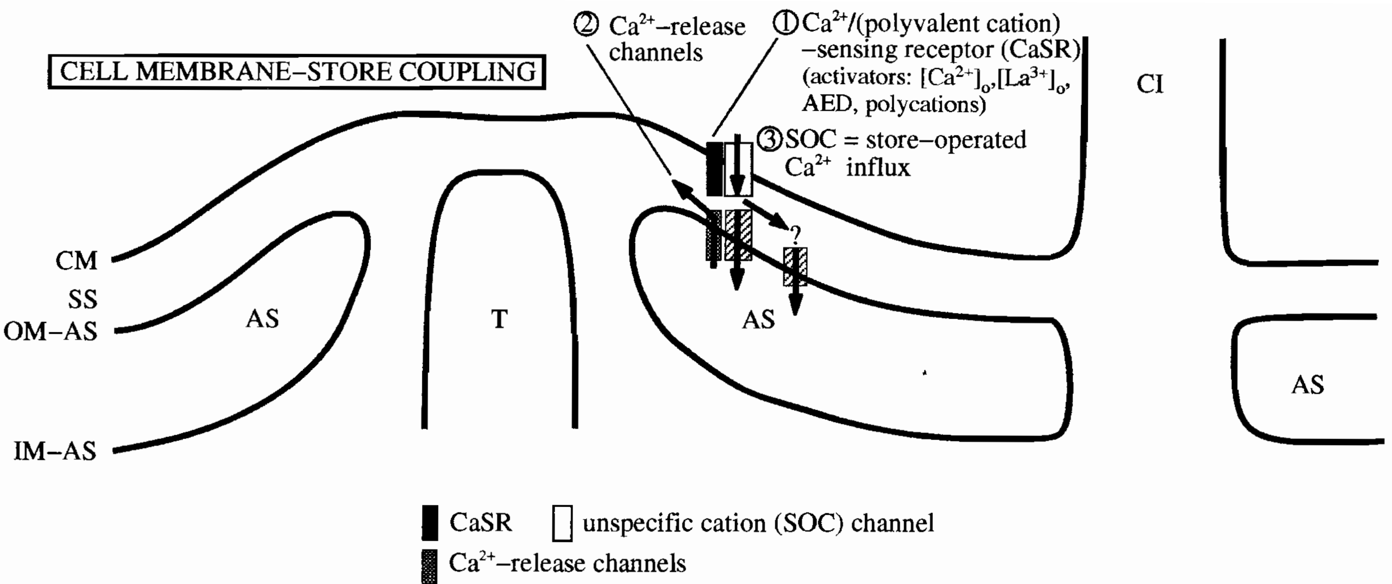



FIG. 4 Hypothetical coupling of $Ca^{2+}$ release from alveolar sacs and $Ca^{2+}$ influx by a SOC-type mechanism, following CaSR activation, in the sequence indicated (1–3). Coarse crosshatch, unknown $Ca^{2+}$ uptake mechanism in alveolar sac membrane. For abbreviations, see Fig. 2.

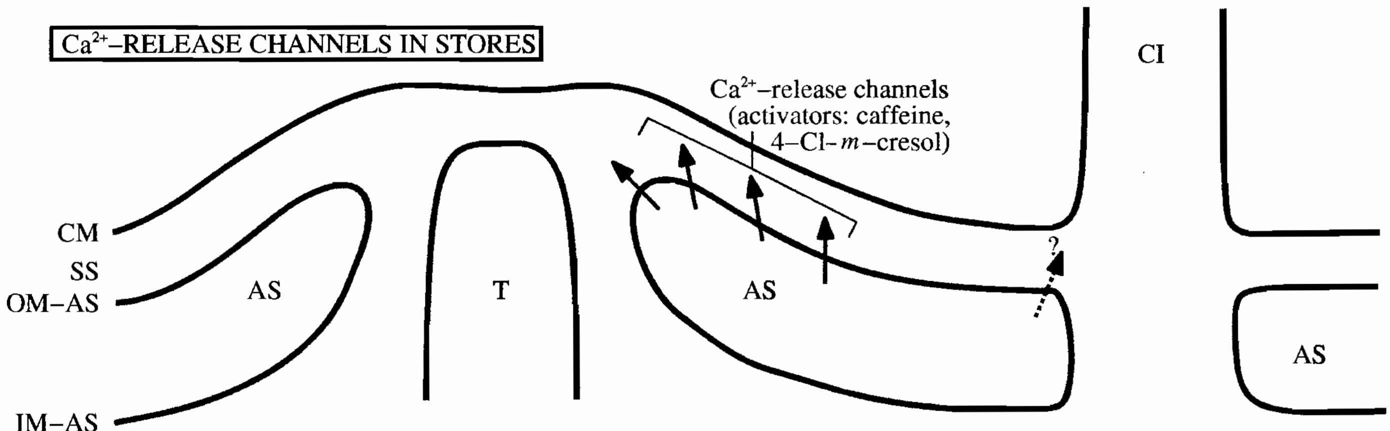



FIG. 5 Assumed localization of $Ca^{2+}$-release channels in alveolar sacs of *Paramecium*.

ryanodine-receptor-type $Ca^{2+}$-release channels even when mutated (Herrmann-Frank *et al.,* 1996; Kabbara and Allen, 1999), and this drug is used in pathophysiology to test patients for malignant hyperthermia, which would entail fatality during anesthesia. In contrast to SR, ryanodine does not mobilize $Ca^{2+}$ from alveolar sacs *in situ* or after isolation (Länge *et al.,* 1995).

We obtain considerable $Ca^{2+}$ influx upon AED stimulation even at $[Ca^{2+}]_o$ = 50 $\mu M$. There would be sufficient driving force for entry into the cytosol, but what would be the situation with alveolar sacs (into which $Ca^{2+}$ may enter either directly or via a very fast indirect uptake mechanism operating at the subsecond level)? Actual $[Ca^{2+}]$ within alveolar sacs is unknown, yet decreases during store activation will further drive reuptake. In ER–SR systems, estimates of $[Ca^{2+}]$ varied over the years by four orders of magnitude (Bygrave and Benedetti, 1996), yet values of ~50 $\mu M$ are now in consideration (Meldolesi and Pozzan, 1998a).

We expect $Ca^{2+}$-release channels to be localized on the outer side of alveolar sacs, i.e., the side facing the cell membrane and/or facing trichocyst docking sites, whereas the inner region has been shown to contain the SERCA-type $Ca^{2+}$ pump heavily enriched (Plattner *et al.,* 1999). This arrangement allows for site-directed $Ca^{2+}$ flux toward trichocyst docking sites and subsequent downregulation of $[Ca^{2+}]_i$ increase. The plasmalemmal pump will "serve" the subplasmalemmal space, whereas $Ca^{2+}$ sweeping into the cell interior will be handled by the organellar $Ca^{2+}$ pump. This has been cloned (Hauser *et al.,* 1998) and characterized pharmacologically and biochemically, particularly with regard to phospho intermediate formation (Kissmehl *et al.,* 1998), as described in Section III.E.4.

In the alveolar sacs of *Paramecium,* calcium is bound to a high-capacity/low-affinity CaBP of the calsequestrin type (Plattner *et al.,* 1997b). We have established this by specific AB binding in immunofluorescence and by immunogold labeling, as well as by Western blot analysis of isolated fractions that also bound $^{45}Ca^{2+}$ in overlays. Interaction with calreticulin has been excluded, and preadsorption with original calsequestrin from SR abolished AB binding.

This explains the high [Ca] = 43 m$M$ found in alveolar sacs by EDX (Hardt and Plattner, 1999, 2000), similar to values detected in the SR. Mobilization of $Ca^{2+}$ occurs during AED-stimulated exocytosis by a signal-transduction pathway to be established [because none of the known second messengers can release $Ca^{2+}$ from alveolar sacs (Länge *et al.,* 1995)]. We assume direct coupling to the cell membrane (Erxleben and Plattner, 1994; Erxleben *et al.,* 1997), which mediates store-operated $Ca^{2+}$ influx (SOC-type mechanisms) via unspecific cation channels (Klauke et al., 2000). In fact, when $[Sr^{2+}]_o$ is substituted for $[Ca^{2+}]_o$ during synchronous (80 ms) AED-stimulated exocytosis in quenched-flow experiments, Sr is detected by EDX in alveolar sacs

after only 80 ms, when ~40% of the Ca has been released (Hardt and Plattner, 2000). Considering the somewhat variable pharmacological characteristics of SOC in different systems (Lewis, 1999), we therefore can reasonably assume it to exist as a functional component in ciliates. This mechanism is much faster than the $I_{CRAC}$-type $Ca^{2+}$-influx current measured in some other secretory cells (Hofer *et al.,* 1998); see Section II.B.

Alveolar sacs are also considered a $Ca^{2+}$ source during GTP- or $Ba^{2+}$-induced chemoresponses (Wassenberg *et al.,* 1997), because this is affected by previous exposure to SERCA inhibitors.

The biogenesis of alveolar sacs is unknown (Capdeville *et al.,* 1993). We presume each sac to be a closed compartment. We could not see any connecting holes, as reported by Allen (1988), after fast freezing and freeze–fracturing (Flötenmeyer *et al.,* 1999). Such connections possibly are snapshots of biogenetic formation by expansion, constriction, and cleavage.

*Conclusions.* Alveolar sacs are ample subplasmalemmal calcium stores containing a high-capacity/low-affinity CaBP of the calsequestrin type. They thus can store Ca in concentrations that occur in the SR of muscle cells. During exocytosis stimulation, alveolar sacs release a large proportion of their calcium, but they do not participate in the regulation of ciliary function.

#### 4. Endoplasmic Reticulum, Golgi Apparatus, and "Ca Crystals"

Throughout a *Paramecium* cell, widely branched cisternae of the ER can be visualized by CLSM (Plattner *et al.,* 1997b) after affinity staining with compound $DiOC_{18}$ (=3,3′-dilinoleyloxacarbocyanine). The nuclear cisterna may be considered a specialized ER portion relevant for $Ca^{2+}$ signaling in the nucleus (Section III.H). However, the structural and biogenetic relationship between ER and alveolar sacs (Section III.C.3) is far from established (Capdeville *et al.,* 1993). Both compartments contain different high-capacity/low-affinity CaBPs, i.e., a calreticulin-like protein occurs in the ER and a calsequestrin-like protein in the alveolar sacs, according to immunological specification with monospecific ABs (Plattner *et al.,* 1997b). This is documented in Fig. 6, which also shows the occurrence of these CaBPs in Ca crystal vacuoles.

Whereas both compartments are expected to possess a $Ca^{2+}$-ATPase, ABs against the cloned SERCA-type pump from *Paramecium* label only its alveolar sacs (Hauser *et al.,* 1998). There are several possible explanations to this. (i) Either the ER is devoid of this pump—an unlikely explanation because it would be without any precedent—or (ii) the ER contains another isoform. Although we have found, in fact, a second SERCA isoform (K. Hauser, N. Pavlovic, N. Klauke, D. Geissinger, and H. Plattner, manuscript submitted), they both contain the same antigenic region used as

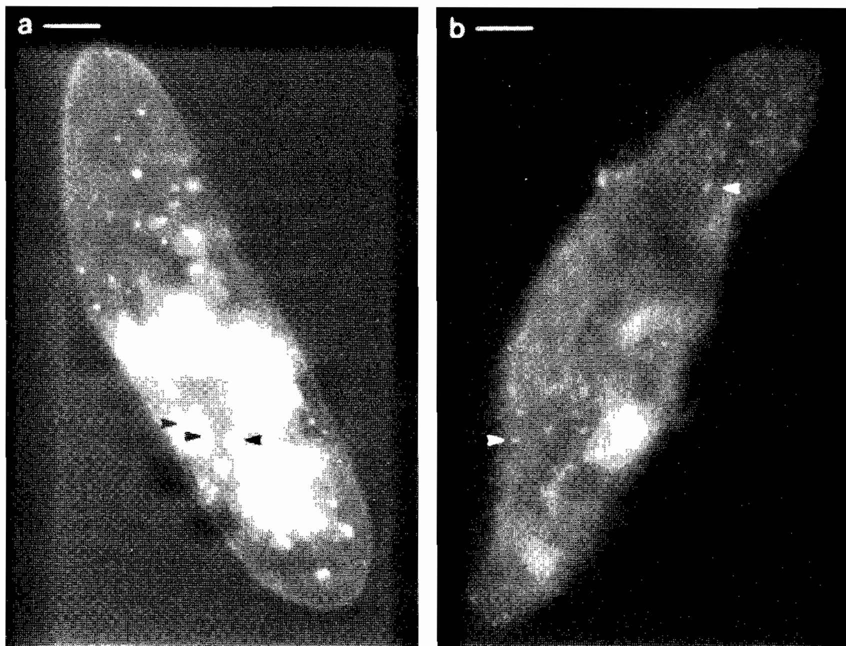



FIG. 6 Immunolocalization in *Paramecium* using ABs against calsequestrin (a) stains the outlines of a cell (indicating presence in alveolar sacs), whereas ABs against calreticulin (b) vaguely stain some diffuse internal structures, probably ER. Both ABs (particularly those against calsequestrin) also stain Ca crystal vacuoles (arrowheads). For more details, see Plattner *et al.* (1997b). Bars = 10 $\mu$m.

peptide for immunization (Hauser *et al.,* 1998). (iii) Pump molecules would be much more abundant in alveolar sacs—the most likely explanation so far (unless additional widely different forms occur) particularly because the phospho intermediate is much more pronounced in alveolar sacs than in ER-enriched "microsomes" (Kissmehl *et al.,* 1998).

The dual distribution of two widely different Ca stores suggests different functions. Whereas alveolar sacs may serve as a rapid $Ca^{2+}$ supply during trichocyst exocytosis and subsequent reestablishment of $Ca^{2+}$ homeostasis in cortical regions (Section III.C.3), the ER may provide $Ca^{2+}$ for intracellular traffic and/or allow for $Ca^{2+}$ homeostasis in deeper cell regions. Enrichment of calcium in the ER has been documented by EDX analysis of nuclear cisternae—the most easily identifiable ER component (Hardt and Plattner, 1999). Microsomes isolated from *Tetrahymena* also store calcium (Muto and Nozawa, 1984, 1985), although contribution by alveolar sacs has not been excluded. However, because in the ER calreticulin participates as a $Ca^{2+}$-dependent chaperone in protein synthesis (Nauseef *et al.,* 1995;

Peterson *et al.,* 1995), one cannot automatically infer its participation in $Ca^{2+}$ signaling and/or homeostasis. More detailed analyses are required.

In yeast, a $Ca^{2+}$ pump unrelated to the plasmalemmal or SERCA-type pump has been detected in the Golgi apparatus (Rudolph *et al.,* 1989; Sorin *et al.,* 1997). Calcium has been visualized in distal parts of the Golgi of mammalian cells by the ESI method (Grohovaz *et al.,* 1996). No similar information is available for ciliates. Because biogenesis of secretory organelles by cotranslational sequestration, lumenal glycosylation, and folding of secretory products requires $Ca^{2+}$-dependent chaperones (Trombetta and Helenius, 1998), the question arises as to how extrusomes can go through all this when trichocyst content, for example, expands as soon as it "sees" $Ca^{2+}$ (see Section III.E.2). The answer may be in the sequential arrangement of the different processing steps (Chilcoat *et al.,* 1996).

Over a long time period, vacuoles containing calcium and phosphorus are known from different ciliates (*Coleps, Euplotes, Paramecium, Tetrahymena*), as found by widely different analysis methods (Rosenberg, 1966; Fauré-Fermiet *et al.,* 1968; Coleman *et al.,* 1974; Dutta, 1974; Hausmann and Walz, 1979). We confirmed Ca content in vacuoles of *Paramecium* by EDX (Hardt and Plattner, 1999). Because we observed labeling with anti-calsequestrin and anticalreticulin ABs in these Ca crystal vacuoles, they may represent a Ca pool amenable to some regulation, despite its unflexible appearance and our ignorance of the uptake mechanism. A most thorough analysis of vacuole content from *Paramecium* by EDX and X-ray diffraction has identified the component struvite (Grover *et al.,* 1997), a Ca,Mg phosphate mineral component of kidney stones.

*Conclusion.* $Ca^{2+}$-mediated or -dependent functions in these organelles deserve much more detailed study.

### D. Ciliary Beat Regulation

Ciliary activity is under the control of $[Ca^{2+}]_i$ within the organelle not only in ciliates (Machemer, 1986, 1988; Preston and Saimi, 1990) but also in some other cells (Tamm, 1994). The most dramatic phenomenon, ciliary reversal, is induced by depolarization with the involvement of voltage-dependent $Ca^{2+}$ channels in *Paramecium* (Brehm and Eckert, 1978; Eckert and Brehm, 1979), *Didinium* (Pernberg and Machemer, 1995b), *Euplotes* (Krüppel and Wissing, 1996), *Stylonychia* (Deitmer, 1986), *Tetrahymena* (Hennessey and Kuruvilla, 1999), etc. These were paradigmatic for other cell systems for a long time. Most authors assume these channels to be restricted to the ciliary membrane (Section III.C.1). Unfortunately, in ciliates they do not respond to the usual inhibitors according to Preston and Saimi (1990), although the wide range of newly developed or discovered

inhibitors, each specific for different $Ca^{2+}$-channel subtypes, may not yet have been tested systematically enough. [Fortunately anti-CaM drugs work well in ciliates (Section III.A).] With cell-free models, ciliary reversal can be activated by applying $[Ca^{2+}] \geq 10^{-6}$ *M* (Natioh, 1995). Though with intact cells simply an increase in $[Ca^{2+}]_o$ does not trigger ciliary reversal, the extent of the response achieved increases with increasing $[Ca^{2+}]_o$ up to millimolar concentrations in sweet water species (Hildebrand and Dryl, 1983) or even up to 50 m*M* with the brackwater species, *Paramecium calkinsi* (Bernal and Ehrlich, 1993).

Depolarization causes an action potential and rapid signal spread over the cell. If this is generated by a local mechanical step–pulse, a response is seen within 10–15 ms over an entire *Paramecium* cell (Machemer and Deitmer, 1985). This is too fast by far to be accounted for by $Ca^{2+}$ diffusion, so that depolarization may be the genuine signal (DePreyer and Machemer, 1978).

Where in a cilium may these $Ca^{2+}$ channels be located? This has been easier to analyze with the flagella of *Chlamydomonas*. During reformation of the flagella after deflagellation, the size of currents attributable to voltage-dependent $Ca^{2+}$ channels strictly parallels the size of the reformed flagellum as it grows (Beck and Uhl, 1994). In the ctenophore, *Mnemiopsis,* ciliary reversal via voltage-dependent $Ca^{2+}$-channel activation causes an intraciliary $[Ca^{2+}]$ increase over the entire length of a cilium within ≤50 ms (Tamm and Terasaki, 1994). All of this might imply equal distribution of channels over the entire ciliary membrane, though precise analyses still have to be executed with ciliates. In the case of ciliates, assembly to functional patches occurring in a kind of maturation process, e.g., at the organelle basis, cannot be excluded. Though merely speculative, this could explain several phenomena. (i) The beat of a cilium propagates from its basis in ciliates (Machemer and Teunis, 1996), as in other cells (Tamm, 1994). (ii) Reestablishment of ciliary $[Ca^{2+}]$ homeostasis after activation may involve components located at or close to the ciliary basis (see discussion to follow). (iii) The membrane of the ciliary basis in ciliated protozoa contains regular protein aggregates (Bardele, 1983), which in *Paramecium* bind $Ca^{2+}$ (Plattner, 1975) and may be engaged in $Ca^{2+}$ regulatory functions, i.e., as channels or pumps. (iv) Careful analysis of airway ciliary epithelium clearly revealed the relevance of a $[Ca^{2+}]$ increase to 350 n*M* just in the ciliary basis, in parallel to accelerated ciliary beat frequency (Lansley and Sanderson, 1999). In sum, the ciliary basis may contain regulatory components as well as target structures involved in $Ca^{2+}$ signaling.

Even more difficult to explain is the way in which normal ciliary beat may be influenced, if at all, by $[Ca^{2+}]_i^{rest}$, and whether the actual intraorganellar $[Ca^{2+}]$ may also be relevant for increased forward swimming in the course of a hyperpolarization reaction. May $Ca^{2+}$ serve as a switch in all

these functions? To what extent may hyperpolarization-induced formation of cAMP, already occurring within one ciliary beat cycle (Schultz *et al.,* 1984; Yang *et al.,* 1997), and depolarization-induced cGMP, formed within 0.5–1 s (H. Plattner, W. Bell, and J. Van Houten, unpublished observation), serve as a signal? How may these signals be connected with any changes in $[Ca^{2+}]$—a problem discussed intensely in reviews (Satir *et al.,* 1993; Satir, 1998)?

The following corollaries, mainly derived from work with *Paramecium,* may be considered. An adenylate cyclase (Schultz *et al.,* 1992), as well as a guanylate cyclase, activity (Schultz *et al.,* 1986) is present in ciliary membranes of *Tetrahymena* and *Paramecium,* where the latter has been cloned (Linder *et al.,* 1999). (Unfortunately this has not yet been achieved with the gene for voltage-dependent $Ca^{2+}$ channels.) Formation of cGMP occurs strictly in parallel to $[Ca^{2+}]_i$ increase (Klumpp and Schultz, 1982; Schultz and Schade, 1989a) and, on the basis of this implication, has been used as an indirect measure of $[Ca^{2+}]_i$ increase (Schultz and Schade, 1989b; Schultz *et al.,* 1997). Ciliary guanylate cyclase is $Ca^{2+}$/CaM-regulated in *Paramecium* and *Tetrahymena* (Schultz and Klumpp, 1991). In these species, $[Ca^{2+}] < 0.1$ m$M$ activates an adenylate cyclase, whereas $[Ca^{2+}] \sim 1$ m$M$ activates a guanylate cyclase (Kudo *et al.,* 1985). This could indicate differential $[Ca^{2+}]$-dependent activation of the respective cyclase (Hasegawa *et al.,* 1999), notably that of guanylate cyclase during ciliary reversal. A cAMP- and a cGMP-dependent protein kinase (PK-A and PK-G) can each phosphorylate different ciliary proteins (Bonini and Nelson, 1990; Ann and Nelson, 1995), which influences ciliary activity *in vitro* (Hamasaki *et al.,* 1991; Satir *et al.,* 1993; Satir, 1998; Hasegawa *et al.,* 1999). Yet the application of cyclic nucleotides *in vivo,* while varying $[Ca^{2+}]_i$, indicated that $Ca^{2+}$ may be the essential second messenger, particularly for ciliary reversal, so that cyclic nucleotides may exert only modulatory effects (Nakaoka and Machemer, 1990). This role would probably be executed dependent on actual $[Ca^{2+}]$. This could signify the acceleration of ciliary beat by cAMP and its shutting off by cGMP, e.g., via phosphorylation processes. In fact, microinjection of GTP and activating or inhibitory analogues has altered $Ca^{2+}$ conductance and behavior in the marine species, *Paramecium calkinsi,* accordingly (Bernal and Ehrlich, 1993). Aspects pertinent to nucleotide effects have been summarized by Schultz and Klumpp (1993), Tamm (1994), and Pech (1995). Beyond these implications, $Ca^{2+}$ may exert some other, perhaps more direct, effects on ciliary beat regulation.

In principle, could a varying $[Ca^{2+}]$ in cilia account for the different ciliary reactions, and how could this be achieved? CaM, known to occur in cilia (Section III.A), possibly plays a key role. The relative amounts of $[Ca^{2+}]$ and $[Mg^{2+}]$ may determine the respective ciliary activity (cell behavior) over the whole range from hyper- to depolarization, as proposed by Mogami

*et al.* (1990) and Machemer and Teunis (1996). They inferred that the $K_d$ of CaM for $Ca^{2+}$ depends on [$Mg^{2+}$] (Potter *et al.*, 1983; Nelson and Chazin, 1998). In cells, [$Mg^{2+}$] is in large excess, namely, between 0.4 and 1.0 m*M* in *Paramecium* (Preston, 1998) and *Stylonychia* (DePreyer and Deitmer, 1980), respectively. According to other analyses, $Ca^{2+}$ affinity decreases with increasing [$Mg^{2+}$], particularly in the $Ca^{2+}$ -binding sites located close to the N-terminus of CaM, for which the [$Ca^{2+}$]/[$Mg^{2+}$] ratio *in vivo* may be of particular relevance (Malmendal *et al.*, 1999). Importantly, this is the region by which CaM binds to target molecules (Klee, 1988). Such different forms of $Ca^{2+}$-activated CaM could serve different functions. (i) They could first increase the activation of $Ca^{2+}$ channels, as suggested by molecular function repair in behavioral mutants (Section III.C.1). (ii) In the end, the $Ca^{2+}$/CaM complex inactivates $Ca^{2+}$ channels (see Section I). (iii) Within a cilium it could activate some of the numerous CaM-BPs (Section III.A) or still other proteins to be determined in future work. (iv) Finally, the principle of [$Ca^{2+}$]/[$Mg^{2+}$] antagonism may also apply to the newly discovered protein kinases with inherent CaM homology loops (Kim *et al.*, 1998). So far this has not been analyzed.

If the hyper- or depolarizing voltage clamp is maintained, this entails the inactivation of $Ca^{2+}$ currents (Hennessey and Kung, 1985) and ciliary motor response (Machemer and Eckert, 1975). Therefore, only a limited number of $Ca^{2+}$ ions enter a cilium during depolarization.

Let us consider the following quantitative aspects of the $Ca^{2+}$ signal in the cilia of *Paramecium,* using values from Erxleben *et al.* (1997). With a diameter of 0.25 $\mu$m and a length of 10 $\mu$m, their individual volume is 0.5 $\mu m^3$. From the number of kinetids, estimated under our conditions, ~3900 cilia may occur on a cell. Cilia would contribute approximately three times more to the total cell surface than the somatic membrane, with values of $3.1 \times 10^4$ $\mu m^2$ and $1.1 \times 10^4$ $\mu m^2$, respectively. But with their total volume of 1950 $\mu m^3$, they would contribute only 2.6% to the total cell volume. These data imply that (i) intraciliary [$Ca^{2+}$] could increase swiftly by influx over a large surface area into a small volume and (ii) diffusion into the cell body, even if it were significant, would entail only a small cortical $[Ca^{2+}]_i$ increase.

By assuming a minimal [$Ca^{2+}$] = $10^{-6}$ *M* to induce ciliary reversal (Nakaoka *et al.*, 1984), 560 $Ca^{2+}$ ions would be required per organelle, but because ~50% of its volume is occupied by axonemal proteins, ~280 $Ca^{2+}$ ions in the soluble space may suffice. Another correction is required due to ≥99% rapid binding to endogenous buffers (Section II.C), yielding a [Ca] value of ≥0.1 m*M*. This correlates with the requirement of $[Ca^{2+}]_o \geq$ 1 m*M* to achieve sufficient driving force for the maximal ciliary reversal reaction (Bernal and Ehrlich, 1993). If $Ca^{2+}$-binding capacity were even 1500–2000, as found at $[Ca^{2+}]_i^{rest}$ = 150 n*M* by *in vitro* simulation experi-

ments (Mogami *et al.,* 1999), an excess of 1.5–2 m*M* over basal [Ca] could be expected. If this holds true, an analysis of actual [Ca] changes during ciliary reversal by EDX would be feasible, despite its limitations discussed in Section II.D.

[$Ca^{2+}$] homeostasis in cilia may be reestablished by different mechanisms, and the following aspects should be considered. (i) Rapid binding to endogenous buffers can explain the recovery of [$Ca^{2+}$] in a cilium only 1 s after reversal induction, at least in the ctenophore, *Mnemiopsis* (Tamm and Terasaki, 1994). Such rapid correction would be impossible by a primary active transporter (pump). (ii) $Ca^{2+}$ may dissociate slowly from CaBPs, like CaM, and the excess $Ca^{2+}$ would then be removed. (iii) Because $Ca^{2+}$ originating from the outside after depolarization does not diffuse rapidly enough to produce any remarkable cortical $[Ca^{2+}]_i$ increase according to fluorochrome analyses in *Didinium* (Pernberg and Machemer, 1995a), how could it be handled by transporters? The SERCA-type pump in alveolar sacs is localized to the side facing the cell center (Plattner *et al.,* 1999) and, thus, could handle this $Ca^{2+}$ only as it gradually diffuses downward. This may also apply to the plasmalemmal $Ca^{2+}$-ATPase because it is probably restricted to the somatic cell membrane and, at the most, to the basis of cilia (Van Houten, 1998). Both of these mechanisms could counteract particularly a $[Ca^{2+}]_i$ increase in the ciliary basis. (iv) Finally, the plasmalemmal $Na^+/Ca^{2+}$ exchanger (Burlando *et al.,* 1999) may serve this purpose, though its localization remains to be established.

*Conclusions.* $[Ca^{2+}]_o$ influx is the source of $Ca^{2+}$-activated ciliary activity. Although its voltage-dependent $Ca^{2+}$ channels long served as a paradigm, any further steps, targets, and effector coupling are still speculative, as is the way in which [$Ca^{2+}$] homeostasis in a cilium may be reestablished.

### E. Stimulated Exocytosis and Endocytosis

Stimulated exocytosis of trichocysts in *Paramecium* is coupled to rapid endocytosis of empty "ghost" membranes (Hausmann and Allen, 1976; Haacke and Plattner, 1984; Plattner *et al.,* 1985a). Similar mechanisms probably also take place in other ciliates when they release extrusomes. An exo-endocytosis process encompasses several steps: (i) exocytotic membrane fusion, (ii) pore expansion, (iii) discharge of secretory contents, (iv) endocytic membrane resealing, and (v) detachment of ghosts. Step iii requires $Ca^{2+}{}_o$ in *Paramecium,* as shown first by Bilinski *et al.* (1981a) and outlined in Section III.E.2. The other steps are driven by a $[Ca^{2+}]_i$ increase in mammalian cells (Neher and Zucker, 1993; Robinson and Martin, 1998; Kasai, 1999), as they are in *Paramecium* (Plattner *et al.,* 1997a). Surprisingly, comparison of time constants shows that *Paramecium* can operate its stimu-

lated exo-endocytotic system faster than any other dense-core-vesicle system. This may be vital for the cell, enabling it to use trichocyst exocytosis for defense against a predator, as first shown by Harumoto and Miyake (1991). Locally released trichocysts may act as a spacer to ward off the attacker, while the cell switches to "back gear" by subsequent ciliary reversal and, thus, escapes with very high efficiency (Knoll *et al.,* 1991b).

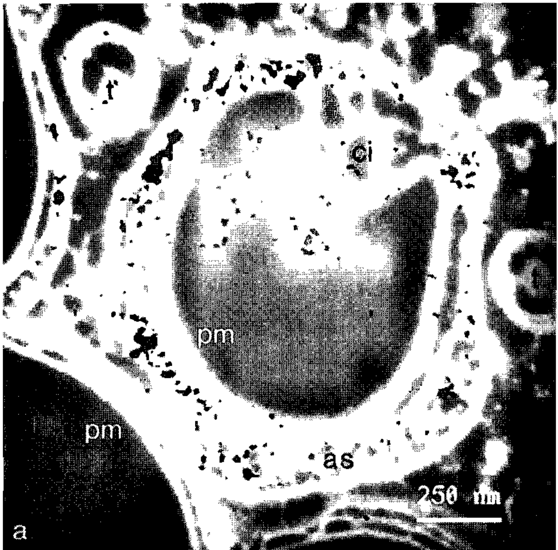
Color pictures in Figs. 7–10 (see color insert) show the following details. In Fig. 7, $Ca^{2+}$ signals are recorded by high-resolution ESI imaging. In a resting cell, such signals are abundant in alveolar sacs, whereas additional signals occur in association with the cell membrane when cells are stimulated with AED for 80 ms. Figure 8 shows trichocyst exocytosis upon contact with a (stained) AED solution and occurrence of an intracellular $Ca^{2+}$–fluorochrome signal emanating from the contact site. In Fig. 9, high-speed CLSM fluorochrome analysis reaveals rapid cortical $[Ca^{2+}]_i$ increase during AED stimulation, followed by internal signal spread. The $[Ca^{2+}]_i$ increase generated by exposure to the SERCA inhibitor, thapsigargicin, is visualized in Fig. 10.

### 1. Exocytotic Membrane Fusion and Pore Expansion

In all secretory cells, exocytosis requires the local assembly of a set of proteins (Burgoyne and Morgan, 1998; Edwards, 1998; Robinson and Martin, 1998; Xu *et al.,* 1999). Whereas this is trivial now, it was not at the beginning when "rosette" particles and "connecting material" at trichocyst docking sites of *Paramecium* integral and peripheral membrane proteins (Westphal and Plattner, 1981; Vilmart and Plattner, 1983) were shown to be mandatory for exocytotic membrane fusion (Plattner, 1981). The strongest argument came indirectly from the absence of membrane fusion in the absence of these structures, even when trichocysts were docked at the cell membrane (Beisson *et al.,* 1976, 1980).

Only in the past decade has a legion of scientists dedicating their work to "higher" eukaryotic cells, like neurons and mast and gland cells, cloned and specified a number of genes and proteins to which varying functions in exocytosis regulation are attributed. In *Paramecium,* systematic mutagen-

FIG. 7 ESI analysis of calcium distribution in a *Paramecium* cell at rest (a) or 80 ms after AED stimulation (b). Cells were prepared by quenched-flow and freeze–substitution under conditions retaining Ca. The false color distribution represents Ca signals identified in the energy loss spectrum (c). Note the enrichment of Ca in alveolar sacs (as) and the absence from cilia (ci) and trichocysts (t) at rest, whereas intense signals occur along the plasmamembrane and in cilia after stimulation (none in the trichocyst ghost, tg). From Knoll *et al.* (1993). (See also color insert).



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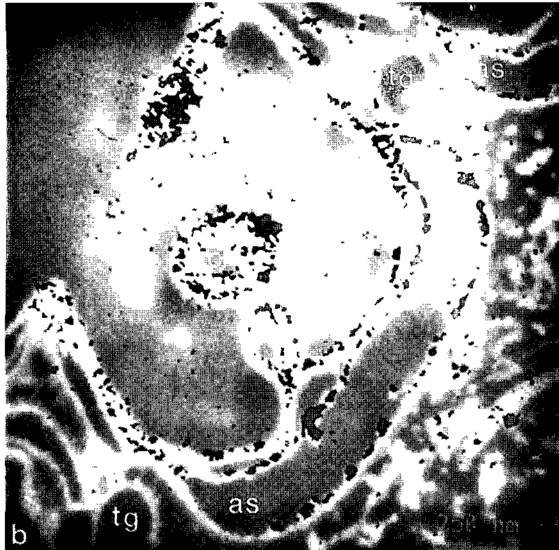

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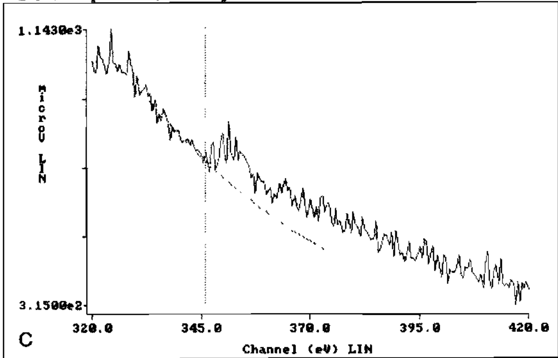

ΔE:346.0 spec:779 μV ref spec: 0 μV
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microV LIN
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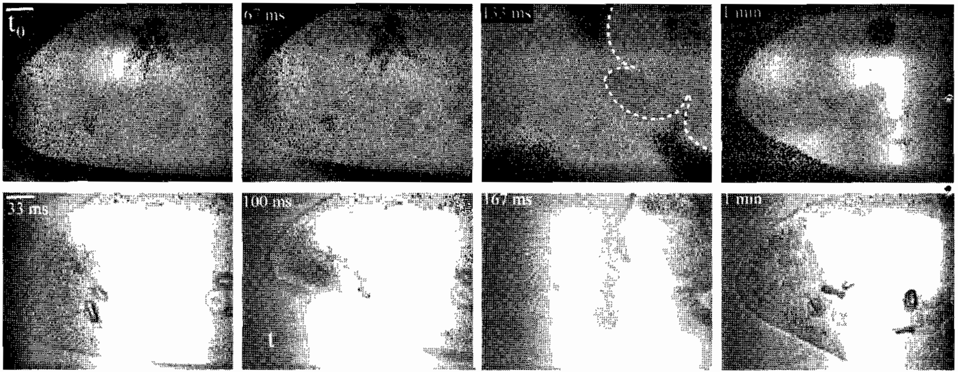


FIG. 8 AED stimulates trichocyst exocytosis in a *Paramecium* cell. Trichocysts (t) are released as soon as AED (2 $\mu M$, $[Ca^{2+}]_o$ = 50 $\mu M$, contact of trigger solution visualized by $F_2$FITC added) touches the surface of the cell loaded with 100 $\mu M$ Fluo-3 for fast CLSM false-color $[Ca^{2+}]_i$ imaging. Bar = 10 $\mu$m. From Klauke and Plattner (1997). (See also color insert).

esis studies suggested that at least 23 gene products may participate, directly or indirectly, in this process (Bonnemain *et al.*, 1992). With few exceptions, it turned out to be difficult to transfer data from higher cells to ciliates, e.g., by using specific antibodies or by designing primers, probably because any sequence identities or similarities are scattered loosely over a gene or a protein.

Hence, we still know nothing about docking and fusion proteins or about synaptotagmin, which is an established vesicle-bound $Ca^{2+}$-sensing protein (Südhof and Rizo, 1996). Colocalization of $Ca^{2+}$-influx channels allows for fast cholinergic neurotransmitter release in motoneurons (Zucker, 1993; Neher, 1998), which is opposite most other cells. Such colocalization also is not known from ciliates, where we still struggle to identify the channels involved. In contrast, strict coassembly of exocytosis sites and cortical stores [in part reminiscent of SR in muscle cells (Länge *et al.,* 1995)] is bluntly evident in this group of Alveolata, whereas it is problematic in higher cells. During trichocyst exocytosis in *Paramecium*, we were unable to pinpoint any of the otherwise established second messengers (see Table VII) except $Ca^{2+}$ (Länge *et al.*, 1995), possibly another primitive characteristic of an early secretory system.

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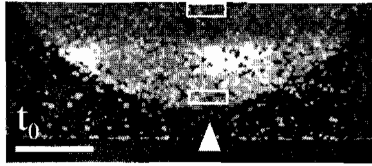
FIG. 9 A *Paramecium* cell (Fluo-3 loaded for false-color CLSM $[Ca^{2+}]_i$ imaging) develops a cortical $Ca^{2+}$ signal even when stimulated by AED in the presence of ultrafast $Ca^{2+}$ chelator, BAPTA, briefly yielding $[Ca^{2+}]_o$ ~ 30 n$M$ during stimulation. Note the time scale from $t_0$ on and the evaluation of framed cell regions in the diagram. For quantitative evaluation, see Fig. 11. Bar = 10 $\mu$m. (See also color insert).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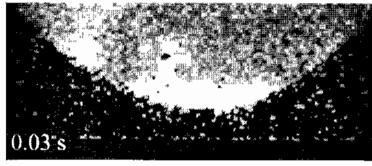
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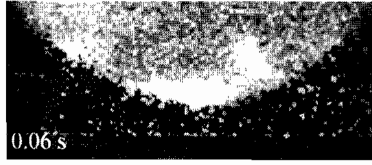
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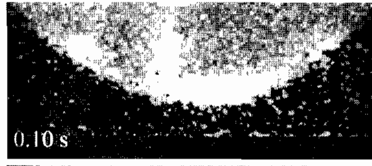
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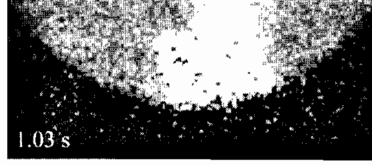
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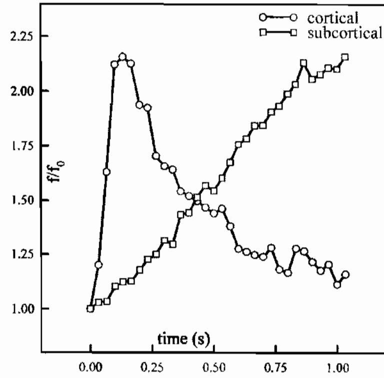
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0.75
1.00

TABLE VII

Compounds of Possible Relevance for $Ca^{2+}$ Dynamics, as tested with *Paramecium*[a]

| Compound/reported activity | Reference | Application/concentration | Effect observed |
|---|---|---|---|
| | SERCA Inhibitors | | |
| Thapsigargicin, p | Du *et al.* (1996a,b) | Extracellular/100 $\mu M$ | ↑ Ca, ++ |
| *tert*-Butyl(benzo)hydroquinone, p | Wictome *et al.* (1992) | Extracellular/100 $\mu M$ | ↑ Ca, ++ |
| Cyclopiazonic acid, p | Du *et al.* (1994, 1996a,b) | Extracellular/500 $\mu M$ | ↑ Ca, ++ |
| | Classical Ca Channel Activators | | |
| Ryanodine, $Ca^{2+}$-release channel activator ($\leq$10 $\mu M$) or inhibitor ($\geq$10 $\mu M$), ip | Meissner (1994) | Intracellular/1–100 $\mu M$ | No effects |
| Inositol 1,4,5-trisphosphate or its thioate form, ip | Safrany *et al.* (1991) | Intracellular/$\leq$50 $\mu M$ | No effects |
| Caffeine [in *Paramecium:* additional SERCA inhibition (Kissmehl *et al.*, 1998)], p | Meissner (1994) | Extracellular/50 m$M$ | ↑ Ca, exo +++ |
| Other Second Messengers or Related Drugs with Store-Activation Effects | | | |
| Cyclic adenosine diphosphoribose (cADPR) | Galione *et al.* (1993a,b) | Intracellular/100 $\mu M$ | No effects |
| Sphingosine 1-phosphate, ip | Olivera and Spiegel (1993) | Intracellular/10 $\mu M$ | No effects |

| | | | |
|---|---|---|---|
| NAADP⁺, ip | Genazzini *et al.* (1997) | Intracellular/100 μM | No effects |
| Cyclic GMP, ip | Galione *et al.* (1993a,b) | Intracellular/100 μM | No effects |
| Dibutyryl cyclic-GMP, p | Rooney *et al.* (1996) | Extracellular/1 mM | No effects |
| 8-Br-cGMP, p | Rodriguez-Pascual *et al.* (1995) | Extracellular/1 mM | No effects |
| Guanylate cyclase inhibitor | | | |
| 6-anilino-5,8-quinolinedione (LY-83583), p | Xu *et al.* (1994) | Extracellular/100 μM | No effects |
| Phosphodiesterase inhibitors, p | | | |
| Quazinone | Holck *et al.* (1984) | Extracellular/100 μM | No effects |
| Isobutylmethylxanthine (IBMX) | Xu *et al.* (1994) | Extracellular/1 mM | No effects |
| Dipyridamol | Tandon and Collier (1994) | Extracellular/100 μM | No effects |
| NO donors, p | | | |
| *S*-Nitroso-*N*-acetylpenicillamine (SNAP) | Stoyanovsky *et al.* (1997) | Extracellular/100 μM | No effects |
| | | Intracellular/100 μM | No effects |
| NO synthase inhibitor, p | | | |
| *N*-Monomethyl-L-arginine (L-NMMA) | Gukovskaya and Pandol (1994) | Extracellular/1 mM | No effects |
| | | Intracellular/1 mM | No effects |

[a] Effects after extra- or intracellular application (microinjection). Symbols: p, permeable; ip, impermeable; ↑Ca, $[Ca^{2+}]_i$ increase; exo, trichocyst exocytosis induction (scaled from − to +++, none to maximal); no effects, no induction of exocytosis and no inhibition of AED-induced exocytosis. Data compiled from Länge *et al.* (1995) and Blanchard (1998).

How can exocytosis of extrusomes be triggered? We refer to Hausmann (1978) for a review on the occurrence of extrusomes in ciliates. Electric stimulation by low-voltage alternating current (Hausmann and Allen, 1976) or picric acid (Jennings, 1906) are unspecific methods. The latter, though a lethal fixative, is useful for fast tests of non-discharge genotypes in *Paramecium*. With *Tetrahymena*, Alcian blue is preferred for this purpose, because it can cause capsule formation from mucocyst materials. Use of $Ca^{2+}$-specific ionophores essentially gave the first hints of the relevance of $[Ca^{2+}]_i$ increase for extrusome exocytosis in ciliates (Plattner, 1974). *Dileptus* and *Didinium* secrete toxicysts in contact with a ciliate as a prey, whereby the stimulative agent, possibly a cell surface component, is unknown. Interaction between the prey and the predator cell causes $[Ca^{2+}]_i$ increase in both cells (Iwadate *et al.*, 1999a).

For work with *Paramecium*, aminoethyldextran (AED), introduced by our laboratory (Plattner *et al.*, 1984, 1985b), has been widely adopted. It mimics different effects observed during "natural" stimulation by contact with *Dileptus* (Knoll *et al.*, 1991b), including $[Ca^{2+}]_i$ increase (Iwadate *et al.*, 1997). Whereas in this case toxicysts may cause a $[Ca^{2+}]_i$ signal by $[Ca^{2+}]_o$ leakage into cells due to toxic effects or mechanical damage by perforation, such a side effect was rigorously excluded with multiple AED applications (Plattner *et al.*, 1985b).

In *Paramecium*, induction of trichocyst exocytosis by AED depends on the formation of a $[Ca^{2+}]_i$ signal that is locally restricted and nonpropagated (Erxleben *et al.*, 1997; Klauke and Plattner, 1997), as is the exocytotic response (Plattner *et al.*, 1984). Both the $[Ca^{2+}]_i$ response and exocytosis are abolished by $Ca^{2+}$ buffer injection (Erxleben and Plattner, 1994; Klauke and Plattner, 1997). Alternatively, photoactivation of caged $Ca^{2+}$ induces local trichocyst exocytosis (Iwadate *et al.*, 1999b). In *Paramecium*, the exocytotic response is inactivated by $Ca^{2+}$ buffer injection, when $[Ca^{2+}]_i$ is quenched to between 1 and 10 $\mu M$ (Klauke and Plattner, 1997). Though this is considered the most reliable approach to reveal the true values required (Section II.D), it also is liable to underestimate the $[Ca^{2+}]_i^{act}$ required, particularly in narrow spaces like organelle docking domains (Kits *et al.*, 1999). On the basis of our data, we assume the requirement of $[Ca^{2+}]_i^{act} \sim 5\ \mu M$ as a rough approximation.

The source of $Ca^{2+}$ during AED stimulation is dual: (i) mobilization from cortical stores and (ii) superimposed influx from the outside, (Plattner *et al.*, 1997a; Klsuke *et al.*, 2000). The first component can be seen at low $[Ca^{2+}]_o$ or during the application of AED or store-mobilizing agents, like caffeine (Klauke and Plattner, 1998) or 4CmC (Klauke *et al.*, 1999). Store mobilization alone causes only partial and slow activation of trichocyst exocytosis (Plattner *et al.*, 1997a). Another stimulant of exocytosis causing a local $[Ca^{2+}]_i$ increase from intra- and extracellular sources is the plant

FIG. 7 ESI analysis of calcium distribution in a *Paramecium* cell at rest (a) or 80 ms after AED stimulation (b). Cells were prepared by quenched-flow and freeze–substitution under conditions retaining Ca. The false color distribution represents Ca signals identified in the energy loss spectrum (c). Note the enrichment of Ca in alveolar sacs (as) and the absence from cilia (ci) and trichocysts (t) at rest, whereas intense signals occur along the plasmamembrane (pm) and in cilia after stimulation (none in the trichosyst ghost, tg). From Knoll *et al.* (1993).

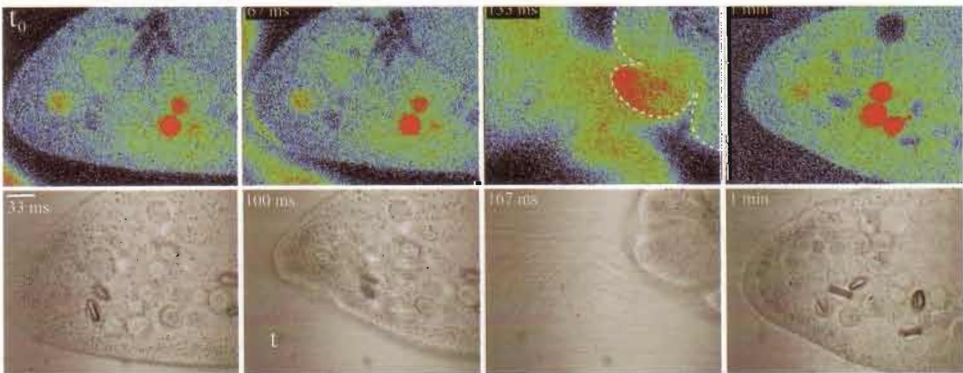



FIG. 8 AED stimulates trichocyst exocytosis in a *Paramecium* cell. Trichocysts (t) are released as soon as AED (2 $\mu M$, $[Ca^{2+}]_o$ = 50 $\mu M$, contact of trigger solution visualized by $F_2$FITC added) touches the surface of the cell loaded with 100 $\mu M$ Fluo-3 for fast CLSM false-color $[Ca^{2+}]_i$ imaging. Bar = 10 $\mu$m From Klauke and Plattner (1997)

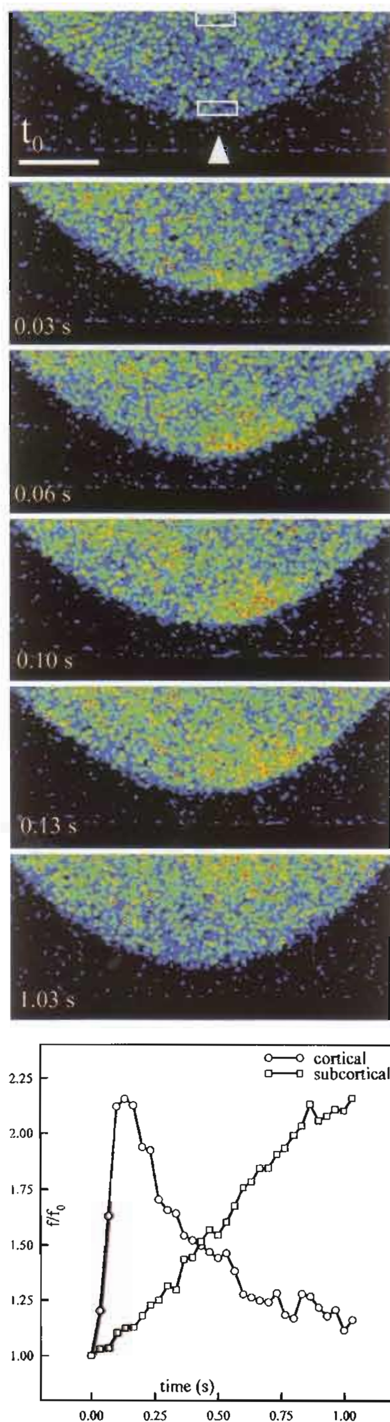



FIG. 9 A *Paramecium* cell (Fluo-3 loaded for false-color CLSM $[Ca^{2+}]_i$ imaging) develops a cortical $Ca^{2+}$ signal even when stimulated by AED in the presence of ultrafast $Ca^{2+}$ chelator, BAPTA, briefly yielding $[Ca^{2+}]_o \sim 30$ n*M* during stimulation. Note the time scale from $t_0$ on and the evaluation of framed cell regions in the diagram. For quantitative evaluation, see Fig. 11. Bar = 10 $\mu$m.

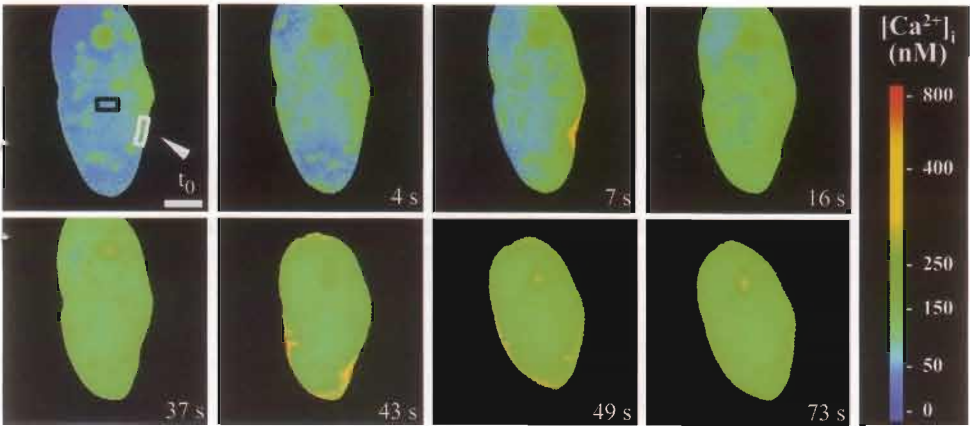



FIG. 10 Fura-red-loaded *Paramecium* cell with extracellular application (at arrowhead) of 100 μ$M$ thapsigargicin at $t_0$. $[Ca^{2+}]_o$ = 50 μ$M$. Note the slow development of a $Ca^{2+}$ signal, first in the cell cortex and then throughout the cell. Bar = 20 μ$m$. From Blanchard (1998).

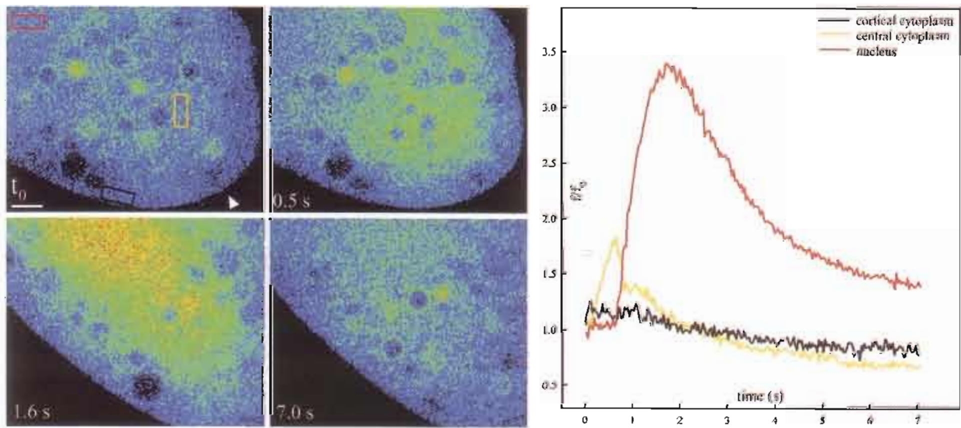
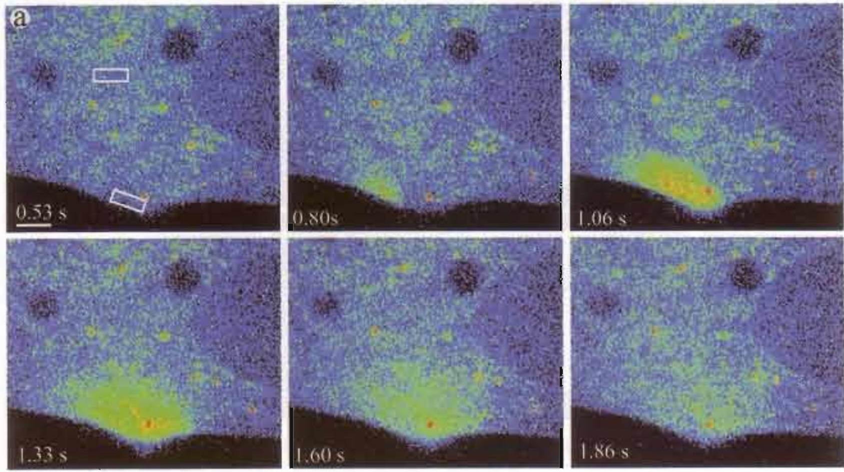
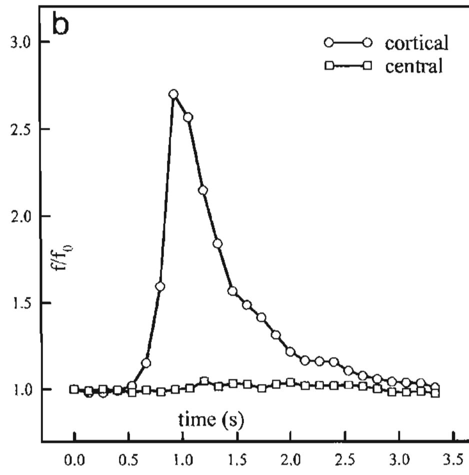



FIG. 18 Fluo-3-loaded *Paramecium* cell during the application of AED (2 μ$M$) + BAPTA (1 m$M$), briefly resulting in $[Ca^{2+}]_o$ ~ 30 n$M$, during stimulation. Frames indicate areas evaluated. This type of nuclear $Ca^{2+}$ signal was also observed, at low $[Ca^{2+}]_o$ with caffeine or 4CmC application (not shown).

FIG. 19 *Paramecium caudatum* strain tnd1 [unpublished results; see Klauke *et al.* (1998) for collaboration with N. Haga and T. Watanabe]. Cells loaded with Fluo-3 for fast $f/f_0$ ratio analysis by CLSM at $[Ca^{2+}]_o$ = 50 μ$M$. Spontaneous $[Ca^{2+}]_i$ oscillations start in the oral region (a) and propagate with a ~8-s period (c); the period shown is evaluated in (b). Remarkably, in this cell population, ~30% of cells underwent conjugation. The periodic signals may be involved in nuclear or any other activities related to this process.



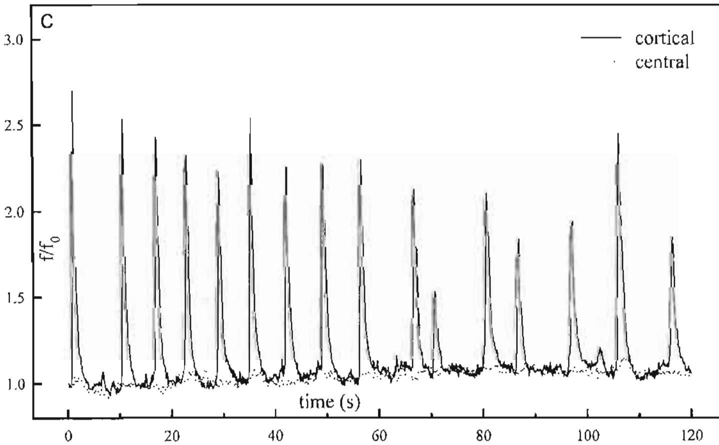




FIG. 19

alkaloid, veratridine (Knoll *et al.*, 1993; Plattner *et al.*, 1994; Blanchard, 1998; Blanchard *et al.*, 1999). Although it normally acts as a $Na^+$ channel agonist, we have found evidence that it may cause stimulation by labilizing membranes, increasing their fluidity and resulting in increased $Ca^{2+}$ fluxes. [Accordingly, veratridine can also activate ciliary $Ca^{2+}$ channels (Schultz and Schade, 1989a).]

Channels involved in $Ca^{2+}$ release from alveolar sacs and quantitative $Ca^{2+}$ fluxes occurring during AED stimulation are discussed in Sections III.C.3 and III.E.4, respectively. For the $Ca^{2+}$ channels involved and a $Ca^{2+}$ sensor in the cell membrane, see Sections III.C.1 and III.C.2, respectively. Briefly, we assume that AED activates a $Ca^{2+}$/(polyvalent cation)-sensing receptor (CaSR; Klauke *et al.*, 1999), which activates ryanodine-receptor-type $Ca^{2+}$-release channels in alveolar sacs. This occurs without the involvement of any of the known second messengers (Länge *et al.*, 1995). $Ca^{2+}$ release from stores activates a store-operated $Ca^{2+}$ influx (SOC-type mechanism).

$Ca^{2+}$ signals occurring upon AED stimulation, with normal or low $[Ca^{2+}]_o$ (~30 n*M*) in the medium, are recorded in Fig. 11. Note that signals still occur, though to a smaller extent, even when $[Ca^{2+}]_o < [Ca^{2+}]_i^{rest}$. This indicates the occurrence of store mobilization independent from influx, which normally is superimposed.

Substitution of $Mn^{2+}$ for $Ca^{2+}$ in the medium during AED stimulation of Fura-2-loaded cells results in fluorescence quenching, indicative of unspe-

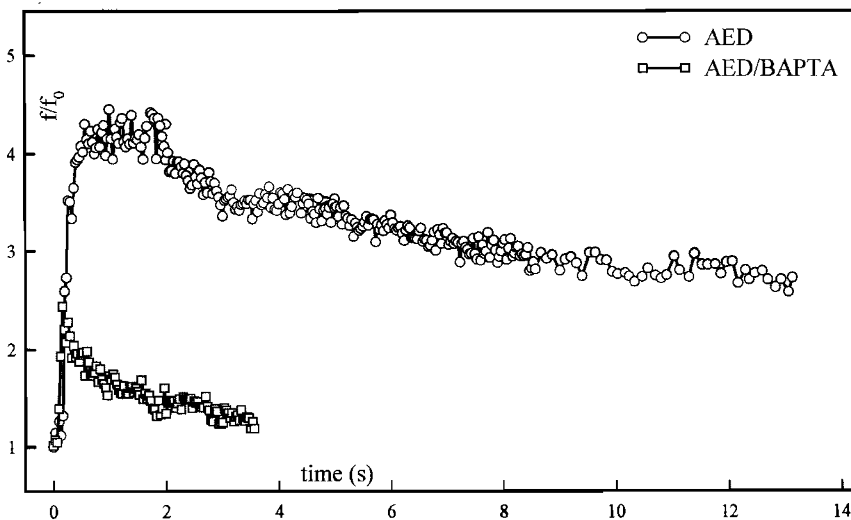



FIG. 11 Quantitative evaluation of the $Ca^{2+}$ signal obtained as in Fig. 9 (AED + BAPTA) or, alternatively, with AED in the presence of $[Ca^{2+}]_o = 50\ \mu M$.

cific cation channels serving for $Ca^{2+}$ influx (Blanchard *et al.*, 1999; Klauke *et al.*, 2000). Because substitution of $Sr^{2+}$ for $Ca^{2+}$ in the medium during 80-ms AED stimulation results in the uptake of $Sr^{2+}$ into alveolar sacs, according to quenched-flow/EDX experiments (Hardt and Plattner, 2000), we conclude that normally $Ca^{2+}$ influx (as mimiced by $Sr^{2+}$) serves to refill alveolar sacs during the time of $Ca^{2+}$ release. This may serve to intensify the signal and to direct $Ca^{2+}$ flux to strategic sites, i.e., the trichocyst release sites. $Ca^{2+}$ (or $Sr^{2+}$) influx seems to take place via unspecific cation channels (Klauke *et al.*, 1999). Such channels occur in *Paramecium* (Saitow *et al.*, 1997), and they are known to mediate a SOC-type mechanism in mammalian cells (Krause *et al.*, 1996). Another similarity is the activation of such $Ca^{2+}$ fluxes by exogenous polyamines (Williams, 1997a,b; Gamberucci *et al.*, 1998). However, we admit that we cannot yet explain the modus operandi of the very rapid delivery of $Ca^{2+}$ (or $Sr^{2+}$) into alveolar sacs, though any pump activity can reasonably be excluded (Section III.E.4).

Does CaM participate in exocytosis regulation? The $K_d$ (Section III.A) for its more sensitive $Ca^{2+}$-binding loops would correspond to the $[Ca^{2+}]_i$ required for trichocyst exocytosis in *Paramecium* cells (Klauke and Plattner, 1997), and CaM has been localized to trichocyst docking sites (Momayezi *et al.*, 1986) whose assembly depends on CaM expression (Kerboeuf *et al.*, 1993). The latter may argue for a specific role in the assembly process. No clear answer is available from higher cells. Yet in catecholamine- or neuropeptide-secreting cells (Hens *et al.*, 1998), just as in *Paramecium* cells (Momayezi *et al.*, 1987b), the activation of PP2B (CaN) has been proposed to be a crucial step during exocytosis on the basis of inhibitory antibody effects in both cases.

The role of the $Ca^{2+}$ signal for membrane fusion and pore expansion is shown schematically in Fig. 12.

The fusion pore formed during trichocyst secretion in *Paramecium* is, as soon it can be recognized, only ~10 nm in size on freeze–fracture replicas, whereby its formation was estimated to require ≤1 ms (Momayezi *et al.*, 1987a; Plattner *et al.*, 1993). In the context of work with other secretory cells, we derived the "focal fusion concept" (Plattner, 1981, 1989; Knoll and Plattner, 1989; Plattner and Knoll, 1993; Plattner *et al.*, 1993), which also included fusion regulation by locally assembled, though unidentified, proteins. In fact, we were aware that these estimates are at the limits of temporal and spatial resolution available (Plattner *et al.*, 1992). According to conductivity measurements by patch-clamp analysis, both values may be ~10 times smaller in systems amenable to this methodology (Almers, 1990).

The expansion of the fusion pore also depends on elevated $[Ca^{2+}]_i$ (Hartmann and Lindau, 1995), possibly by the mediation of a large 145-kDa CaBP called CAPS [from calcium-dependent activator protein for secretion. (Elhamdani *et al.*, 1999)], which so far is known only from neuronal and

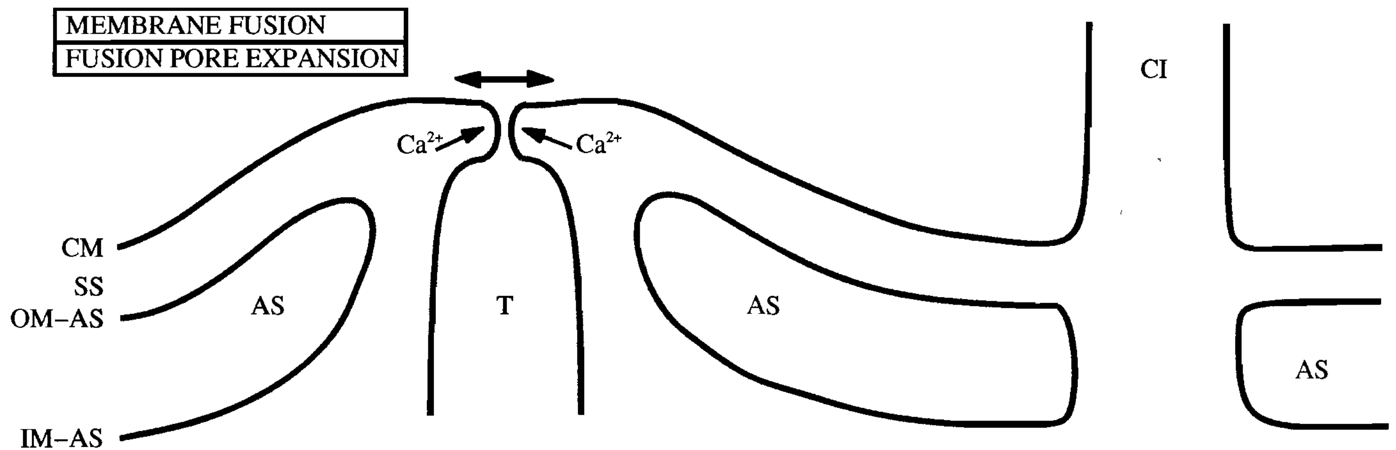



FIG. 12 $Ca^{2+}$ requirement for exocytotic membrane fusion and pore expansion is indicated by the respective arrows. For abbreviations, see Fig. 2.

neuroendocrine cells. Because during trichocyst release an exocytotic opening expands to the diameter of its body (Olbricht *et al.,* 1984), we should be prepared for a similar mechanism.

*Conclusions.* Although exocytotic membrane fusion requires a $[Ca^{2+}]_i$ increase to $\sim 5\ \mu M$ in ciliates, their $Ca^{2+}$ sensor is unknown. We have evidence that increased $[Ca^{2+}]_i$ drives fusion pore expansion. For both of these (and the subsequent) processes to take place optimally, mobilization from alveolar sacs has to precede $Ca^{2+}$ influx occurring by a SOC-type mechanism.

## 2. Secretory Content Discharge

The content of secretory organelles frequently is acidic, with a lumenal pH of 5–6 (Mellman, 1992), and enriched in $Ca^{2+}$, with $[Ca] \leq 50$ m$M$ (Winkler, 1977; Nicaise *et al.,* 1992). Both of these properties were considered to contribute to the maturation and condensation of the content, organelle targeting, and release upon contact with extracellular medium. However, we assume that this cannot be generally applicable to all dense-core vesicles.

Trichocysts of *Paramecium* are not remarkably acidic compartments (Lumpert *et al.,* 1992; Garreau De Loubresse *et al.,* 1995), and no Ca signals are detected in resting trichocysts by EDX (Schmitz *et al.,* 1985; Schmitz and Zierold, 1989; Zierold *et al.,* 1989), as we have confirmed (Hardt *et al.,* 1998; Hardt and Plattner, 2000). On the contrary, access of $[Ca^{2+}]_o$ to trichocyst content after the formation of an exocytotic pore is prerequisite to normal release (Bilinski *et al.,* 1981a) by vigorous decondensation. This is visible in the light microscope by stretching severalfold, from carrot (inside) to needle shape (outside cell). This decondensation process is $Ca^{2+}$-dependent and can be used to test the integrity of trichocyst membranes after organelle isolation (Lima *et al.,* 1989; Glas-Albrecht and Plattner, 1990). Alternatively, in cells, the formation of exocytotic pores can be induced, whereas trichocyst content is retained in condensed form when $[Ca^{2+}]_o$ is kept $\leq 10^{-6}\ M$ (Matt and Plattner, 1978; Plattner *et al.,* 1985b, 1997a). A schematic outline of this process is presented in Fig. 13.

The molecular basis of trichocyst or mucocyst decondensation is the occurrence of CaBPs in secretory content whose modification in *Tetrahymena* impedes content release (Chilcoat *et al.,* 1996), similar to the spontaneous non-discharge mutant tnd1 in *P. caudatum* (Watanabe and Haga, 1996; Klauke *et al.,* 1998). In tnd1 cells, $^{45}Ca^{2+}$ binding of several bands is reduced in gel overlays, the precise genetic basis remaining to be established. In *Tetrahymena,* this $Ca^{2+}$ sensitivity develops during proteolytic processing of mucocyst content (Verbsky and Turkewitz, 1998). Any $Ca^{2+}$-dependent biosynthetic processes during maturation thus can be exeuted without internal "explosion." More data are required to understand the incapability of

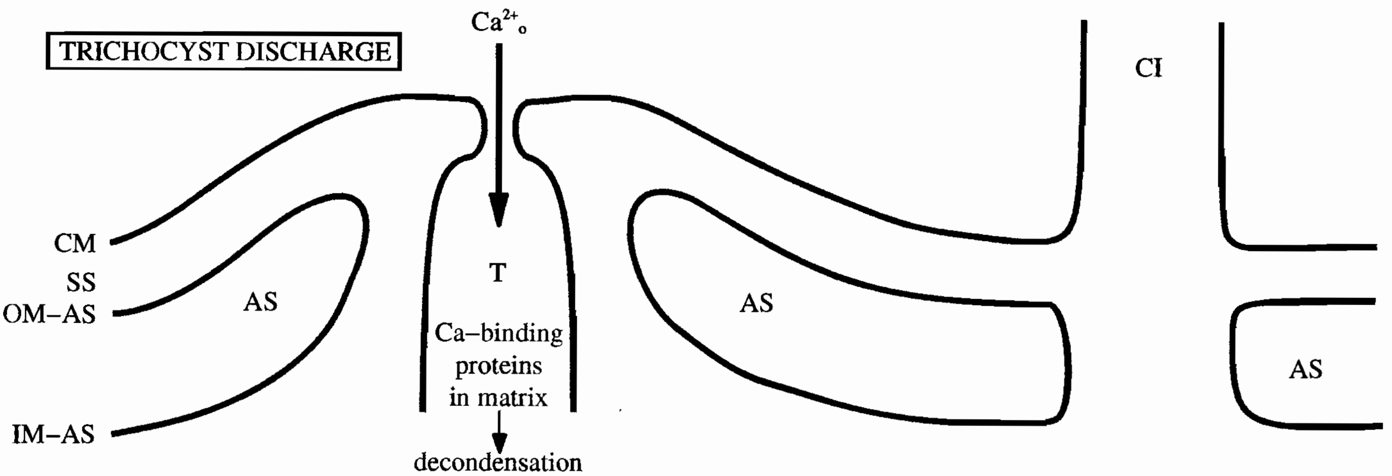



FIG. 13 An influx of $Ca^{2+}$ from the outside is required to trigger content decondensation via binding to some secretory CaBPs. For abbreviations, see Fig. 2.

the trichocysts of some *P. tetraurelia* strains to perform decondensation of their trichocysts content (Pouphile *et al.,* 1986).

Even within the cell, trichocyst content can be forced to undergo decondensation. This artifactual "internal decondensation" takes place when trichocyst membranes become permeable while $[Ca^{2+}]_i$ is high, e.g., in the course of ionophore application. A similar artifact is decondensation toward the outside in absence of membrane fusion—an artifact described by us as "pseudo-exocytosis" (Matt *et al.,* 1980). As a consequence, one cannot consider the absence of trichocyst release as an indicator of membrane fusion, because this may occur independently (Section III.E.1).

The secretory lectins detected in the trichocyst tips of *Paramecium* are another $Ca^{2+}$-binding component (Haacke-Bell and Plattner, 1987), yet their $Ca^{2+}$ requirement for ligand binding may be involved in functions to be determined.

*Conclusion.* In ciliates, the release of secretory proteins by "decondensation" is activated by binding $[Ca^{2+}]_o$, which gains access after the formation of an exocytotic opening.

### 3. Endocytotic Membrane Fusion and Detachment of "Ghosts"

Frequently exocytosis is coupled with subsequent endocytosis of empty "ghost" membranes. This exo-endocytosis coupling requires elevated $[Ca^{2+}]_i$ in a variety of cell types (Heinemann *et al.,* 1994; Henkel and Almers, 1996; Vogel *et al.,* 1999). It counteracts increase of the cell surface and maintains membrane specificity. In some cases retrieved membranes may be refilled.

Exo-endocytosis coupling also occurs after trichocyst release in *Paramecium* (Hausmann and Allen, 1976). After AED stimulation, this coupling is very fast [350 ms under standard conditions, i.e., at $[Ca^{2+}]_o = 500\ \mu M$ (Knoll *et al.,* 1991a)] and, therefore, operates without clathrin coat attachment (Plattner *et al.,* 1985a). More careful analysis by the quenched-flow method at variable $[Ca^{2+}]_o$ revealed that coupling speed increases with increasing $[Ca^{2+}]_o$ (Plattner *et al.,* 1997a). This implies increased efficiency of both membrane resealing during ghost detachment and increased internalization of ghosts with increasing $[Ca^{2+}]_o$, which causes more intense cortical $[Ca^{2+}]_i$ signals (Klauke and Plattner, 1997). Both of these processes can be detected on freeze–fracture replicas as follows. In the resting state, the particle "ring" delineating an exocytosis site contains a "rosette" of particles, which decays into subunits upon pore formation (Section III.E.I), whereas the ring collapses only as a ghost is detached (Olbricht *et al.,* 1984). In agreement with earlier analyses of ultrathin sections (Plattner *et al.,* 1985a), under standard conditions this occurs with $t_{1/2}$ = ~10–20 min,

depending on the strain, but it is much faster as $[Ca^{2+}]_o$ is increased (Plattner *et al.*, 1997a). Evidently sole $Ca^{2+}$ mobilization from alveolar sacs without additional influx yields $[Ca^{2+}]_i$ signals that are too weak to drive exo-endocytosis coupling to a maximal extent.

*Conclusion.* Endocytotic membrane fusion and "ghost" detachment are accelerated by increased $[Ca^{2+}]_i$ resulting from mobilization by alveolar sacs and from $Ca^{2+}$ influx.

### 4. $Ca^{2+}$ Fluxes and Reestablishment of $Ca^{2+}$ Homeostasis

By combining fluorochrome and EDX analyses with $^{45}Ca^{2+}$ flux measurements, we can estimate total Ca flux in *Paramecium* during exo-endocytosis stimulation (see discussion to follow). As we shall see, total Ca flux exceeds the values expected from $[Ca^{2+}]_i$ imaging by orders of magnitude. Balance calculations indicate that cytosolic CaBPs contribute considerably to the reestablishment of $Ca^{2+}$ homeostasis and that mitochondria may serve only briefly as an intermediary $Ca^{2+}$ buffering compartment, whereas kinetic properties of $Ca^{2+}$ pumps imply only long term effects. For comparison, in the SR, SERCA activity increases with $[Ca^{2+}]$ and, at resting $[Ca^{2+}]$, overcompensates for leakage by about one order of magnitude (Berman, 1999).

Though in a simpler way, $Ca^{2+}$ flux and reinstallation of homeostasis accompanying ciliary activity also face some complications. A particularly fascinating aspect will be how the activities of both of these target structures can be regulated differentially. These aspects will be dealt with separately in Section III.D.

Let us first consider $Ca^{2+}$ flux during stimulated exo-endocytosis and then the counteracting mechanisms, notably the activity of $Ca^{2+}$ pumps.

As outlined in Sections III.E.1 and III.E.3, exocytotic membrane fusion, fusion pore expansion, endocytotic membrane fusion, and ghost detachment are driven by a cortical $[Ca^{2+}]_i$ increase to $\sim 5\ \mu M$ (Klauke and Plattner, 1997) resulting from the mobilization from alveolar sacs and superimposed influx (Plattner *et al.*, 1997a). For a pilot calculation of these two components of $Ca^{2+}$ flux, values are available only from AED stimulation of *Paramecium*. Because $[Ca]_i$ largely depends on $[Ca^{2+}]_o$ in *Paramecium* (Browning and Nelson, 1976), we normally keep cells at $[Ca^{2+}]_o = 50–500\ \mu M$ before starting experiments. Under these conditions, cells had a volume of 0.733 $\times 10^{-10}$ l (73,300 $\mu m^3$) and a surface area of 10,703 $\mu m^2$. From this we derive (between the cell membrane and outer alveolar sac membrane, width $\sim$15 nm) a volume of 161 $\mu m^3$ for the subplasmalemmal space (Erxleben *et al.*, 1997). We assume that not only $Ca^{2+}$ influx over somatic channels (Section III.C.1) but also efflux from alveolar sacs will occur primarily across the subplasmalemmal space (Section III.C.3). From this we can

calculate theoretical $[Ca^{2+}]_i$ (dissolved) or $[Ca]_i$ (total) increase, either (i) for this space with its strategic targets or (ii) for the entire cell due to centripetal spread and (iii) for $[Ca^{2+}]_i^{act}$ reduction by binding to cytosolic CaBPs, sequestration, and expulsion.

Let us first consider theoretical values obtained by EDX analysis (for which Fig. 14 is an example) without considering counteracting mechanisms. Alveolar sacs contain [Ca] = 43 m*M* (referenced to organelle volume). From their thickness of 98 nm (Hardt and Plattner, 2000), a total volume of 1052 $\mu m^3$ is derived. If all $Ca^{2+}$ were mobilized upon stimulation, [Ca] in the subplasmalemmal space would theoretically result in [Ca] = 281 m*M* (disregarding gradient reversal), whereas in reality $Ca^{2+}$ is diluted over the entire cell to a global $[Ca]_i$ = 0.617 m*M* (assuming equal distribution, which is not entirely fulfilled; see below discussion to follow). Considering that

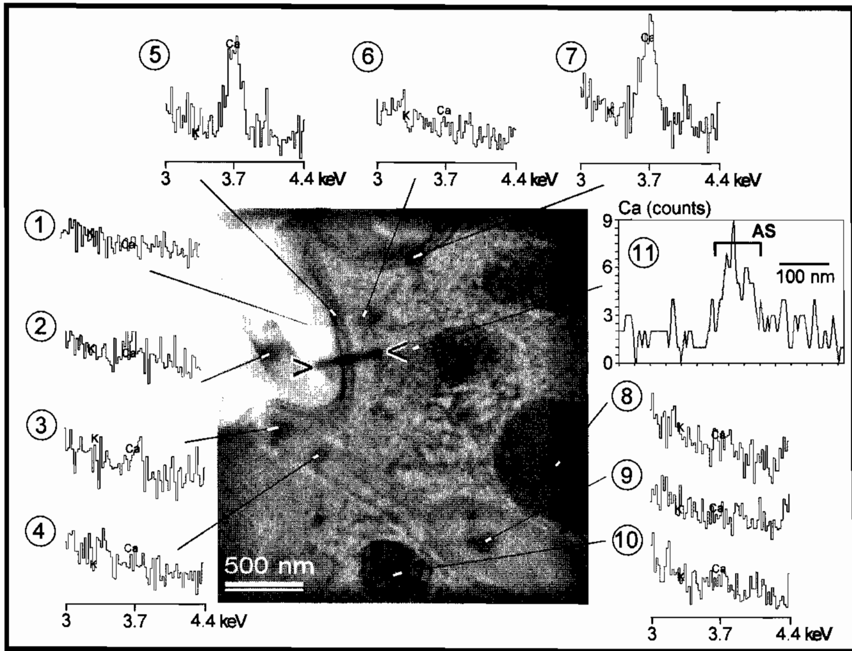



FIG. 14 Example of EDX analysis (in conjunction with imaging in the scanning transmission mode) of a resting *Paramecium* cell processed by quenched-flow and freeze–substitution under conditions retaining Ca. Only a part of the energy spectrum (containing the $Ca_{K\alpha}$ signal) is shown for the registration sites recognizable by black contamination spots or the line scan (for the $Ca_{K\alpha}$ signals, position 11). Positions 1–10 are as follows: extracellular medium (1); cilium (2); trichocyst tip (3, 4); alveolar sac (5, 7, and line scan in 11); cytosol (6); mitochondrium (8, 10); and trichocyst matrix (9). Note the selective occurrence of Ca signals in alveolar sacs. From Hardt *et al.* (1998).

only 40% of Ca is mobilized from alveolar sacs during the 80 ms required for synchronous exocytosis (Hardt and Plattner, 2000), the preceding data would have to be corrected to theoretical values of 112 and 0.247 m*M*, respectively. These estimations may now be compared with similar pilot calculations for the $Ca^{2+}$-influx component.

During AED stimulation, $Ca^{2+}$ influx amounts to $5 \times 10^{-15}$ mol/cell (Kerboeuf and Cohen, 1990). Referenced to the cell volume ($0.733 \times 10^{-10}$ l; Erxleben *et al.,* 1997), the resulting global [Ca] increase would be 0.068 m*M* or, theoretically, 4.74 m*M* in the subplasmalemmal space (again disregarding gradient reversal and counterregulations). However, the influx value obtained by Kerboeuf and Cohen (1990) at $[Ca^{2+}]_o = 40\ \mu M$, whereas we determined a swift acceleration of exo-endocytotic mechanisms with increasing $[Ca^{2+}]_o$ beyond this value (Plattner *et al.*, 1997a). Therefore, a linear activation increase may be assumed for the $[Ca^{2+}]_o = 500\ \mu M$ that we used, e.g., for quenched-flow and quantitative EDX analyses (Hardt and Plattner, 2000). A similar relationship has been ascertained for HeLa cells (Bootman *et al.,* 1996). The preceding values may be corrected accordingly. Then the global $[Ca]_i$ increase may reach 0.85 m*M,* whereas the theoretical value in the subplasmalemmal space would amount to 59.3 m*M* (disregarding gradients), if centripetal $Ca^{2+}$ spread and cytosolic binding were not rapid enough (though they evidently are).

Comparison of these theoretical values (though disregarding counteracting mechanisms) with each other and with real $[Ca^{2+}]_i^{act}$ measured is appropriate to derive the following conclusions. (i) The amount of Ca contributed by mobilization from cortical stores is comparable to that provided by influx, e.g., at $[Ca^{2+}]_o = 500\ \mu M$. (ii) If one assumes that $Ca^{2+}$ influx serves to refill aveolar sacs as they release their $Ca^{2+}$ upon AED stimulation, the preceding data can well explain that [Ca] in alveolar sacs does not change during the 80 ms required for synchronous trichocyst exocytosis (Hardt and Plattner, 2000). Entry into alveolar sacs would not be against a concentration gradient because most of the Ca will be in bound form (Sections III.C.3 and III.C.4) and free $Ca^{2+}$ will decrease during release, thus driving refilling. This intense coupling of release from stores and refilling is without precedent in secretory systems, although cooperation of internal and external $Ca^{2+}$ sources is widely distributed (Section II.B). (iii) Only a small fraction of $[Ca^{2+}]_i$ remaining in free form serves to activate the exo-endocytotic process.

In reality, $Ca^{2+}$ from internal and external sources will spread rapidly in centripetal fashion and become bound to cortical CaBPs, notably to CaM on the cell membrane and at trichocyst docking sites, to cortical annexin-related proteins, and to copines (Section III.A), as well as to cortical contractile filaments (Section III.B) whose contraction accompanies massive exocytosis stimulation. Because of these mechanisms all acting in concert,

free $Ca^{2+}$ is downregulated rapidly. In fact, in all cells analyzed so far, $[Ca^{2+}]_i^{act}$ is much lower than $[Ca]_i$, as was discussed in Section II.C.

Another rapid counterregulation process is sequestration into mitochondria. We found by EDX that about one-half of the cortical $[Ca]_i$ increase is fed rapidly into mitochondria (Hardt and Plattner, 2000). Mitochondria are known from mammalian systems to act as Ca stores when cells are heavily loaded with Ca (not just in pathological events, as has been previously assumed). This operates via a very fast $Ca^{2+}$ uniporter uptake (secondary active) mechanism and a $Na^{+}/Ca^{2+}$ antiporter release mechanism (Duchen *et al.,* 1998; Rutter *et al.,* 1998; Chakraborti *et al.,* 1999; Csordás *et al.,* 1999; Korzeniewski, 1999). The cortical enrichment of mitochondria in *Paramecium* allows for rapid $Ca^{2+}$ buffering, though they subsequently release $Ca^{2+}$ again quite rapidly (Hardt and Plattner, 2000). The retention time seems to depend on $[Ca^{2+}]_o$, i.e., on the amount of $Ca^{2+}$ influx. In the end, cytosolic binding may predominate.

The complex interplay between $Ca^{2+}$ mobilization from stores, $Ca^{2+}$ influx from the outside, and $Ca^{2+}$ dispersal within the cells is summarized in Fig. 15.

Because only $[Ca^{2+}]_i^{act} = 5\ \mu M$ is required to drive exo-endocytosis (Klauke and Plattner, 1997), actual [Ca] values calculated previously surpass these requirements by far (Plattner *et al.,* 1997a). This is not unique to *Paramecium* (and probably to other ciliates); a similar excess also occurs during exocytosis stimulation in other cells (Section II.C). One could exaggerate mildly and say that the cell body could only serve as a sink to accommodate this large excess of Ca during stimulation. However, realistic reasons may be (i) the necessity to overcome the highly efficient counterregulating mechanisms mentioned that are activated as soon as $[Ca^{2+}]_i$ increases and that normally may impede exocytosis to occur in the absence of a stimulus. (ii) Later steps of the exo-endocytotic cycle, up to "ghost" retrieval, also require increased $[Ca^{2+}]_i$ (Plattner *et al.,* 1997a)—another reason for the cell to work with a large excess. (iii) It cannot yet be excluded that $Ca^{2+}$ waves spreading into the cell center are important to activate some internal processes (see Section III.G).

Downregulation of $[Ca^{2+}]_i$ by $Ca^{2+}$ pump activities is probably the slowest recovery process, as one may derive from the subsequent data. Just like other eukaryotic cells (Section II.C), ciliates posses two types of $Ca^{2+}$ pumps (ATPases), one in the plasmalemma and another in Ca-storage organelles (SERCA-type pump). Both are P-type ATPases, i.e., in an activity cycle a phospho intermediate is formed, and their respective sizes are ~130 and 105–110 kDa, respectively (Carafoli, 1991, 1994; Martonosi, 1992, 1995).

Either type of pump has been characterized and cloned in *Paramecium,* the large 130- to 135-kDa pump of the cell membrane (Elwess and Van-Houten, 1997), as well as the 105- to 110-kDa-sized pump of Ca stores (Hauser *et al.,* 1998). Their respective structures have some canonical basic

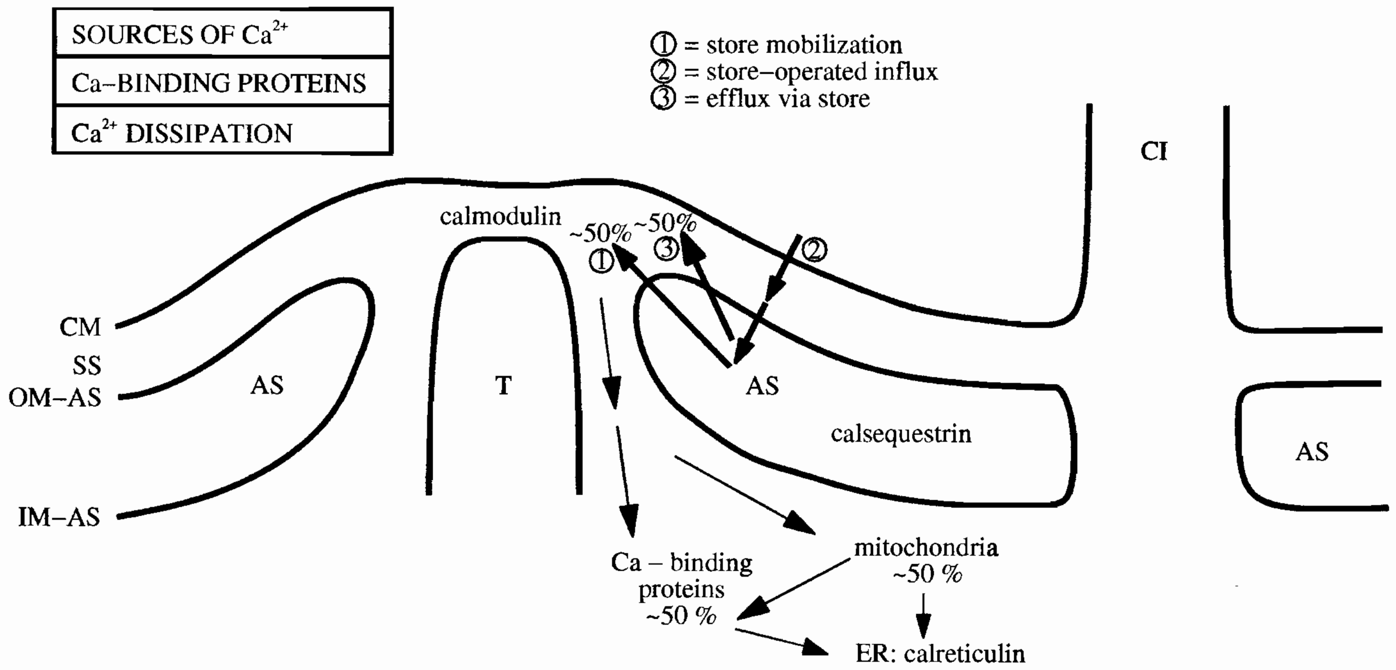



FIG. 15 Schematic representation of $Ca^{2+}$ flux occuring during AED-stimulated exocytosis in the presence of $[Ca^{2+}]_o$, as calculated and outlined in the text. Note the sequence of events, 1–3, and occurrence of specific CaBPs, such as CaM at exocytosis sites, calsequestrin in the alveolar sac lumen, and calreticulin in the ER. For abbreviations, see Fig. 2.

properties (see Section II.C), as they contain a nucleotide-binding site and a phosphorylation site, with arginine phosphorylation. Only the plasmalemmal pump possesses a CaM-binding domain for activation (Section III.A), opposite the store pump. So far, this SERCA-type pump has been localized only to alveolar sacs, though it should also occur in the ER (Section III.C.4).

Careful analysis of the SERCA pump of *Paramecium* (characterized in Fig. 16) has revealed several noncharacteristic features. It has low sensitivity to some classical inhibitors, like cyclopiazonic acid (Demaurex *et al.,* 1992), *tert*-butylhydroquinone (Nakamura *et al.*, 1992), and thapsigargin (Thastrup *et al.,* 1990; Inesi and Sagara, 1994), and some rare earth metal ions. Particularly striking is the low sensitivity to the plant sesquiterpene toxin, thapsigargin, which is highly active in most other systems even in nanomolar concentrations. In *Paramecium*, phospho intermediate formation may even be enhanced, rather than reduced, by $La^{3+}$, depending on the incubation protocol, and phospho intermediate formation is reduced by caffeine in concentrations used to stimulate the release of $Ca^{2+}$ from stores (Section II.B), as we found (Kissmehl *et al.,* 1998). The latter corresponds to the noncompetitive inhibition of $^{45}Ca^{2+}$-pumping activity of isolated alveolar sacs by caffeine (Länge *et al.,* 1996). With regard to the effect on chemosensory responses, Wassenberg *et al.* (1997) found decreasing efficiency in the order *tert*-butylhydroquinone, cyclopiazonic acid, and thapsigargin. Whereas this SERCA is insensitive to CaM, CaM antagonists, ruthenium red, or vanadate (Kissmehl *et al.,* 1998), the opposite holds true for the plasmalemmal $Ca^{2+}$ pump (Wright and VanHouten, 1990; Wright *et al.*, 1993), as expected.

The kinetic properties of these pumps are as follows. For the plasmalemmal $Ca^{2+}$-ATPase of *Paramecium,* Wright and VanHouten (1990) reported $K_m = 0.09\ \mu M$ and $v_{max} = 0.024\ \mu\text{mol} \times \text{mg}^{-1}$ protein $\times\ \text{min}^{-1}$ for $Ca^{2+}$ and $K_m = 0.075$ m$M$ for ATP, all under optimal conditions with maximal stimulation at $[Ca^{2+}] = 0.3\ \mu M$ ($pH_{opt} = 6.9$, $[Mg^{2+}] = 3$ m$M$). Because the authors largely relied on fractionation protocols previously published for pellicles (Bilinski *et al.,* 1981b; Doughty and Kaneshiro, 1985), they took great pains to exclude any significant contributions by $Ca^{2+}$-ATPase activity from contaminating stores. Refined methods to isolate somatic plasma membranes by Smith and Hennessey (1993) resulted in compatible values for specific activities of $0.0294\ \mu\text{mol} \times \text{mg}^{-1}$ protein $\times\ \text{min}^{-1}$ at $[Ca^{2+}] = 1\ \mu M$ for maximal stimulation. For isolated alveolar sacs, we determined the following values: $K_m = 5.8\ \mu M$ and $v_{max} = 0.33$ nmol $\times$ mg$^{-1}$ protein $\times$ min$^{-1}$ for $Ca^{2+}$; $K_m = 13.2\ \mu M$ and $v_{max} = 0.15$ nmol $\times$ mg$^{-1}$ protein $\times$ min$^{-1}$ for ATP (Länge *et al.,* 1996). [Kinetic data determined for alveolar sacs fractions by Stelly *et al.* (1991) are in between those reported here for these stores and cell membranes, possibly due to the contribution of activities they can inhibit by anti-CaM drugs.) Comparison of the preceding data for $Ca^{2+}$ kinetics of the alveolar sacs and the plasma-

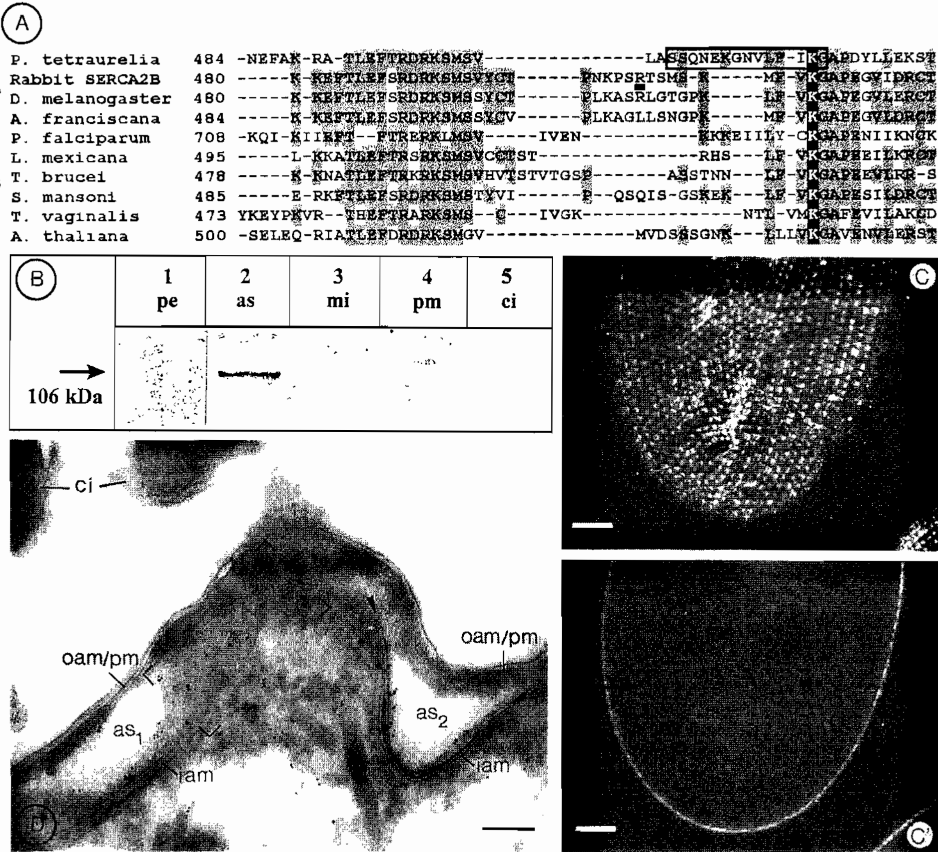



FIG. 16 A SERCA-type $Ca^{2+}$ pump occurs in the alveolar sacs of *Paramecium*. Its gene has been cloned. The amino acid sequence derived for one of the transmembrane domains shown in (A) reveals wide variation between species. Because this region is reported to bind the inhibitor, thapsigargin, this wide abberration from the mammalian form in *Paramecium* and some of the Apicomplexa may explain the low sensitivity to this drug. The framed region has been used to prepare ABs. Their application in Western blots (B) to pellicles (surface complex), alveolar sacs (as), microsomes (mi), plasma membrane fractions (pm), and cilia (ci) shows enrichment of this pump (106 kDa) in alveolar sacs. Immunolocalization in CLSM (C) or EM (D) analysis also shows the occurrence of the SERCA-type pump in alveolar sacs [patches in *en face* view (C), outlines in median view (C′)], notably their inner membrane [using a SERCA-specific AB, as specified in Plattner *et al.* (1999), which also recognizes SERCA in nearby ER]. Note the absence of gold label from the plasma membrane (pm) and the outer alveolar sac membrane (oam), in contrast to particularly heavy labeling at a site where an inner alveolar sac membrane (iam) is met in a tangential section ($as_1$), and also the occurrence of label along a cross-cut inner alveolar sac membrane (iam in $as_2$). Bars = 10 $\mu$m (C), 0.1 $\mu$m (D). (A–C) from Hauser *et al.* (1998); (D) from Plattner *et al.* (1999).

lemmal $Ca^{2+}$-ATPase clearly indicates that the latter is much more sensitive and efficient. We attribute this to the requirements of permanently counteracting $Ca^{2+}$ leakage on the cell surface and downregulating any $[Ca^{2+}]_i$ increase in the narrow subplasmalemmal space. To correct for such leakage, the SERCA pump could contribute little, as it is localized on that part of aveolar sacs that faces the cell interior (Plattner *et al.*, 1999). Also, filling of these stores *in vitro* takes place rather slowly (Stelly *et al.*, 1991; Länge *et al.*, 1995, 1996).

To what extent could, for example, the SERCA-type pump of alveolar sacs contribute to reestablishing $[Ca^{2+}]_i$ homeostasis in a *Paramecium* cell after AED stimulation? To give a baseline, in skeletal muscle SR, the rate of $Ca^{2+}$ reuptake is ~13 times slower than its release (Saiki and Ikemoto, 1999). Assume a global $[Ca]_i$ increase to 1.47 m*M*, i.e., 0.62 m*M* from stores and 0.85 m*M* from influx (under conditions specified earlier) referenced to cell volume; this would result in a total of $1.1 \times 10^{-13}$ mol of Ca (above basal level) in a cell. Its alveolar sacs would contribute 1070 $\mu m^3$ (cell surface area and compartment thickness given earlier), i.e., $10^{-12}$ l, and $10^{-7}$ mg of cell protein would be SERCA molecules (assuming the pump would contribute 10% to the organelle). To downregulate the $[Ca]_i$ increase mentioned in 1 min, the SERCA system of a cell would have to operate with an efficiency of $1.1 \times 10^{-13}$ mol of Ca/$10^{-7}$ mg of SERCA protein/min, whereas the $v_{max}$ value ($Ca^{2+}$) given previously requires much more protein to achieve similar pump activity (even if data were referenced to SERCA protein in the fraction). This is reasonable because even after stimulation the pump sees only ~0.1% of Ca in dissolved form (activating $Ca^{2+}$) that it has to handle. Hence, this pump (as well as the plasmalemmal one) may serve only for long-term regulation. This is in striking contrast to the very rapid $Ca^{2+}$ replenishment in alveolar sacs during mobilization on a subsecond time scale in the course of synchronous AED-triggered trichocyst exocytosis, as we could show by substituting $[Sr^{2+}]_o$ for $[Ca^{2+}]_o$ in quenched-flow/freeze substitution and EDX analyses (Hardt and Plattner, 1999, 2000). The underlying mechanism is unknown, but a primary active transport process appears to be out of the question.

The localization of these pumps in *Paramecium* faces some unexpected problems. As mentioned, the SERCA pump could be localized to the inner side of alveolar sacs (Plattner *et al.*, 1999), whereas its occurrence in the ER still has to be established. Equally intriguing is the distribution of the plasmalemmal $Ca^{2+}$ pump. Whereas its presence in the somatic membrane is well-established (see discussion to follow), does it also occur in ciliary membranes?

Cilia have been removed several times, particularly from *Paramecium* cells, and their membranes isolated. Thus, a $Ca^{2+}$-ATPase, represented by 60- to 68-kDa bands in gel electrophoresis, with a very high $K_m$ =

5.17 m$M$ for [$Ca^{2+}$] has been identified (Andrivon *et al.,* 1983), and similar data have been presented by other groups (Doughty and Kaneshiro, 1985; Travis and Nelson, 1986). This activity has been assigned to an ecto-ATPase and would have masked any subtle $Ca^{2+}$-pump activity in a variety of other publications, if it were to occur in ciliary membranes. In fact, this has been denied on the basis of biochemical (Wright *et al.,* 1993) and immunolabeling experiments using ABs against a peptide segment of the cloned molecule (Van Houten, 1998).

Though aspects pertinent to ciliary reversal were considered more thoroughly in Section III.D, the following mechanisms may be recalled in the present context. (i) Some $Ca^{2+}$ will be bound to CaBPs, including effector–target molecules in cilia (Section III.D). (ii) $Ca^{2+}$ may diffuse slowly into the cell body, followed by the respective inactivation mechanisms (see previous discussion). (iii) A $Na^+/Ca^{2+}$ exchanger, as described for *Euplotes* (Burlando *et al.,* 1999), could serve rapid $Ca^{2+}$ extrusion. In fact, the authors present evidence of its occurrence in the cell membrane on the basis of inhibitor studies (bepridil), deciliation, and electrophysiology. Considering the frequent ambiguity of drug effects in general, and of $Na^+/Ca^{2+}$ antiporter inhibitors in particular (Blaustein and Lederer, 1999), more work clearly is needed to settle this important question, as well as the localization of this antiporter to determine whether it may operate solely in the somatic membrane. As a rule, we see no activation of exocytosis in parallel to a ciliary reversal reaction, whereas the opposite effect does occur (Section III.D). In cardiac myocytes, for example, the $Na^+/Ca^{2+}$ exchanger is approximately four times more effective than the plasmalemmal $Ca^{2+}$ pump (Choi and Eisner, 1999).

For ciliates, no information is available on the occurrence of acidocalcisome-like organelles, which in *Trypanosoma* are endowed with a $H^+/Ca^{2+}$ exchanger (Scott *et al.,* 1997). Also unknown is the $Ca^{2+}$-uptake mechanism operating in calcium crystal vacuoles (Section III.C.4).

*Conclusions.* $Ca^{2+}$ flux occurring during an exo-endocytotic cycle (as during ciliary reversal) surpass the amount actually required for activation by orders of magnitude. This may be due to the rapid downregulation, notably by binding to cytosolic CaBPs and also by the $Na^+/Ca^{2+}$ exchanger, and, though much more slowly, by the $Ca^{2+}$ pump in the cell membrane and in the stores (Fig. 17).

### F. Constitutive Exo-endocytosis, Vesicle Traffic, and Internal Fusion Processes

For cell membrane growth and renewal of its constituents by the elimination of old ones and insertion of new ones, exo-endocytosis must occur perma-

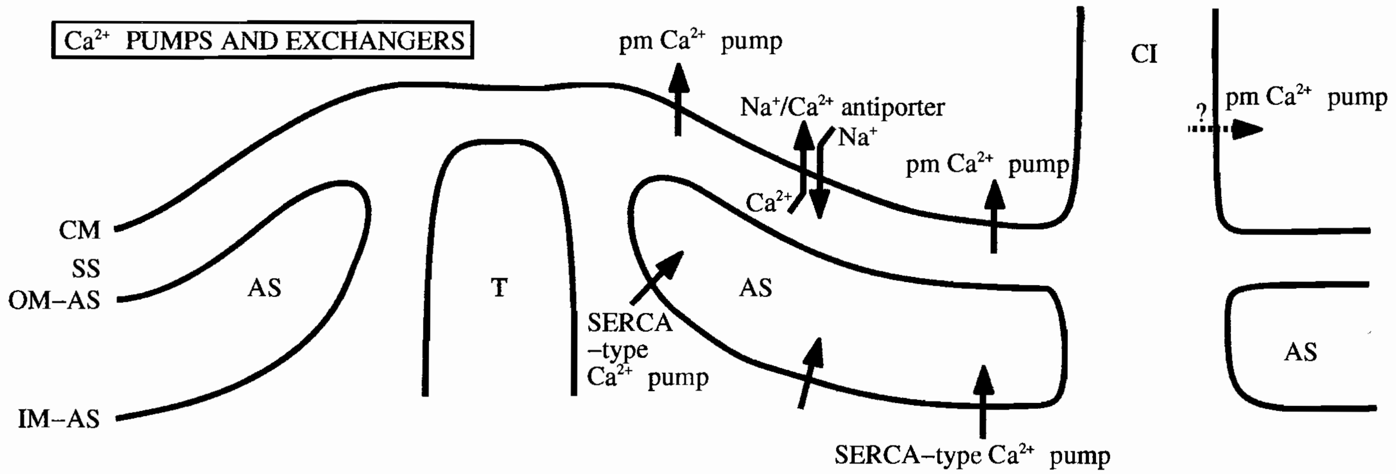



FIG. 17 Distribution of $Ca^{2+}$ pumps and a $Na^{+}/Ca^{2+}$ antiporter. The plasma membrane (pm) $Ca^{2+}$ pump may be absent from ciliary membranes, whereas the SERCA-type pump is enriched unilaterally within alveolar sacs, i.e., in their inner membrane regions. The localization of the $Na^{+}/Ca^{2+}$ exchanger is not known; it may occur in the somatic membrane (as drawn) and/or possibly also in the ciliary membrane. For abbreviations, see Fig. 2.

nently. Very little specific detail is known from ciliates, e.g., whether calcium may be required and where the respective exo- and endocytotic processes may take place (Capdeville *et al.*, 1993). Some details of the biosynthetic and degradative pathways of cell surface components (glycocalyx) in *Paramecium* cells have been elucidated using immunolabeling (Flötenmeyer *et al.,* 1999). Internalization occurs in part via food vacuoles. In addition, terminal cisternae, located below ciliary basal bodies, seem to be center stage for exo-endocytotic vesicle input and output activity via parasomal sacs. $Ca^{2+}$ here may exert a control function much as in higher eukaryotic cells. Pinching off of clathrin-coated vesicles requires dephosphorylation of the monomeric GTP-binding protein (G-protein), dynamin, by $Ca^{2+}$/CaM-activated protein phosphatase type 2B (PP2B = calcineurin, CaN) (Robinson *et al.,* 1994). CaN is characterized in more detail in Section III.A.

Detachment of nascent food vacuoles may depend on $Ca^{2+}$, as one may infer from the involvement of actin, probably actomyosin (Section III.B.1). With regard to vesicle traffic and internal fusion processes, e.g., during the digestive cycle in *Paramecium* (Allen and Fok, 1993; Fok and Allen, 1993) or *Tetrahymena* (Tiedtke *et al.*, 1993), few data are available from ciliates. In this context, calcium stores in the widely branched ER (Section III.C.4) may be important. Work with isolated vacuoles from yeast has revitalized an old paradigm, as it showed that release of $Ca^{2+}$ from the organelles may drive membrane fusion (Peters and Mayer, 1998). In *Tetrahymena,* early phagosomes are covered by a 25-kDa protein that, according to partial sequencing, represents a CaBP (Maicher and Tiedtke, 1999). More extensive analysis of such precisely timed vacuolar stages will yield important clues to the relevance of CaBPs for intracellular trafficking. The elaborate fusion processes occurring in the Golgi apparatus, e.g., of *Paramecium* (Garreau De Loubresse, 1993), may be brought about by $Ca^{2+}$ from the organelle itself. This speculation is founded on the occurrence of a $Ca^{2+}$ pump (Sorin *et al.*, 1997; Dürr *et al.,* 1998) and the relatively high [Ca] in this organelle (Grohovaz *et al.,* 1996).

Although the site and mode of constitutive release of lysosomal hydrolases in *Tetrahymena* remain to be established, release is known to be $Ca^{2+}$-dependent (Florin-Christensen *et al.,* 1990) and, therefore, can be stimulated by $Ca^{2+}$-mobilizing agents (Tiedtke *et al.,* 1988, 1993).

*Conclusion.* We still have a lot to learn about $Ca^{2+}$ regulation of internal fusion processes, not only in ciliates. In ciliates, any role of CaN- and dynamin-mediated processes still has to be established.

## G. Nuclear Functions

In higher eukaryotes, $Ca^{2+}$ is causally engaged in (i) in gene transcription and (ii) apoptosis. Any comment on the effects of $Ca^{2+}$ on nuclear functions

in ciliates is elusive at this time. Nevertheless, we address this topic not only because of theoretical considerations but also because we occasionally observed [$Ca^{2+}$] signals in the nucleus of *Paramecium* cells when stimulated at low [$Ca^{2+}$]$_o$ (Fig. 18; see also color insert).

A paradigm to transcription activation is the activation of T-lymphocytes, resulting in the rejection of heterotransplants. This involves the activation of calcineurin (CaN), with the participation of a FK506-binding protein, as reviewed by Guerini (1997) and Klee *et al.* (1998). This drug, as well as cyclosporin A, suppresses $Ca^{2+}$/CaM-activated protein phosphatase 2B (PP2B) activity of CaN and tissue rejection, whereas another CaN antagonist, rapamycin, inhibits CaN effects without inhibiting its PP2B activity. Yet CaN regulates gene transcription in different mammalian cells (Shibasaki *et al.,* 1996) and in yeast (Jiang and Cyert, 1999). In *Paramecium,* CaN has been shown to occur by Western blot (Momayezi *et al.*, 1986) and biochemical analyses (Kissmehl *et al.*, 1997b) and by cloning the catalytic A-subunit (Hinrichsen *et al.,* 1995; EMBL accession number AF014922). However, no systematic studies have been done so far on drug effects, intracellular localization, or effects on the nuclear function of CaN.

Another aspect possibly related to a nuclear $Ca^{2+}$ signal is the induction of gene transcription after massive AED-stimulated exocytosis (Haddad and Turkewitz, 1997). It remains to be seen whether a nuclear [$Ca^{2+}$] increase might serve as a potential signal, whether CaN may be involved, and whether genes transcribed encompass $Ca^{2+}$ targets, channels, or pumps. It also appears worth testing whether $Ca^{2+}$ and/or CaN would be involved in encystment.

A rise in [$Ca^{2+}$]$_i$ initiates micronuclear migration as the earliest nuclear event in the fertilization of *Paramecium* cells (Xianyu and Haga, 1993) for reasons to be elucidated. In *Tetrahymena,* the 25-kDa CaBP, TCBP-25 (Takemasa *et al.,* 1989), is concentrated at cell–cell contact sites during conjugation (Hanyu *et al.*, 1995).

Apoptotic effects may be envisaged even in ciliates, if one considers disruption of the macronuleus in the course of autogamy or conjugation as such a process with which it shares the diagnostic aspect of "DNA ladder" formation. However, as opposed to higher eukaryotes, no information is available on the involvement of a nuclear $Ca^{2+}$ signal in macronuclear disruption.

*Conclusion.* $Ca^{2+}$ effects on nuclear functions in ciliates are an open field for future research.

## H. Cell Division and Pattern Formation

Ciliates are important models to study cell surface pattern formation. Concerning the topic of this review, we are only at the very beginning of an

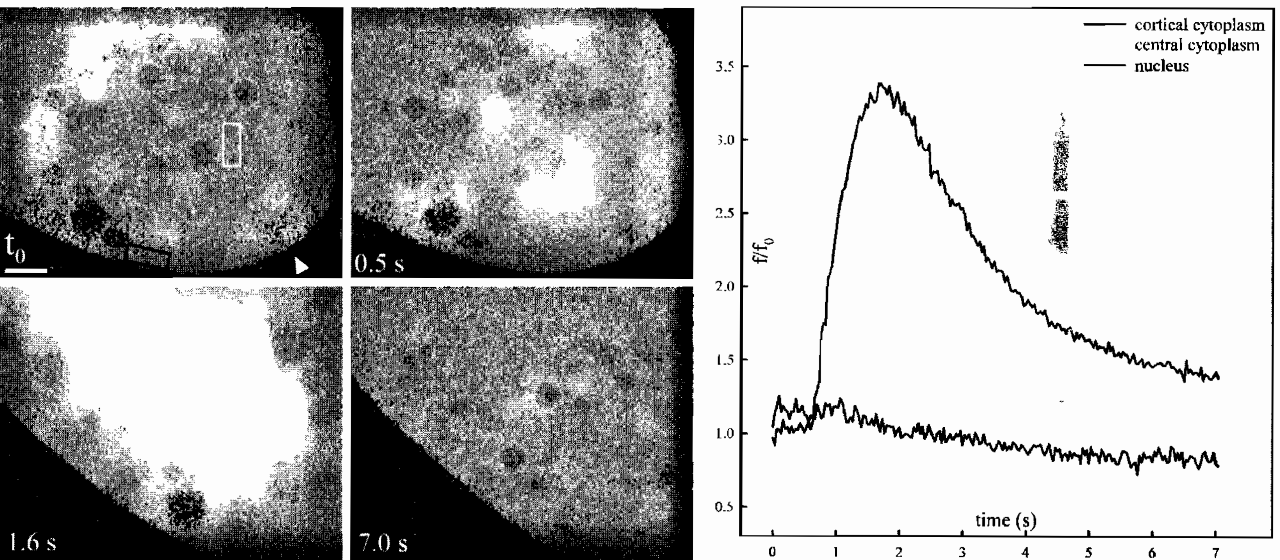



FIG. 18 Fluo-3-loaded *Paramecium* cell during the application of AED (2 $\mu M$) + BAPTA (1 m$M$), briefly resulting in $[Ca^{2+}]_o \sim 30$ n$M$, during stimulation. Frames indicate areas evaluated. This type of nuclear $Ca^{2+}$ signal was also observed, at low $[Ca^{2+}]_o$ with caffeine or 4CmC application (not shown). (See also color insert).

understanding of potential causal connections. Also without any further functional analysis, we have seen such spontaneous $[Ca^{2+}]_i$ oscillations in a *Paramecium* strain with a duration of ~8 s (Fig. 19; see also color insert). Interestingly, waves emanate from that site that has been identified by

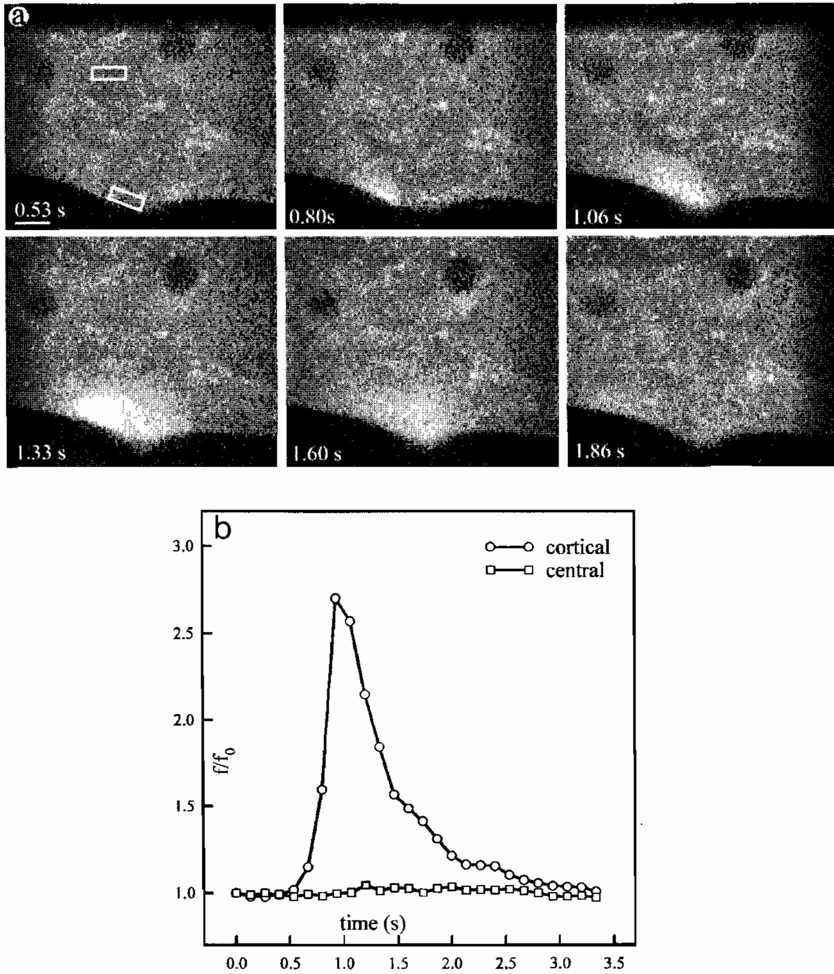



FIG. 19 *Paramecium caudatum* strain tnd1 [unpublished results; see Klauke *et al.* (1998) for collaboration with N. Haga and T. Watanabe]. Cells loaded with Fluo-3 for fast $f/f_0$ ratio analysis by CLSM at $[Ca^{2+}]_0 = 50\ \mu M$. Spontaneous $[Ca^{2+}]_i$ oscillations start in the oral region (a) and propagate with a ~8-s period (c); the period shown is evaluated in (b). Remarkably, in this cell population, ~30% of cells underwent conjugation. The periodic signals may be involved in nuclear or any other activities related to this process. (See also color insert).

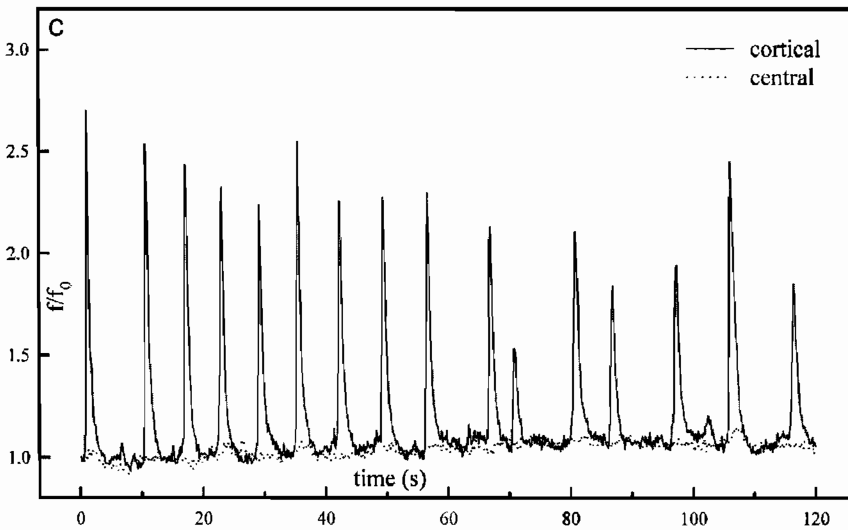



FIG. 19 *(continued)*

Ogura and Machemer (1980) as the site where sensitivity to de- and hyper-polyrization stimuli is equal.

In *Paramecium,* $[Ca^{2+}]_i$ oscillations (periods of ~2 min) emanating from a cortical region tentatively were brought in connection with morphogenetic restructuring of the cell cortex (Prajer *et al.,* 1997). In mammalian cells, involvement of protein de-/rephosphorylation processes is well-established, not only for some intracellular but also for some superficial restructuring and biogenetic processes. One now may speculate about the participation of second messengers, like cyclic nucleotides and/or $Ca^{2+}$, or about protein phosphorylation/dephosphorylation processes with the involvement of cytoskeletal elements, including centrin. Could $Ca^{2+}$ interfere along these lines in ciliates?

The puzzle is far from complete. CaN would be a candidate for phosphatases (Sections III.A and III.G). Whereas CaM kinases could not be identified, a kinase with inherent CaM-like domains has been cloned from *Paramecium* (Kim *et al.,* 1998). On the one hand, a *Paramecium* cell cortex contains more than 15 protein substrates (ranging from ~20 to 200 kDa) for phosphorylation, as shown on Western blots by antibodies against seryl/threonyl phosphorylation sites (Keryer *et al.,* 1987). On the other hand, the cortical enrichment of centrin in different ciliates is well-established (Section III.B.2), and its role in morphogenesis in different eukaryotes, depending on its phosphorylation state, emerges slowly (Martindale and

Salisbury, 1990; Sullivan *et al.,* 1998). This and other candidates could now be analyzed, notably with species amenable to synchronization, for any $Ca^{2+}$-dependent effects, including dephosphorylation and their variation during morphogenesis. In *Paramecium,* during morphogenesis, phosphorylation of ciliary rootlet proteins (30–36 kDa) spreads from the oral apparatus, and their hyperphosphorylation causes disassembly of these cortical structures (Sperling *et al.,* 1991).

Unfortunately, dissection of mechanisms by drug application faces considerable problems in ciliates (Section III.C.1), but mapping of phosphorylation sites on a cellular and molecular level is possible.

*Conclusion.* The aspects mentioned here are among the most poorly understood, in contrast to their eminent functional importance.

## IV. Concluding Remarks—Outlook on Open Questions for Future Research

In ciliates, $Ca^{2+}$ acts as a second messenger of paramount importance. This probably holds for almost as many functions as in higher eukaryotes, though little is known, for example, about its role during cell–cell interactions (conjugation). $Ca^{2+}$ that regulates exocytosis of extrusomes comes primarily from alveolar sacs, superimposed by store-operated $Ca^{2+}$ influx. Only both components in concert can adequately accelerate all steps of an exo-endocytotic cycle. *Paramecium* represents the fastest operating "dense-core vesicle" system known, probably because of its vital role in predator defense, whereas in predatory ciliates an equally rapid activity would be favorable. No second messengers other than $Ca^{2+}$ and no electrical signals are known to be involved. With regard to secretory activity, ciliates therefore are nonexcitable cells. Depolarization entails ciliary reversal, triggered by $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels. Only in this sense are ciliates excitable cells (though they are always exciting). In cilia, signal transduction by $Ca^{2+}$ is coupled to cyclic nucleotide effects. One hypothesis says that CaM may serve as a switch.

Though important discoveries have been made on $Ca^{2+}$ in ciliates, many important questions remain to be settled. For example, what is the role of annexins and copines (largely unknown in other cells)? What is the role of actin and myosin for cortical functions? What is the function of a variety of ion channels inasmuch as they so far have been characterized only electrophysiologically?

The molecular identity and localization (even function) of many $Ca^{2+}$-sensitive proteins remain to be established, such as for the voltage-dependent $Ca^{2+}$ channels, the protein kinases with CaM-like $Ca^{2+}$-binding

motifs, the $Ca^{2+}$ sensor molecules and $Ca^{2+}$-influx channels in the cell membrane, and the $Ca^{2+}$-release channels in alveolar sacs. What are the respective roles of a $Ca^{2+}$-inhibited (Kissmehl *et al.,* 1997a) and a $Ca^{2+}$-activated protein kinase in *Paramecium* (Gundersen and Nelson, 1987)? Also, what is the functional role of the phosphoprotein, PP63/parafusin? This is dephosphorylated rapidly [probably by CaN (Kissmehl *et al.,* 1997b)] upon exocytosis induction, depending on exocytosis site assembly (Zieseniss and Plattner, 1985), and multiply rephosphorylated by different (in part $Ca^{2+}$-sensitive) kinases (Kissmehl *et al.,* 1996; Kussmann *et al.,* 1999).

Does a $PInsP_2$ cycle, with the formation of $InsP_3$ and DAG (followed by PK-C activation), exist in ciliates, as suggested by Fabczak *et al.* (1998, 1999) on the basis of work with the green-algae-containing species, *Blepharisma?* Both gene cloning and functional–enzymatic analysis are mandatory for a final judgment, considering the nonenzymatic morphogenetic functions of some PK-C isoforms, e.g., during neurite outgrowth (Zeidman *et al.,* 1999). What is the implication of the alternative (plantlike) inositol hexakisphosphate hydrolysis pathway, as described for *Paramecium* (Freund *et al.,* 1992), with regard to the potential occurrence of $InsP_3$/DAG signaling?

Is there any functional equivalent of phospholipase-C, as suggested by molecular cloning with *Euplotes* (Klobutcher *et al.,* 1991)? Would there be a $Ca^{2+}$-activated isoform, if any? Are there trimeric G-proteins in ciliates, as suggested by Forney and Rodkey (1992) on the basis of molecular cloning with *Paramecium?* (Consider the absence of ligand-activated nucleotide cyclases.) May Tyr-phosphorylation (whatever the implication of $Ca^{2+}$ would be) definitely be excluded for protozoans except myxomycetes, as suggested by Darnell (1997)?

The $Ca^{2+}$ sensor operating at exocytosis sites remains to be identified. Is dynamin, if involved in endocytosis via coated pits in ciliates, regulated by CaN in these organisms? Does CaN regulate any other functions in the cell? How is $[Ca^{2+}]_i$ homeostasis reestablished after ciliary reversal? Which role does the multitude of CaM-binding proteins play? Which additional cytosolic CaBPs may be important? In which membranes or cell membrane regions may a $Na^+/Ca^{2+}$ exchanger be localized? Finally, aspects of microdomain regulation might be studied favorably in ciliates because of their highly regular "design." This concerns $Ca^{2+}$-dependent morphogenetic effects, with the result of microdomain formation.

A related question is how a cell manages to separately regulate such widely different functions in its cortex as ciliary reversal and exocytosis, because both processes are $Ca^{2+}$-dependent. Different reasons may be envisaged. A kinetid may be designed just to avoid functional overlap by taking into account site-directed $Ca^{2+}$ flux from different sources, signal attenuation along diffusion barriers, and different sensitivities of the targets involved. Specifically, $[Ca^{2+}]_i^{act}$ required for exocytosis may be ~5 $\mu M$, but

only ~1 $\mu M$ for ciliary reversal. Many of these questions will keep us busy for quite a while.

## Note Added in Proof

In the context of Section II.A, several isoforms of the plasmamembrane-bound CaM-binding protein, PMC1, were recently cloned in *Paramecium* (Chan *et al.,* 1999). Meanwhile we also localized CaN in *Paramecium* by semiquantitative EM-gold labeling. The label was concentrated on the complex formed by a plasma membrane and alveolar sacs on the infraciliary lattice, rims of heterochromatic areas of the macronucleus and parasomal sacs (Momayezi *et al.,* 2000). This largely reflects the distribution of the biochemically defined substrate molecules reported in the literature.

In agreement with the view presented in Section III.C.2, the Hennessey group recently stressed the occurrence in *Tetrahymena* of an general polycation receptor, rather than of a dedicated lysozyme receptor (Kuruvilla and Hennessey, 1999).

As to Section III.C.4, the overexpression of a SERCA-GFP (green fluorescent protein) in *Paramecium* reveals signal in the ER and transfer to alveolar sacs (Hauser, *et al.,* 2000). This transfer occurs only when GFP is attached at the C-terminal end of the SERCA molecule, but not when GFP is integrated between the nucleotide-binding site and the phosphorylation site, possibly because the SERCA molecule then would exhibit an overt ER retention signal. We conclude that there is no patent connection between ER and alevolar sacs, and that their biogenesis occurs by vesicular transport from the ER.

As described in Section III.D, in the context of ciliary beat regulation, an antagonism between $Ca^{2+}$- and $Mg^{2+}$-mediated CaM effects is postulated by some authors. This may also occur at trichocyst-docking sites, where CaM is known to be of functional importance (Section III.E.1). In *Paramecium,* we can detach trichocysts from the cell membrane after inducing membrane fusion under conditions of inhibited contents release ("frustrated exocytosis"), by including in the medium increased [$Mg^{2+}$] and/or a CaM-antagonistic drug, calmidizolium (Klauke and Plattner, 2000).

In the context of Section III.H, additional information became available on the involvement p85, which interacts in a $Ca^{2+}$-dependent manner with CaM at the cleavage furrow in dividing *Tetrahymena* (Numata *et al.,* 1999). CaM and p85 are colocalized and cell division is reportedly inhibited by the CaM antagonist, W7 (Numata *et al.,* 2000a). The *p85*gene has been cloned (Gonda *et al.,* 1999). Also in *Tetrahymena,* CaM and the elongation factor-1$\alpha$ are colocalized in the cleavage furrow (Numata *et al.,* 2000b).

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