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Calmodulin is essential for assembling links necessary for exocytotic membrane fusion in *Paramecium*

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Calmodulin has long been suspected to be involved in calcium-regulated exocytosis but its precise site(s) of action has not yet been identified. In *Paramecium*, a genetic approach to the problem is possible as *in vivo*-selected mutations in the calmodulin gene that prevent the activation of some channels have been characterized. Three of these calmodulin mutants were examined for exocytotic capacity and the mutant *cam*^{1} was found to be defective for exocytosis at 35°C. The loss of exocytotic capacity in *cam*^{1} cells can be restored by transformation with the wild-type calmodulin gene, demonstrating that its exocytotic lesion is indeed due to the mutation in the calmodulin gene. The *cam*^{1} mutant displays abnormal exocytotic sites at the non-permissive temperature: it lacks the links ('rosettes' of intramembranous particles in the plasma membrane and the fibrous 'connecting material') which normally connect plasma and trichocyst membranes. Upon shift of *cam*^{1} cells from the permissive to a non-permissive temperature, preformed sites remain functional. These results demonstrate that calmodulin is necessary for the assembly of these links at the exocytotic site. These results do not, however, exclude the possibility of calmodulin also being involved in Ca^{2+}-dependent steps of the stimulus-exocytosis coupling.

Key words: membrane fusion/mutant/regulated secretion/trichocyst

Introduction

Stimulus-exocytosis coupling is generally dependent on a transient increase of intracellular free calcium (Douglas, 1968, 1974; Cheek, 1989; Knight *et al.*, 1989), most probably through the action of some calcium binding proteins. Calmodulin, the most ubiquitous calcium mediator in eukaryotic cells (Means and Dedman, 1980; Stoclet *et al.*, 1987; Means, 1988), has been shown to be involved in exocytosis in various systems by different means: use of inhibitors (e.g. Krausz *et al.*, 1980; Burgoyne, 1982; Ganguly *et al.*, 1992), anti-calmodulin andibodies (Steinhardt and Alderton, 1982), a calmodulin binding inhibitory peptide (Birch *et al.*, 1992) and, in permeabilized cell models, by addition of exogenous calmodulin (Okabe *et al.*, 1992).

An original approach to the role of calmodulin in exocytosis takes advantage of the properties of the unicellular organism *Paramecium*. Indeed, *Paramecium* not only displays a pathway of regulated exocytosis which is calcium dependent (Plattner, 1974; Matt *et al.*, 1978, 1980; Satir and Oberg, 1978; Gilligan and Satir, 1983; Cohen and Kerboeuf, 1993) and possibly calmodulin dependent (Tiggemann and Plattner, 1982; Garofalo *et al.*, 1983; Momayezi *et al.*, 1986, 1987; see Discussion) but also possesses a single calmodulin gene in which a number of point mutations have been characterized which confer phenotypes detectable *in vivo* (Kung *et al.*, 1992).

The exocytosis of the secretory vesicles of *Paramecium*, the trichocysts (defensive organelles), can be synchronously triggered by external stimuli (see reviews by Adoutte, 1988; Plattner *et al.*, 1991; Cohen and Kerboeuf, 1993) and many mutations that interfere with the process are available (Pollack, 1974; Sonneborn, 1974; Cohen and Beisson, 1980). Trichocysts are normally docked beneath the plasma membrane, awaiting an external stimulus for the fusion of trichocyst and plasma membranes. This pre-fusion state is characterized by the presence of fibrous material connecting the plasma and trichocyst membranes, visible in transmission electron microscopy, which is anchored in the plasma membrane through a 'rosette' of 15 nm intramembranous particles, visible in freeze-fracture electron microscopy (Plattner *et al.*, 1973; Beisson *et al.*, 1976). These structures (rosette and connecting material) are missing in most mutants whose trichocysts are docked but cannot be secreted (Beisson *et al.*, 1976; Pouphile *et al.*, 1986).

Concerning *Paramecium* calmodulin, *in vivo* mutagenesis and selection of behavioural mutants affected in their swimming response to various ionic stimuli permitted the identification of mutations in the structural gene for calmodulin (*cam* mutants, Kink *et al.*, 1990). Genetic and molecular dissection of calmodulin have defined precise zones in this molecule which interact with different calmodulin binding proteins: mutations localized in the N-terminal lobe of the molecule prevent the interaction of calmodulin with a Ca^{2+}-dependent Na^+ channel, whereas mutations in the C-terminal lobe prevent interactions with Ca^{2+}-dependent K^+ channels (reviewed by Kung *et al.*, 1992).

If calmodulin is involved in exocytosis, it is possible that some mutations in the calmodulin gene affect exocytosis. Therefore, we wondered whether some of the existing *cam* mutants were defective for exocytosis, at least under some culture conditions. Three calmodulin mutants, *cam*^{1}, *cam*^{6} and *cam*^{7}, were examined for exocytotic capacity at different temperatures and we found that the mutant *cam*^{1} is unable to undergo trichocyst exocytosis at 35°C. The loss of exocytotic capacity in *cam*^{1} cells can be repaired together with the behavioural defect by transformation with the wild-type calmodulin gene, and therefore arises from the mutation in the calmodulin gene. In addition, the site of action of the mutation could be identified, as the *cam*^{1} mutant at 35°C displays abnormal exocytotic sites: absence of rosettes of

Table I. Phenotypes of three *cam* mutants

Mutant	Growth temperature (°C)	Growth	Behaviour	Exocytosis
cam^1	18	+	pnt	+
	28	+	pnt	+
	33	+	pnt	+
	35	+	pnt	−
cam^6	18	+	pnt	+
	28	+	pnt	+
	33	+	pnt	+
	35	+	pnt	+
cam^7	18	+	pnt	+
	28	+	pnt	+
	33	+	pnt	+
	35	−		

Cells of each *cam* strain used were grown for at least 2 days, i.e. 6−10 fissions, at the indicated temperatures and stationary and log-phase cells were tested for behaviour and exocytotic capacity, with the same result. 'pnt' indicates pantophobiac behaviour.

particles and of underlying connecting material. Therefore, calmodulin plays an essential role in linking the trichocyst membrane to the plasma membrane at the exocytotic site.

Results

A Paramecium calmodulin mutant has a thermosensitive exocytotic capacity

Three calmodulin mutants, *cam*^{1}, *cam*^{6} and *cam*^{7}, were cultured at 18, 28, 33 and 35°C and then submitted to behavioural and exocytotic tests. In addition to their known pantophobiac swimming behaviour (exaggerated response to ionic stimuli), we detected new phenotypes: inability to undergo exocytosis at 35°C for *cam*^{1}, and thermolethality for *cam*^{7} (Table I). *cam*^{6} presented a wild-type phenotype for growth and exocytosis at all temperatures.

The exocytotic defect of cam^1 *is restored by the wild-type calmodulin gene*

To check whether the exocytotic deficiency of the *cam*^{1} mutant and the thermolethality of *cam*^{7} were actually due to the mutated calmodulin gene, the plasmid pCAMTEL carrying the wild-type calmodulin gene was microinjected into the macronucleus of these mutants and the phenotype of the transformants analysed (Table II). In this experiment, the recovery of the behavioural defects given by pCAMTEL in *cam*^{1} cells was always accompanied by a recovery of the exocytotic capacity, indicating that both defects arise from the mutation in the calmodulin gene. Concerning *cam*^{7}, the transformation by pCAMTEL restored only the behavioural defect at permissive temperatures and not the thermolethality. Most probably, the thermolethality of this strain is due to a mutation in a different gene.

cam^1 *has abnormal exocytosis sites at the non-permissive temperature*

In *Paramecium*, the step in exocytosis affected by mutation can be determined by cytological observation. The trichocysts are highly refringent carrot-shaped organelles

Table II. Transformation experiment on two *cam* mutants with the wild-type calmodulin gene

Recipient strain	Behaviour	Exocytosis at 35°C	Survival at 35°C	Number of clones in each class
cam^1 (17)	pnt	−		11
	pnt	+		0
	+	−		0
	+	+		6
cam^7 (8)	pnt		−	2
	pnt		+	0
	+		−	6
	+		+	0

Seventeen *cam*^{1} cells and eight *cam*^{7} cells that survived microinjection of the plasmid pCAMTEL into their macronucleus were cultured and the resulting clones tested for behaviour and exocytotic ability or growth at 35°C. The number of clones of each possible phenotypic class are given.

visible by phase contrast microscopy. They are normally docked beneath the plasma membrane. Various types of exocytotic defects have long been characterized in the mutants: no trichocysts, morphologically normal or abnormal trichocysts not docked at the cortex, normal and docked trichocysts unable to fuse with the plasma membrane (Pollack, 1974; Sonneborn, 1974; Cohen and Beisson, 1980). *cam*^{1} cells grown at 35°C display normal trichocysts, docked at the cortex but not secretable, indicating that the calmodulin-dependent step blocked in this mutant concerns the late events of membrane fusion. We analysed *cam*^{1} cells by freeze-fracture and transmission electron microscopy in order to look for ultrastructural lesions, characteristic of many exocytotic mutants (Beisson *et al.*, 1976; Pouphile *et al.*, 1986). Examination of the plasma membrane by freeze-fracture electron microscopy revealed that the rosette of particles normally present at wild-type exocytotic sites is lacking in the mutant (Figure 1 and Table III). Correlatively, the underlying connecting material observed by transmission electron microscopy is also missing (Figure 2).

Preformed sites of the mutant are stable at the non-permissive temperature

Temperature shifts from non-permissive (35°C) to permissive (20°C) conditions and vice versa were performed on log-phase and stationary *cam*^{1} cultures and the change of their exocytotic performance followed (Figure 3). *cam*^{1} populations of exo$^-$ phenotype, either growing or stationary, recover notable exocytotic capacity within 2 h after transfer to the permissive temperature (Figure 3a): this recovery is thus independent of cell growth and, in particular, can occur without site turnover, as observed in stationary cells. *cam*^{1} cells of exo$^+$ phenotype shifted to non-permissive temperature behave differently according to their physiological state (Figure 3b): log-phase cells significantly lose their exocytotic capacity within 7 h while stationary cells remain exo$^+$ during the same period at 35°C (although a shift towards the exo$^-$ phenotype can be detected after 24 h). Thus, *cam*^{1} exocytotic sites assembled at the permissive temperature are stable at the non-permissive temperature, in the absence of site turnover, as observed in stationary but not in log-phase cells.

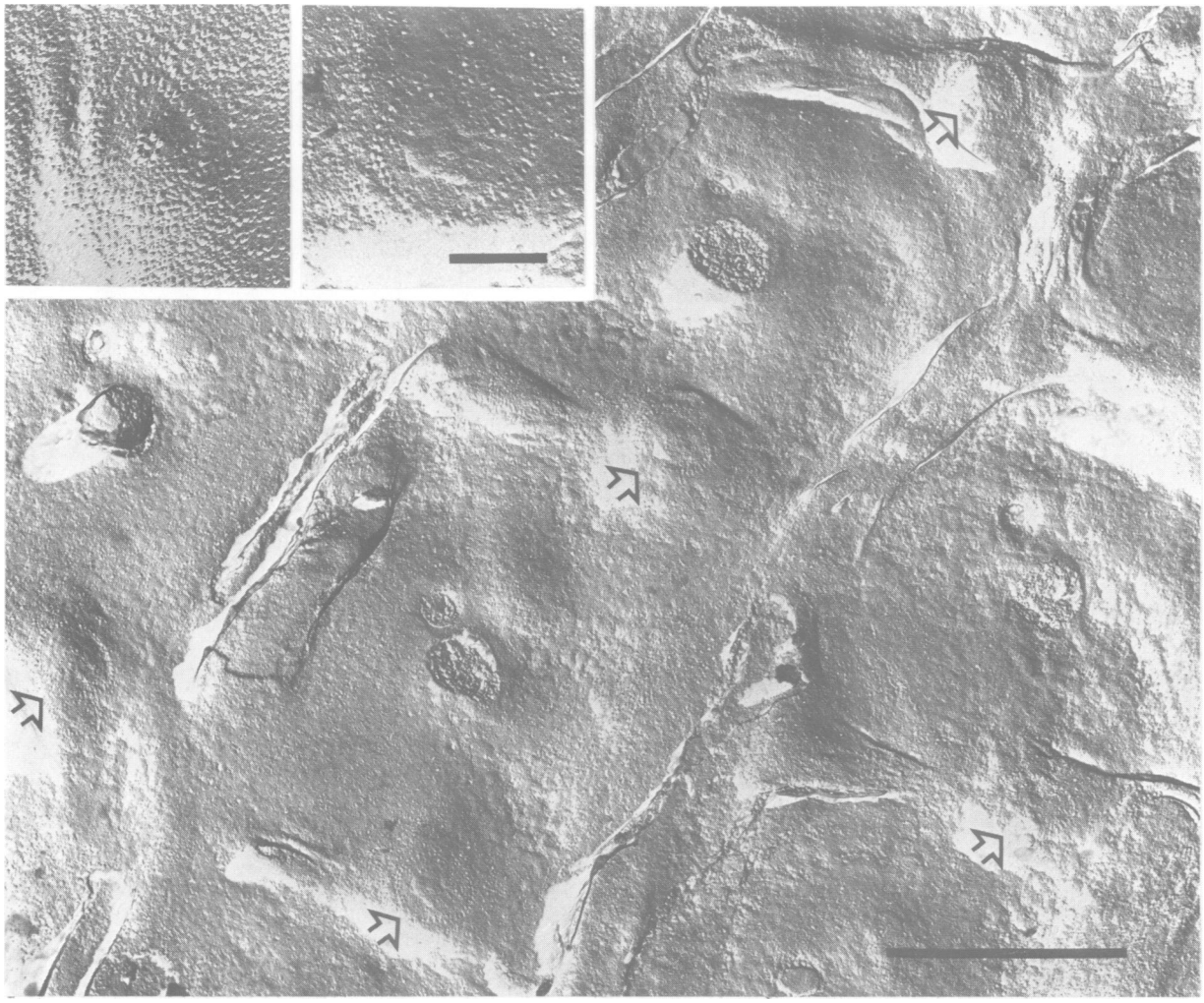

Fig. 1. Exocytotic sites of *cam*^{1} cells in freeze-fracture. Image of the plasmic face of the freeze-fractured plasma membrane of a *cam*^{1} cell grown at 35°C. Five exocytotic sites, visible on this replica, are pointed by arrows and display no rosettes. Bar, 1 μm. Inset left: enlargement of an exocytotic site of the wild-type, as a control, showing a rosette of 15 nm particles in the centre of the site. Inset right: enlargement of a *cam*^{1} site without rosette particles. Bar, 0.2 μm.

Discussion

Calmodulin is necessary for exocytosis in Paramecium

The prominent role of calmodulin in exocytosis in *Paramecium* has been suggested by cytological localization (Momayezi *et al.*, 1986), inhibition of secretion by anti-calmodulin drugs (Garofalo *et al.*, 1983), and some biochemical evidence that calmodulin activates an ATPase (Tiggemann and Plattner, 1982) and a calcineurin-like phosphatase (Momayezi *et al.*, 1987) thought to be involved in membrane fusion. However, these authors tried to prove that calmodulin was indeed essential for exocytosis by microinjection of anti-calmodulin antibodies into cells, but with no effects on trichocyst discharge. This indicated that calmodulin was either not involved in exocytosis or was inaccessible to the antibody or too concentrated in the cell compared with the quantity of microinjected antibody. Similarly, preliminary attempts to prevent exocytosis by calmodulin antisense oligonucleotides, according to Hinrichsen *et al.* (1992), were mostly inconclusive as they did not give clear inhibition of trichocyst discharge (D.Fraga, J.Cohen and R.Hinrichsen, unpublished).

In the present work, using the analysis of calmodulin mutants and rescue transformation, we could definitely demonstrate that calmodulin is an essential protein for exocytosis in *Paramecium:* we found that one allele of the calmodulin gene, *cam*^{1}, prevents trichocyst exocytosis at 35°C (Table I) and that this exocytotic defect was due to the calmodulin mutation since it could be repaired by microinjection with the wild-type calmodulin gene (Table II).

Table III. Number of particles per rosette in *cam*^{1} exocytotic sites

Strain	No. of particles per rosette											Total sites	Mean no. particles/ rosette
	0	1	2	3	4	5	6	7	8	9	10		
Wild type[a]	–	–	–	–	–	–	6	12	11	2	–	31	7.3
cam^{1} (35°C)	25	7	3	2	–	–	–	–	–	–	–	37	0.5

[a]Data for the wild-type are from Bonnemain *et al.* (1992).
Fields of plasmic faces of freeze-fracture replicas presenting a sufficient number of visible exocytotic sites were photographed and the number of particles per rosette was counted in sites occupied by a trichocyst, recognizable by the presence of a 'ring' of particles and not 'parentheses' (Beisson *et al.*, 1976). See also Figure 1.

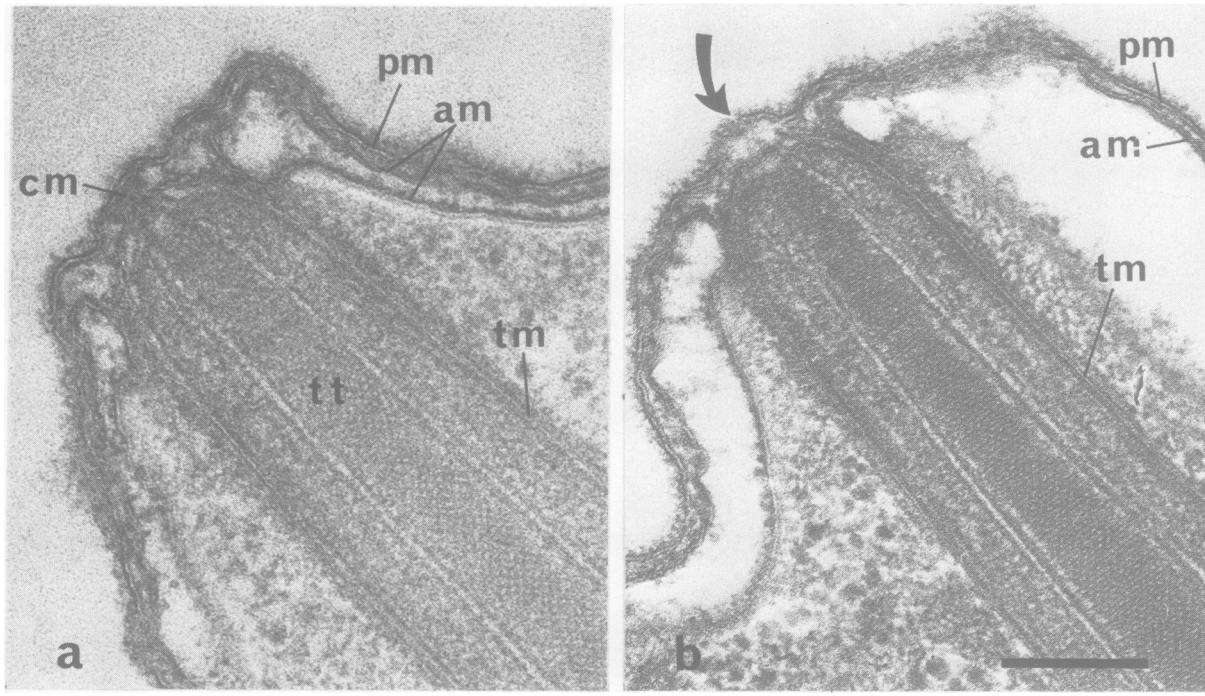

Fig. 2. Exocytotic sites of *cam*^{1} cells on thin sections. Thin sections through the attachment site of a trichocyst at the plasma membrane in a wild-type cell (**a**) and a *cam*^{1} cell grown at 35°C (**b**). The 'connecting material' (cm) clearly visible in the wild-type is absent in *cam*^{1} (arrow). pm, plasma membrane; am, alveolar membrane; tm, trichocyst membrane; tt, trichocyst tip. Bar, 0.2 μm.

Calmodulin-dependent step in trichocyst exocytosis

We were also able to determine the step disturbed by the mutant calmodulin in the secretoy pathway as the membrane linking necessary for membrane fusion. In most secretory cells, where granules are stored within the cytoplasm far from the plasma membrane, this membrane linking is very transient and hard to detect. In contrast, in *Paramecium*, the trichocysts are docked in a stable prefusion state, as long as the cell remains unstimulated. This state is characterized by the presence of links (rosette and connecting material) between the membranes to be fused. In other cell systems, despite their transience, such links have been visualized in some cases (Aunis *et al.*, 1979; Nakata *et al.*, 1990) and various kinds of proteins have been assumed to participate in membrane-to-membrane interactions during exocytosis: synexin (Creutz, 1981), calpactin (Burgoyne, 1988; Drutz and Creutz, 1988; Ali *et al.*, 1989; Sarafian *et al.*, 1991), GTP binding proteins (Gould *et al.*, 1988; Nadin *et al.*, 1989; Fischer von Mollard *et al.*, 1990; Gomperts, 1990), etc., suggesting that connections between the membranes to be fused is a general feature of exocytosis.

The *cam*^{1} mutant, cultured at the non-permissive temperature, is devoid of these links (Figures 1 and 2, Table III), demonstrating that calmodulin is an essential protein for their construction. Moreover, in stationary cells, where the turnover of exocytotic sites is supposed to be low, sites formed at the permissive temperature remain functional for at least 7 h at the non-permissive temperature. As the non-permissive temperature most likely provokes conformational changes in the calmodulin molecule rather than preventing its synthesis, these experiments show that, once built, the connecting material and the rosette remain structurally and functionally intact, even in the presence of the inactive *cam*^{1} calmodulin molecules. Therefore, the role of calmodulin identified here is restricted to the assembly (and not to the maintenance) of the structures that link the membranes to be fused.

However, anti-calmodulin drugs block exocytosis of wild-type cells (Garofalo *et al.*, 1983), which have normal exocytotic sites, suggesting that the level of action of calmodulin revealed by the *cam*^{1} mutation is not the only one in the exocytotic pathway. In fact, the various phenotypes detected so far in calmodulin mutants are allele specific: only the interaction of calmodulin with one or a few of its numerous targets in the cell is disturbed by each mutated allele (see for instance the demonstration for calmodulin-dependent channels by Kung *et al.*, 1992), so that the *cam*^{1} mutation could be deleterious for constructing the membrane links but not for other putative calmodulin-dependent steps in the exocytotic pathway. It is therefore possible that other calmodulin mutants reveal other calmodulin-dependent steps in trichocyst exocytosis, for instance in stimulus–exocytosis coupling, so that the complete *cam* allelic series needs to be examined. It is also possible that *cam* mutant alleles with no behavioural phenotype will be needed to identify other calmodulin-dependent events in exocytosis.

In conclusion, we have demonstrated here that the Ser101Phe substitution in *Paramecium* calmodulin not only prevents the activation of Ca^{2+}-dependent K^{+} channels but also the building of the links that enable membrane fusion during exocytosis. The involvement of calmodulin in this process indicates that calmodulin binding proteins are also likely to participate in the exocytotic membrane linkage. One way to identify the genes that encode such calmodulin binding proteins is to look for interactions in double mutants containing the *cam*^{1} mutation associated with different

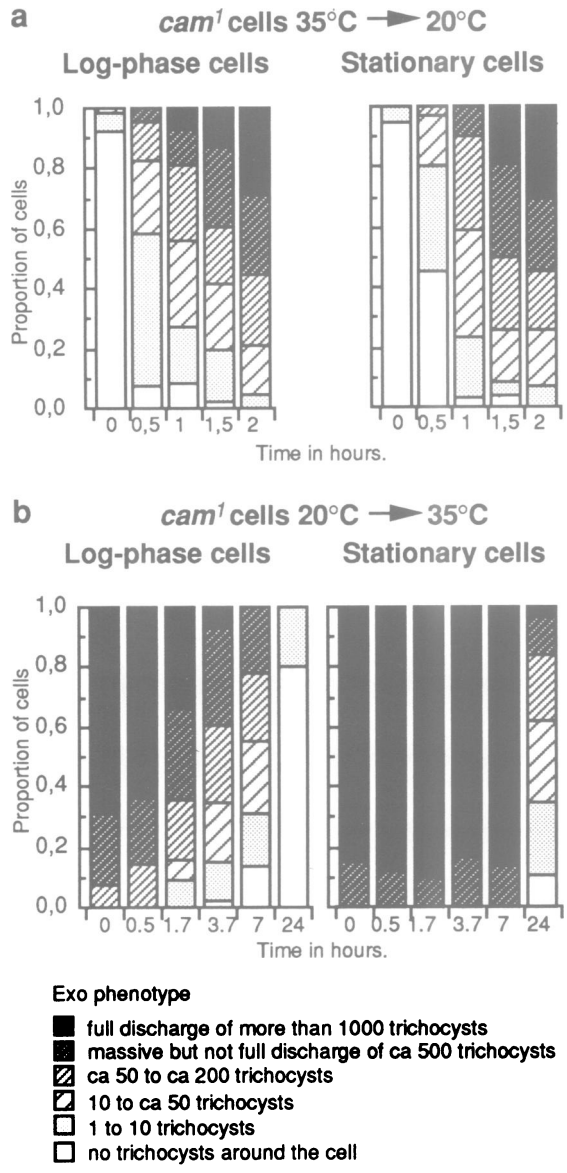

Fig. 3. Exocytotic phenotype of *cam*^{1} cells upon temperature shift. Log-phase and stationary cultures of *cam*^{1} were grown at 35°C and shifted to 20°C (**a**) or grown at 20°C and shifted to 35°C (**b**). At each time shown on the abscissa, samples of 40–80 cells were treated with picric acid and the distribution of exocytotic abilities within the population scored.

mutations known to affect exocytosis. Such an approach previously led to the suggestion that the products of three known exocytotic genes that interact in double mutants may be components of the connecting material (Bonnemain *et al.*, 1992).

Materials and methods

Strains and culture conditions

The strains used in this study were derivatives of stock 51 of *Paramecium tetraurelia* (Sonneborn, 1975) carrying mutations in the calmodulin gene: *cam*^{1} which has a Ser101Phe substitution (d4-622; Kink *et al.*, 1990), *cam*^{6} and *cam*^{7} (d4-656 and d4-657 respectively) which are alleles of *cam*^{1} and also have point substitutions in the calmodulin gene (Y.Saimi *et al.*, personnal communication).

Wild-type and mutant cells were grown at the indicated temperature in grass or wheat grass powder (Pines International, Lawrence, KS) infusion, bacterized the day before use with *Klebsiella pneumoniae* and supplemented with 0.4 μg/ml beta-sitosterol according to Sonneborn (1970).

Test of exocytotic ability

Exocytotic ability of *cam* cells was determined by mixing a sample of cells with an equal volume of saturated picric acid, after cultivation for at least 2 days at the desired temperature. By observation under 10 × dark field light microscopy, cells thus fixed are either surrounded by a 'crown' of secreted trichocysts, if they are able to undergo exocytosis (exo^{+}), or appear 'naked' if not (exo^{-}). Exocytotic ability can also be monitored without sacrificing the cells using the synthetic polyamine aminoethyldextran (Plattner *et al.*, 1985; Kerboeuf and Cohen, 1990). After mixing cells with the secretagogue, the trichocysts are discharged into the medium, forming a 'cloud' visible under the microscope, while the cells continue to swim.

Behavioural tests

The phenotype of the *cam* mutants used in this work is 'pantophobiac' (pnt), characterized by exaggerated response to several ionic stimuli (Kink *et al.*, 1990). To distinguish clearly pnt from wild-type phenotypes, we used a 'Na-test' (Hinrichsen *et al.*, 1985): cells were briefly washed in 1 mM HEPES, 1 mM $CaCl_2$, 4 mM KCl pH 7.0 and transferred to the same solution containing 10 mM NaCl. In this solution, [pnt^{+}] cells swim backwards briefly once or a few times, lasting altogether <5 s, whereas [pnt] cells swim backwards for >40 s.

Transformation by microinjection of DNA

A volume of 5–10 pl of pCAMTEL plasmid DNA, at 5 μg/μl in water, was microinjected into the macronucleus of recipient cells. This plasmid contains a functional copy of the wild-type calmodulin gene of *Paramecium* (Kanabrocki *et al.*, 1991). The microinjection was performed according to the principle developed by Knowles (1974) using a microinjection device comprising a Diaphot inverted microscope (Nikon, Japan), MM188 and NT88 micromanipulators (Narishige, Japan) and microinjection and medium removal air circuits driven by screw syringes (Alcatel, France). Microinjected cells were then placed into fresh medium and cultured at 28°C for 1 day, then subcloned at different temperatures and cultured for 2 days before being tested for behaviour and exocytotic ability. Transformation efficiency can vary according to various factors, including the skill of the manipulator.

Freeze-fracture and transmission electron microscopy

Freeze-fracture of stationary cell pellets and examination of the replicas were performed according to Bonnemain *et al.* (1992) except that 1% instead of 0.5% glutaraldehyde was used for fixation. Inclusion into resin, thin sectioning and examination of the sections under the electron microscope were as described by Jerka-Dziadosz *et al.* (1992).

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