Cell and Molecular Biological and Fluorochrome Analysis of the Mechanism Involved in the Calcium Dynamics during Exocytosis in Paramecium Cells
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"He who makes no mistakes makes nothing"

(Magee William, 1868)

"To My Mama, Papa, Wife, Son, Daughter,
All My Family Members And Egypt"
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AED</td>
<td>Aminoethyl-dextran</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxy-methyl ester</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1, 2-bis(o-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid.</td>
</tr>
<tr>
<td>[Ca]</td>
<td>Total calcium (free and bound)</td>
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<tr>
<td>[Ca$^{2+}$]_{i/o}</td>
<td>Intra/extracellular free calcium concentration</td>
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<td>Ca$^{2+}$-ATPase</td>
<td>Calcium-activated adenosine triphosphatase (= Ca$^{2+}$-pump)</td>
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<td>CaSR</td>
<td>Calcium sensing receptor</td>
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<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<tr>
<td>CICR</td>
<td>Ca$^{2+}$-induced Ca$^{2+}$-release</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>4CmC</td>
<td>4-chloro-meta-cresol</td>
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<tr>
<td>EDX</td>
<td>Energy-dispersive x-ray</td>
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<td>EGTA</td>
<td>Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>f/f$_0$</td>
<td>Fluorescence increase/ Fluorescence at rest</td>
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<td>Fig.</td>
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<td>hsp</td>
<td>Heat shock protein</td>
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<td>IMP</td>
<td>Intramembranous particles</td>
</tr>
<tr>
<td>InsP$_3$</td>
<td>Inositol-tris-phosphate</td>
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<tr>
<td>Mag.</td>
<td>Magnification</td>
</tr>
<tr>
<td>PC12</td>
<td>Rat pheocromocytoma cell line 12</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis</td>
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<tr>
<td>SOC</td>
<td>Store-operated Ca$^{2+}$ influx</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<tr>
<td>STEM</td>
<td>Scanning-transmission electron microscope</td>
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1. Introduction

1.1 Paramecium cell as a model system

The ciliate protozoan Paramecium is an important eukaryotic cell used for a wide range of studies in cell and molecular biology. However, of all eukaryotic cells being investigated, Paramecium has numerous features that warrant a very special niche for it. Many of these features are shared with other protists, especially the ciliates, but many are unique. The large size of Paramecium makes it particularly suited for the use of different extensive physiological and microinjection techniques as well as light and electron microscopic investigations. Of all living forms, only Paramecium (along with the other ciliates) has a cell cortex so rich in structure and complex in development, a micronucleus, and a macronucleus with different structures and functions and with amazing complex patterns of formation, mating type substances of wide variety and specificity, life cycle stages of immaturity, maturity and senility so well defined in single-celled organisms, primitive behavioural mechanisms so suited for analysis (Görtz, 1988) etc.

Additionally, the ease of growth of Paramecium allows to obtain rapidly a large number of cells, with the convenience of avoiding contamination with various cell types, as it is the case with cell systems arising from multicellular organisms. Biochemical studies are also favoured by the possibility of growing axenic cultures (as an alternative to bacterized cultures) which, in addition, often yield very high population densities required for some types of analysis. The fascination Paramecium exercised over the 19th century microscopists may have resulted from the animal-like swimming behaviour of the organism in response to stimuli such as food, other Paramecia or danger. Paramecium retreats from toxic substances such as acids, from electric shock or from certain predators by swimming backwards, leaving behind a trail of
insoluble needle-shaped secretory organelles “trichocysts” that are released by a process called “exocytosis” (Vayssié et al., 2000).

These trichocysts are secretory products which result from a pathway of synthesis, storage and stimulus-dependent release that, not only on a phenomenological level, is analogous to the regulated secretory pathway that allows specialized metazoan cells to deliver secretory products (hormones, neuropeptides, digestive enzymes, histamine, etc.) to the extracellular space in response to physiological stimuli (Arvan and Castle, 1998; Burgoyne and Morgan, 1998). Hence, there is ample evidence that exocytosis regulation follows similar principles in all cells – from protozoan to mammalian – though with variations of the basic theme (Plattner, 1989; Burgoyne and Morgan 1993). Therefore, the abundance of trichocysts docked at the cell membrane in a position for expulsion by exocytosis (Plattner et al., 1973; 1985; 1991; Allen, 1978; Hausmann, 1978; Bilinski, et al., 1981; Pape and Plattner 1985; smith and Hennessey, 1993; Erxleben and Plattner, 1994) makes Paramecium cell attractive as a model system for studying the synchronous exocytosis, the mechanism involved in sensory transductions and intracellular \( \text{Ca}^{2+} \) regulation. In addition, regulated exocytosis represents a conspicuous cell function in the ciliated protozoan Paramecium, since, when Paramecium is attacked by a predator, such as the carnivorous ciliate Dileptus, it discharges the trichocysts which are considered as defensive extrusomes, around the attacked portion of the cell providing an efficient escape mechanism (Hara and Asai, 1980; Harumoto and Miyake, 1991; Knoll et al., 1991; 1993; Harumoto, 1994; Iwadate et al., 1997; 1999).

Exocytosis could be stimulated within about 0.1 s (Knoll et al., 1991b) in response to different secretagogues, such as polyamines (Plattner et al., 1984; 1985; Plattner 1987; Klauke and Plattner, 1997; Klauke et al., 2000), veratridine (Knoll et al., 1992; Plattner et al., 1994; Blanchard et al., 1999), or caffeine (Klauke and Plattner, 1998; Kissmehl et al., 1998; Klauke et al., 1998).
Therefore, synchronous exocytosis of almost all trichocysts within a very short time allows to analyze some aspects of exocytosis more reliably than in some other systems, as previously discussed (Plattner, 1989).

1.2 Trichocysts and related structures

The cell cortex of *Paramecium* sp. is a highly organized structure. Directly beneath the plasma membrane resides a large uninterrupted array of flat membrane bounded vesicles (except in the cytopharyngeal region), designated as cortical alveoli, which is underlain by an amorphous layer named epiplasm. These three closely apposed layers constituting the pellicle are regularly folded, thus displaying a pattern of hexagonal or polygonal unit fields, the kinetids, over the whole cell surface. Cilia, single or in pairs, anchored in the pellicle by their basal bodies, emerge from the depression of each kinetid, arranged in longitudinal rows over the entire cell surface. Internally, numerous dense-core vesicles named “trichocyst” are docked, tip first, within the pellicular ridges at the plasma membrane, i.e., alternating with cilia. Wild type *Paramecium* cells contain approximately $10^3$ trichocysts, 95% of which are mature and firmly attached to the cell cortex, whereas 5% occur free in the cytoplasm.

The biogenesis and ultrastructure of trichocysts (Adoutte, 1988) and their possible functions (Haacke-Bell et al., 1990) have been described. Trichocysts are 5 µm long spindle-shaped organelles that can be isolated in their intact form (Lima et al., 1989; Glas-Albrecht and Plattner, 1990). They are composed of two parts, a body and a tip (Bannister, 1972; Allen, 1988). The conical tip of the trichocysts is a complex structure of ~1.6 µm in length constituted of several successive layers covered by a sheath, while the carrot-shaped body is 3-4 µm long and 0.5-1 µm wide and displays a paracrystalline matrix (Steers et al., 1969; Adoutte et al., 1984; Tindall et al., 1989). This matrix essentially made up of a family of proteins of probably dimeric trichynins of low
molecular weight (Adoutte, 1988), in addition, soluble and insoluble, partly glycosylated proteins of higher molecular weight occur (Glas-Albrecht et al., 1990). The body and the tip are enclosed in the trichocyst membrane which comes in close apposition to both the membrane of the alveoli and the plasma membrane. Attachment of trichocysts at the cell membrane, as well as exocytosis, occur at defined sites whose ultrastructure has previously been described (Plattner et al., 1973; Beisson et al., 1976). These sites are composed of elements which assemble as new trichocysts are docked. They undergo several modifications during trichocyst exocytosis, as documented in Fig. 1. The trichocysts are firmly attached to the cell membrane in a regular distribution pattern (Jurand and Selman, 1968; Sonneborn, 1970; Ehret and McArdle, 1974).

By the freeze-fracture technique, Bachmann, Schmitt and Plattner (1972) and Janisch (1972) presented the first evidence that the cell membrane contains regular arrays of intramembranous particles (IMP) forming “rosettes” at the docking sites of trichocysts (the point of membrane fusion), a double ring of IMPs delineates a docking/release site (Plattner et al., 1980). In more detailed analysis a variety of different membrane specializations were described to occur at these docking sites (Plattner et al., 1973; Pape and Plattner, 1985; Knoll et al., 1991b, 1993). The rings persist in the absence of a docked trichocyst but assume the shape of “parentheses” (Beisson et al., 1976).

Recently, the changes in the membrane structures, during exocytosis were explained precisely by Plattner et al. (1997a) who concluded that for exocytosis, fusion occurs between trichocysts and cell membranes at the docked sites and the rosette particles decay to smaller IMPs as a focal exocytotic opening is formed. Then a “filled ring” is formed for resealing during endocytosis of an empty ghost. Finally “oval rings” and subsequently a “parenthesis” is formed when the ghost is completely detached from the cell surface by internalization (i.e., parenthesis indicates a non occupied potential docking site); see Fig. 1.
Fig. 1. A schematic drawing showing the ultrastructure of the trichocyst docking sites in freeze-fracture replicas (top) and in ultrathin sections (bottom). Mature docking sites are characterized by the occurrence of a “rosette” of “intramembranous particles”, IMPs (a). Note that the ring matches the rims of the alveolar sacs (AS), which are also connected to the cell membrane (CM). The dark green zone around the tip of the trichocyst represents connecting material constituting the collar. Similar material forms a contact between alveolar sacs membrane and cell membrane (represented in yellow). Upon stimulation, cell membrane and trichocyst membrane (TM) fuse and a focal exocytotic opening is formed (b). The expansion of the original opening - up to the ring particles - allows the extrusion of the trichocyst matrix (c). After resealing of the plasma membrane, ghosts corresponding to empty trichocysts remain attached to the alveolar sacs membrane but progressively detach from the cell membrane (d). Then, the oval ring of IMPs collapses to a parenthesis stage (e). The formation of a plug of connecting material occurs before docking of a new trichocyst (f). The appearance of a new rosette indicates a releasable docked trichocyst (g). According to (Pape and Plattner, 1985).
Paramecium cells possess an extended subplasmalemmal calcium storage alveolar system or “alveolar sacs” surrounding the trichocyst docking sites. They underlie almost all of the non-ciliary (i.e., somatic) cell surface, except for sites occupied by cilia or trichocysts (Adoutte, 1988; Plattner et al., 1991; Stelly et al., 1991; 1995; Knoll et al., 1993; Länge et al., 1995). The alveolar sacs were claimed to be joined laterally to each other by membrane bounded pores which cross the space (the septum) between them (Allen, 1971). This would make the lumen of the alveolar sacs as well as their membranes continuous. Yet no such connections were found by Plattner et al. (1999) after fast freezing. In electron micrographs the volume of this luminal space varies from cell to cell, being at times flattened and at other times greatly distended (Allen, 1988). Again, after fast freezing, only flat sacs are seen (Plattner et al., 1999).

1.3 Role of Ca$^{2+}$ in exocytosis

Generally, Ca$^{2+}$ plays a pivotal role in the regulation of the exocytotic membrane fusion symphony (Cheek, 1989; Clapham, 1995; Klauke and Plattner, 1997; Berridge et al., 1998; Iwadate et al., 1999; Patterson et al., 1999; Alderton et al., 2000; Plattner and Klauke, 2001). Many studies proved that Ca$^{2+}$ acts as a second messenger in dense-core vesicle exocytosis, specifically in membrane fusion (Berridge, 1997; Barritt, 1999; Berridge et al., 2000). This also holds true of ciliated protozoa, including Paramecium (Plattner and Klauke, 2001). In Paramecium, exocytosis of dense-core vesicles (“trichocysts”) can be stimulated by the non-permeable polyamine secretagogue, aminoethylxdran “AED” (Plattner et al., 1984; 1985) which is paralleled by an increase in intracellular free Ca$^{2+}$ concentration, [Ca$^{2+}$], according to electrophysiological recordings of Ca$^{2+}$-activated currents (Erxleben and Plattner, 1994; Erxleben et al., 1997) and fluorochrome analyses (Klauke and Plattner, 1997). Ca$^{2+}$ buffer injection indicates requirement of a free Ca$^{2+}$ concentration, [Ca$^{2+}$]$_{act}$, of ~5 µM for
membrane fusion to occur. Ca$^{2+}$ signals are much weaker when extracellular Ca$^{2+}$ is chelated briefly, within $<$1 s, e.g. to $[\text{Ca}^{2+}]_o = 30$ nM, a value slightly below resting values, $[\text{Ca}^{2+}]_i^{\text{rest}} \approx 65$ nM (Klauke and Plattner, 1997). These data fit precisely the observation of $^{45}\text{Ca}^{2+}$ influx during AED stimulation which drives exocytosis down to $[\text{Ca}^{2+}]_o = 300$ nM (Kerboeuf and Cohen, 1990). Later on, using wild type cells, Ca$^{2+}$ release from alveolar sacs has been shown to occur during AED stimulation by electron spectroscopic imaging (Knoll et al., 1993), secondary ion mass spectroscopic imaging (Stelly et al., 1995) and quantitative energy-dispersive x-ray microanalysis, “EDX” (Hardt and Plattner, 2000).

In *Paramecium*, this store activation can be dissociated from Ca$^{2+}$ influx component by stimulation with AED at $[\text{Ca}^{2+}]_o \approx 30$ nM (Klauke and Plattner, 1997) and by stimulation with permeable activators of these Ca$^{2+}$ stores, like caffeine (Klauke and Plattner, 1998) or 4-chloro-meta-cresol, “4CmC” (Klauke et al., 2000), also at low $[\text{Ca}^{2+}]_o$. Since *Paramecium* is sensitive to low $[\text{Ca}^{2+}]_o$, this is produced by adding a chelator to the stimulant. While caffeine has to be applied in $\sim$50 mM concentration to achieve maximal stimulation in *Paramecium* (Klauke and Plattner, 1998), just as in higher eukaryotic cells (Cheek et al., 1993), only 0.5 mM of 4CmC is required in *Paramecium* (Klauke et al., 2000) or up to 1 mM in higher eukaryotic cells (Zorzato et al., 1993; Herrmann-Frank et al., 1996; Westerblad et al., 1998; Kabbara and Allen, 1999).

1.4 Models of Ca$^{2+}$ signalling pathway in exocytosis (CICR and SOC)

Normally, in *Paramecium* Ca$^{2+}$ mobilization from alveolar sacs is superimposed by Ca$^{2+}$ influx and both components have to cooperate for maximal stimulation of the exo-endocytotic cycle (Plattner et al., 1997a; Plattner and Klaauke, 2001). This could imply different mechanisms between which we now try to discriminate using cells with and without Ca$^{2+}$ influx, respectively.
1.4.1  **(CICR) mechanism**

Theoretically Ca$^{2+}$ could enter from outside (Cohen and Kerboeuf, 1993) and cause a Ca$^{2+}$-induced Ca$^{2+}$-release “CICR”. Whereas, it has also been argued that the inward Ca$^{2+}$ current occurring via the hyperpolarization-sensitive Ca$^{2+}$ channels (Ca$^{2+}_{\text{hyper}}$) could account for a rapid increase of the [Ca$^{2+}$] beneath the cell membrane (Kerboeuf and Cohen, 1990) causing a Ca$^{2+}$ release from the alveolar sacs by a Ca$^{2+}$-induced Ca$^{2+}$-release “CICR” mechanism (Fig. 2a).

1.4.2  **(SOC) mechanism**

Alternatively, stores could be activated by coupling to the cell membrane via a signal other than Ca$^{2+}$ influx, e.g. by some other chemical or by structural coupling, so that Ca$^{2+}$ primarily released from the internal stores could then secondarily entail a store-operated Ca$^{2+}$ influx “SOC” mechanism (Fig. 2b), as outlined in recent reviews (Berridge, 1997; Barritt, 1998; 1999; Berridge et al., 1998; 2000; Gregory et al., 1999; Patterson et al., 1999; Alderton et al., 2000; Elliott, 2001). A SOC-type mechanism can include activation of a “Ca$^{2+}$/polyvalent cation) sensing receptor”, CaSR, by AED (Klauke et al., 2000). Increasingly such sensors are found widely distributed, up to higher eukaryotes (Zaidi et al., 1995; Chattopadhyay et al., 1998). CaSR can mediate a [Ca$^{2+}$]$_i$ increase in response to an [Ca$^{2+}$]$_o$, increase or to extracellular polyamines. In *Paramecium* where exocytosis induction by polyamines is well established (Plattner et al. 1984; 1985) CaSR may be functionally coupled to the alveolar sacs which are also physically connected to the cell membrane by protein bridges over a subplasmalemmal space of only ~15 nm width (Plattner et al., 1991). Functional coupling does not involve any known second messenger (Länge et al., 1995). Although alveolar sacs resemble the Sarcoplasmic Reticulum “SR”, since in both cases Ca$^{2+}$ can be released by caffeine- and 4CmC- sensitive channels (Klauke and Plattner, 1998; Klauke et al., 2000), superimposed influx in *Paramecium* may occur through cation channels of low sensitivity (Klauke et al., 2000), like those described by Saitow et al. (1997).
Since the molecular identity of the components envisaged is not yet established, this scenario is hypothetic.

**Fig. 2.** A schematic drawing showing the two models of possible mobilization pathways of Ca$^{2+}$ from the subplasmalemmal stores during Paramecium cell stimulation (e.g., by AED). The left side of the scheme represents the Ca$^{2+}$ release from the alveolar sacs that could be evoked by a hyperpolarization of the plasma membrane. Following activation of the hyperpolarization-sensitive Ca$^{2+}$ channels by AED (orange arrows, 1a), a Ca$^{2+}$ influx in the subplasmalemmal space would result in a local [Ca$^{2+}$] elevation that in turn would cause a Ca$^{2+}$ release from the stores (CICR, 2a). Released Ca$^{2+}$, in excess in the cortical space, would be re-sequestrated in the alveolar sacs by the Ca$^{2+}$-ATPase (3a). Alternatively, on the right side of the scheme, the mobilization of intracellular stored Ca$^{2+}$ would occur prior to the influx of the external Ca$^{2+}$. The direct coupling of the cell membrane with the store membrane at the level of the ring particles (represented by the large grey arrow, 1b) would allow the transduction of the external signal directly into a Ca$^{2+}$ release from the alveolar sacs (2b). The decrease of the [Ca$^{2+}$] in the subplasmalemmal stores would induce a secondary Ca$^{2+}$ influx from the extracellular space required for stores refilling (3b).
1.5 *Paramecium* mutants and analysis of Ca\textsuperscript{2+} dynamics

Many mutations available in *Paramecium* may facilitate a more detailed analysis of Ca\textsuperscript{2+} dynamics during exocytosis. For instance, the non-discharge mutant, nd12, is unable to release trichocysts by exocytosis after cultivation at 35°C (Cohen and Beisson, 1980), in contrast to 25°C, and it has no \textsuperscript{45}Ca\textsuperscript{2+} influx upon AED stimulation (Kerboeuf and Cohen, 1990). Therefore, the nd12 mutant lends itself to analyze selective components of the Ca\textsuperscript{2+} signaling pathway, e.g. to discriminate between CICR- and SOC-type mechanisms.

In the present study, to avoid any interference of ciliary Ca\textsuperscript{2+} channels, the analyses has been performed with the double mutant, pwA-nd12, for which Kerboeuf and Cohen (1990) have shown absence of \textsuperscript{45}Ca\textsuperscript{2+} influx when cultivated at 35°C. The d4-500r, a pawnA (pwA) mutant, is devoid of any functional voltage-dependent ciliary Ca\textsuperscript{2+} channels (Satow and Kung, 1980; Haga et al., 1982). In pwA-nd12 (35°C) cells, any Ca\textsuperscript{2+} influx must pass the somatic (non-ciliary) cell membrane. In contrast to 35°C cells exocytosis in response to AED is normal in double mutant cells when cultivated at 25°C (Kerboeuf and Cohen, 1990), as in d4-500r cells (Plattner et al., 1984).
1.6 **Aim of the work**

Bearing in mind the introductory remarks given previously, the present work has been carried out on the double mutant “pwA-nd12” mainly to discriminate between CICR- and SOC-type mechanisms used in calcium signalling for exocytosis.

Since the cellular calcium imaging is one of the most reliable biological techniques (Brownlee, 2000), therefore, this study included fluorochrome analysis, while stimulating cells with AED, caffeine, 4CmC and Ca²⁺ respectively, under conditions of variable [Ca²⁺]₀. The applied fluorochromes were fura-red/double wavelength (2λ) and eventually fast fluo-3/1λ confocal laser scanning microscopy (CLSM) analysis to monitor changes in [Ca²⁺]ᵢ, in response to the different stimuli, at different [Ca²⁺]₀ values. In addition, EDX was used to analyze the total (free and bound) calcium concentration [Ca] in alveolar sacs and their capacity to release Ca²⁺ in response to AED, caffeine, 4CmC and Ca²⁺ respectively. Normally bound calcium dominates over free calcium over thousandfold, in the cytosol as well as in stores, also in *Paramecium* (Plattner and Klauke, 2001). Also, membrane fusion capacity has been analyzed by fast freezing and quantitative freeze-fracture analysis. Finally, protein electrophoresis has been carried out for the total protein occurring in the double mutant pwA-nd12 (25°C and 35°C) cells.
2. Methods and Materials

2.1 Cell cultures

The double mutant *Paramecium tetraurelia* cells with combined pwA-nd12 genotype were obtained by crossing nd12 mutant and d4-500r mutant as described by (Kerboeuf and Cohen, 1990). These cells were kindly received from CNRS, Gif-sur-Yvette, Cohen Lab, France.

The used medium for culturing the pwA-nd12 cells was dried lettuce medium which was monoxenically inoculated with *Enterobacter aerogenes* as feeding bacteria (Plattner et al., 1980) and contained \([\text{Ca}^{2+}]_o \geq 50 \ \mu\text{M}\) which is required for normal cells growth and physiological activities, since \([\text{Ca}^{2+}]_i\) depends largely on \([\text{Ca}^{2+}]_o\) in *Paramecium* (Browning and Nelson, 1976; Plattner and Klauke, 2001). About 25 ml of well grown cells from the stem culture of the pwA-nd12 were put into 250 ml of the culturing medium in a 500 ml glass flask to allow the presence of a sufficient amount of oxygen for the growing cells culture. Then, the pwa-nd12 cell cultures were grown under permissive condition at 25°C i.e., they could perform exocytosis of trichocysts upon stimulation e.g. by AED (Plattner et al., 1984; 1985). After 4 days of inoculation at 25°C the cell cultures were transferred to non-permissive conditions at 35°C i.e., they could not perform exocytosis (non-discharge mutant) upon AED application.

Stock cultures of cells at 25°C could be renewed between the time period of 4 to 10 days after filtration and concentration of cells by low–speed centrifugation. At every second inoculation of the cultured pwA-nd12 cells at 25°C, stigmasterol (5 mg/L) was added to the culturing medium as an additional supply for nutritional compounds. Cell culturing and incubation were performed under sterilized conditions.

Both non-discharge and pawn properties of the crossed strains cultured either at 25°C or 35°C were tested before any experimental work by adding
0.02% AED (~2 µM final conc.) to cells (1+1) in suspension, according to Plattner et al. (1984; 1985) to test the discharge capability of trichocysts. Parallel to AED test, Saturated aqueous solution of picric acid was added to cells (1+1) to check the exocytosis capacity. The pawn character was tested by adding 40 mM KCl solution to cells (1+1), for chemical depolarization of the membranes, i.e., to check the ciliary reversal property. Only cells cultivated at 25°C performed exocytosis in response to AED, while cells from both 25°C and 35°C cultures were unable to perform ciliary reversal according to chemical depolarization test. It was found that at least ≥8 divisions (≥3 days) were required to acquire non-discharge properties upon transfer from 25°C to 35°C.

2.2 Testing the growth rate of the pwA-nd12 cells

This test was done to investigate the growth rate of cells under the permissive and non-permissive conditions and to check the tolerance degree of the pwA-nd12 grown at 35°C. Thus, from the culture incubated at 25°C, 5 ml cells were taken daily and mixed with 5 ml acetate buffer, then 9 ml from the mixture were taken on a counting glass slide to count the cells. After the fourth day at 25°C the cell cultures were transferred to 35°C and the same procedure has been done to trace the growth rate of pwA-nd12 at 35°C. Using Sigma plot 4.0 software, a growth curve was obtained.

2.3 Testing the exocytotic capacity

2.3.1 Exocytosis and reversibility test

Testing the exocytosis capacity and reversibility of pwA-nd12 cells at 35°C was planned in order to control the condition and time period under which all the further experiments on pwA-nd12 at 35°C have been done and to check if the cells at 35°C could undergo reversibility of the non-discharge characters after returning them back to a 25°C incubator and to see how long was the required time for reversibility.
To perform this test 10 ml cells were taken daily from pwA-nd12 cultured at 35°C, starting at day 0, then concentrated by low-speed centrifugation (800 rpm). 10 µl concentrated cells were put on a glass slide then 10 µl of 0.02% AED (~2 µM final conc.) were added to stimulate the exocytosis. The exocytosis capacity was examined and evaluated under phase contrast light microscope (Plattner et al., 1984; 1985).

When the cells reached to the day at which no more any exocytosis could be performed, some cultures were returned back for incubation at 25°C. Then every hour, the exocytosis capacity was again tested as mentioned above.

The same test has been carried out again with the pwA-nd12/35°C, but with adding 1 mM Ca\(^{2+}\) to the culturing medium at the fourth day. Addition of Ca\(^{2+}\) to the medium was to ensure enough concentration of extracellular Ca\(^{2+}\) which may be needed for exocytosis and reversibility as well.

### 2.3.2 Exocytosis capacity upon different triggering agents

In order to check the exocytosis capacity with different materials, 10 ml cells at 35°C were taken daily, starting at day 0, concentrated at 800 rpm for each of the following experiments. Then 10 µl concentrated cells were put on a glass slide and 10 µl triggering agent were added. Testing the exocytosis capacity has been taken place under the phase-contrast microscope. Saturated aqueous solution of picric acid was used as a routine triggering agent as in genetic studies, in comparison to the effect of AED (2 µM final conc.) at [Ca\(^{2+}\)]\(_{o}\) = 50 µM. Eventually, [Ca\(^{2+}\)]\(_{o}\) was increased to 1 mM ensuring the Ca\(^{2+}\) influx force or chelated by EGTA to ~30 nM alternatively with the triggering materials as follows.

1- AED (2 µM final conc.) + 1 mM Ca\(^{2+}\) or 1 mM EGTA
2- Caffeine (50 mM final conc.) + 1 mM Ca\(^{2+}\) or 1 mM EGTA
3- 4CmC (0.5 mM final conc.) + 1 mM Ca\(^{2+}\) or 1 mM EGTA
4- Ionophore A23187 (33 µM final conc.) + 1 mM Ca\(^{2+}\) or 1 mM EGTA (Ionophore A23187 was dissolved in dimethylsulfoxide “DMSO”).
2.4 Cell stimulation and microfluorometric calcium dynamics analysis

Recently, the development of new fluorescent dyes by Tsien and others provided the ability to investigate ionic activity in single cells (Tsien, 1980; 1988; Gryniewicz et al., 1985; Minta et al., 1989). It is interesting to mention that the rapid local calcium transients $[\text{Ca}^{2+}]_{i}^{\text{act}}$ during activation in Paramecium cells by AED was shown for the first time by Klauke and Plattner (1997). Ca$^{2+}$ fluorochromes’ loading becomes one of the most important and useful tools used for the time- and space-resolved analysis of $[\text{Ca}^{2+}]_{i}$ during activation in Paramecium cells (Erxleben et al., 1997; Plattner et al., 1997b; Klauke et al., 1998; Klauke and Plattner, 1998; 2000; Blanchard et al., 1999; Hauser et al., 2000; Plattner and Klauke, 2001). This technique could be divided into the following three major steps:

1- microinjection to load the fluorochrome into a single cell,
2- triggering the cells using different triggering agents,
3- fluorometry and image analysis for individual cells.

In the present work, to perform the fluorochrome analysis of triggered individual pwA.nd12 cells, previously injected with a Ca$^{2+}$ fluorochrome, the next protocols used were according to Klauke (1995) and Blanchard (1998), with few modifications.

2.4.1 Microinjection

It was discussed by (Takahashi et al., 1999) that this method is invasive and requires specialized instruments and practice. In addition, the number of cells that can be loaded with probes is limited. In spite of these difficulties, many scientists rely on microinjection (Blinks et al., 1982; Endoh and Blinks, 1988), because it gives precise and more detailed results.

Since the Paramecium pellicle prevents the passage of the cell–permeable ester form of the fluorescent Ca$^{2+}$ indicators, in the present work, microinjection of individual cells is used to load the cell with the non-permeable fluorochrome salt directly into the cytosol under light microscope control. Thus,
the next few preparations were used to establish the microinjection of the double mutant pwA-nd12 cells.

2.4.1.1 Preparation of siliconated slides

Since the oil immersion was used for the microinjection and the observation in both, transmitted light and fluorescence modes, it was necessary to use cover slips (24 x 60 mm) as cell carriers. Their 0.17 mm thickness allows the 63x oil objective lens to focus through a 50 µm thick object such as *Paramecium* cells. One slip surface of the slide was siliconated by application of a mixture solution of silicon and isopropyl alcohol 1+10 respectively, then the slides were kept one to two hours at 100°C for drying. Overlying one side of the slide with silicon solution is to increase the convexity of the microdrops. Therefore, the pressure on the cells was reduced and the microdrop is kept as a swimming pool providing normal conditions for the cell. It should be taken into consideration that the other side of the cover slip will be above the oil lens in the inverted microscope used and a droplet of oil will be applied on it for the 63x oil immersion objective. Therefore, this side should not be covered with silicon to allow the dispersion of the oil droplet.

2.4.1.2 Preparation of different types of capillaries used

**Equipment and tools used**

I- Soda-lime glass tubes were used, without a filament for sucking capillaries or with an inner filament for finer capillaries. The tube was 100 mm long, with 1.2 mm external diameter and 0.96 mm internal diameter.

II- A micropipette thermal puller consisting of a thermal coil (heater), a potentiometer to control the electrical current passing in the thermal coil, in addition to an attached unit which is able to hold and pull a capillary at either end. There is a scale to control the stretching grade of the capillary. These structural constituents allow control of numerous parameters, such as temperature, duration and force of pull, each of which dictates in part the shape, diameter and fineness of the microcapillary.
III-A microforge is needed to bend the microcapillary. It consists of a chrome-nickel wire (0.5 mm in diameter) bent into a fine hairpin loop. The electrical current through the wire is controlled by a potentiometer at ~6 A. there is an attached light microscope focused on the wire to measure and adjust the microcapillary’s diameter. A simple mechanical micromanipulator is present to clamp and position the capillary near to the wire. The position and bending the capillary is controlled by a dissecting microscope.

**Capillaries preparing protocol**

I- **Sucking capillaries**

The glass tube without filament was fixed into the micropipette puller and pulled in two stages. First, a current of ~25 A was applied to the heater coil and the tube was stretched at 1 grade only of the puller scale. Secondly, the current was readjusted at 15 A and the capillary was heated up for ~25 s until it was broken into two identical capillaries. The external diameter of each capillary at the breaking point was reduced from 1200 µm to 100 µm. this capillary could be used to prepare any of the following ones;

1- Isolation capillary used to transmit single cells into single droplets on the cover slip. It was prepared by the microforge to bent the 100 µm capillary downward to 45°. Therefore, its tip could be placed obliquely in the medium droplets facilitating the isolation process.

2- Medium sucking capillary used to immobilize the isolated single cells incompletely under the inverted microscope, preparing them for microinjection. The 100 µm capillary was held on the micromanipulator of the microforge to reduce its diameter into 10-20 µm by softening the glass close to the red-hot chrome-nickel wire. This diameter reduction was controlled by a scaled lens of the attached light microscope. Then the capillary was bent two times, first to 45° downwards, then the tip was bent to 45° forwards carefully. This double bending helps to absorb or add the medium from or into the isolated cells droplets for the microinjection process.
II- Finer capillaries

The glass tube with incorporated filament was pulled two times on the puller, both were carried out at ~25 A to break into two similar capillaries. Each had a smooth needle-like tip with 1 or 2 µm inner diameter. The inner filament of the finer microcapillaries improved the capillary attraction so that, when the injected solution was introduced at the rear open end of the capillary, it moved down to its very thin tip. At the level of narrowing, the tip of the finer capillary was bent ~90° on the microforge. These finer capillaries could be used as;

1- Injecting microcapillary that was loaded with the Ca$^{2+}$ fluorochrome, then introduced into the cell.

2- Triggering capillary containing the trigger agent needed for the cells stimulation. If the tip of this capillary was clogged, it was possible to open it carefully by touching the glass slide gently and breaking the extremity of the needle with the sucking capillary under the inverted microscope.

3- Fine sucking capillary was used to immobilize the single cells completely by fine sucking of the medium before trigger application. It could be used to add medium into the individual cell droplets after cell microinjection to allow recovery of the cell before triggering. It is placed just touching the glass of the slide in a parallel manner.

2.4.1.3 Microloader preparation

A long polyethylene tube (10 cm long, 5 mm diameter) was softened in the middle over the short flame of a Bunsen burner and pulled, outside the flame, to a 15 cm long tube of ~1 mm in diameter. This tube was cut in the middle to give two microloaders. The 5 mm large end of the microloader was fixed to a pipette to suck in 4 µl of the used solution. The thin end of the microloader was then introduced into the rear opening of the capillary and the filling solution is then pumped into it. The fluorochromes and the triggering agents were freshly loaded for each experiment. The medium sucking and isolating capillaries were completely filled with a fresh culturing medium or
distilled water carefully without presence of air bubbles while, the fine sucking capillary was used empty.

2.4.1.4 Evacuation of the injecting or triggering materials

The injecting and triggering capillaries were inserted separately into two tubes which were carried by 2 micromanipulators and connected to an air pump to evacuate the solution during microinjection or triggering under ~5 bars pressure. The isolating, sucking and fine sucking capillaries were inserted into a paraffin oil filled tube of Hamilton syringes and set up in the holders of micromanipulators. Evacuation and sucking of the medium (or cells transmission during isolation process) were carried out by moving carefully a microwheel, which was connected to the Hamilton syringe.

2.4.1.5 The micromanipulators

All micromanipulators used were capable of moving the capillary in 3 planes (x, y, z). The manipulator used for the single cell isolation was a simple mechanical one (micropositioner D10/S10 from Brindi). While, the other micromanipulators used in the immobilization, injection and triggering processes were of a mechanical type from Leitz. They were mounted and carefully adjusted in a suitable manner and close to the sides of the chassis of the inverted microscope (Fig. 3a, b). finally, the fine sucking capillary for complete immobilization of the cells was held on a M3301 manual micromanipulator which was fixed to the slide stage of the microscope.

2.4.1.6 Preparing the cells for microinjection

Single cell isolation procedure

Six droplets of ~1 μl lettuce medium were laid down in two regular rows on the center of a previously silicon covered slide and then individually covered with paraffin oil to prevent evaporation. Cells were obtained from the culture after filtration (to get rid of bacteria) and concentration to have a high density of cells. From this concentrated suspension, a ~25 μl drop of cells was put on a second cover slip by using a micropipette. Then sucking a single cell up
into the isolating sucking capillary and dropped into one of the 6 pre-laid medium droplets on the siliconated cover slide. By the same way all the other 5 droplets were filled with single cells, as well. The number of cells was restricted to 6 only to perform the 6 trials within a time not exceeding more than one hour for the double mutant pwA-nd12 (35°C) cells, i.e., not to allow the reversion of the 35°C cells. Sucking the single cells was carried out under binocular dissecting microscope (Fig. 4a, b) which aids to choose the cells in their elongation phase, since this is the stage presenting the larger surface and the start of disorganization of the cytoskeletal structures of the cortex. Therefore, the resistance opposed to the penetration of the injecting micropipette into the cell is highly reduced (Itfode, et al. 1989).
Fig. 3. Photos showing (a) the used inverted microscope (2λ) with its attached tools for the microinjection such as the fluorescence and transmitted cameras, monitors, and the Leitz micromanipulators which are set close to the microscope. Each manipulator (b) could be moved in 3 planes (x, y, z) and used for microinjection and triggering experiments. These photos are taken in the cell biology lab, “AG. Plattner”, University of Konstanz, Germany.
Fig. 4. A photo showing the single cell isolation procedure (a) under the microscopic control, using the micropositioner “D10”. The schematic representation (b) of the isolation process was reproduced according to Koizumi (1974). The photo (a) is taken in the cell biology lab, “AG. Plattner”, University of Konstanz, Germany.
Cells immobilization

Under the inverted conventional or confocal laser scanning (CLSM) microscope, the cells were immobilized incompletely by aspirating the excess fluid from the cell droplet using the twofold bent sucking capillary (~10 µm ø). To facilitate the microinjection, the volume of the droplet should be reduced to a minimum in order to slow the swimming velocity of the cells according to the method described by Knowles (1974) and Koizumi (1974), adapted to the inverted microscope by (Kersken et al., 1986) as reported in Fig. 5.

Fig. 5. A photo showing the procedure of the Paramecium cells immobilization, using the different microcapillaries under the inverted microscopic control, preparing the cells for the microinjection with fura-red and the further triggering experiments. The photo is taken in the cell biology lab. “AG. Plattner”, University of Konstanz, Germany.
Microinjection of the individual cells

Loaded microinjection capillary with a fluorochrome was adjusted above the slowly moving (immobilized) cell by controlling the capillary shadow under the phase contrast, 10x lens of the inverted microscope, using the higher magnification phase contrast 63x and the Leitz micromanipulators (Figs. 3, 5); the tip of the injecting capillary was positioned in a perpendicular direction over the middle of the cell near the cytostome (Fig. 6). Injection in this area facilitates the rapid and equal distribution of the fluorochrome all over the cell body by the movement of the cytoplasm in a time period between 0.5 to 2 minutes (Fig. 7). Then, the capillary was lowered slowly until it reached the surface of the slide, therefore, penetrating the cell membrane at the contact point. Once the opening of the microcapillary was inside the cell, the injecting material was pressure ejected out of the capillary by opening the pressure valve that controls the passage of the air into the rubber tube connected to the capillary. The applied air pressure was adjusted at 5 bars by a complete set of valves. By using black-and-white CCD camera connected to the microscope and a 14 inch monitor one can watch the delivery of the injected solution that was manifested as a change in the phase density spreading out of the needle. The volume injected was controlled by the pressure valve and was estimated according to Klauke (1995) to equal about 10% of the cell volume, i.e., \( \sim 10^{-11} \) l injected volume (Erxleben et al., 1997). As soon as the microinjection was completed, the injecting microneedle was pulled out of the cell quickly and the microdroplet was carefully enlarged by adding \( \sim 2 \mu l \) medium, so that the cell could swim again and recover from the injecting process.
Fig. 6. The injecting process of pwA-nd12 Paramecium (P) cell with fura-red. (a) shows adjusting the tip of the capillary (arrow) before injection, (b) shows the tip of the capillary inside the cytoplasm of the Paramecium cell. Mag. = 630x
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Fig. 7. A fluorescent image of a Paramecium (pwA-nd12) cell (P), injected with fura-red, at 490 nm excitation wave length. Mag. 400x

Fig. 8. A transmitted light microscope image of a Paramecium (pwA-nd12) cell (P), injected with fura-red, showing adjustment of the position of the triggering capillary (arrow). Mag. 630x
2.4.2 Triggering the cells

The fine triggering microcapillary (~1-2 µm Ø) was loaded with the different triggering materials upon the applied experiment. Then, it was adjusted by the help of Leitz micromanipulator horizontally over the glass slide and near the droplet containing the injected cell with a fluorochrome. Complete immobilization of the cell was performed by sucking the medium using two fold bent sucking and fine sucking microcapillaries, respectively. Then the triggering capillary was lowered to touch the cover slip surface and positioned close and opposed to the anterior end of the cell with a distance of approximately 10 µm (Fig. 8). The triggering solution was pressure ejected from the micropipette using 5 bar air pressure for ~0.1 s controlled by the pressure valves and the controlling monitor. The final concentration of triggering material reaching the cell surface is estimated, using a dilution factor, as 10% of the initial concentration of the trigger inside the microcapillary (Klauke, 1995; Blanchard, 1998; Klauke and Plattner, 1998). Some of the applied triggering agents were permeable and others were impermeable to the cell owing to their own properties (Table 1). All the triggering agents were used in Pipes buffer (5 mM, pH = 7.0), as normally used for extracellular application (Klauke, 1995).

Table 1. Triggering agents for extracellular application.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Final extracellular concentration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AED</td>
<td>2 µM or 10 µM</td>
<td>(Plattner et al., 1984)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>50 mM</td>
<td>(Klauke and Plattner, 1998)</td>
</tr>
<tr>
<td>4CmC</td>
<td>0.5 mM</td>
<td>(Klauke et al., 2000)</td>
</tr>
<tr>
<td>Calcium</td>
<td>1 mM or 10 mM</td>
<td>(Plattner et al., 1997a)</td>
</tr>
</tbody>
</table>

The triggering experiments have been performed usually, with \([\text{Ca}^{2+}]_o\) ~50 µM, since, in strictly monoxenic cultures, as routinely used, routine measurements by a \(\text{Ca}^{2+}\)-selective electrode always yielded \([\text{Ca}^{2+}]_o\) close 50 µM; these are the “standard conditions” whereby any deviation can easily be
corrected. Eventually $[\text{Ca}^{2+}]_o$ was reduced by adding BAPTA (1 mM final conc.) to each of the stimulants (in a mixture 1+1). BAPTA is functioning as an extracellular or intracellular calcium chelator (Tsien, 1980) producing lower $\text{Ca}^{2+}$ values. The resulting $[\text{Ca}^{2+}]_o$ as derived from calculation is $\sim 30$ nM, as published previously (Knoll et al., 1991b). Evidently it is important to work with these extremes, i.e. with and without a potential $\text{Ca}^{2+}$ influx, influx being impossible when $[\text{Ca}^{2+}]_o$ is reduced to slightly below $[\text{Ca}^{2+}]_{\text{rest}}$ by the BAPTA chelator as it will be shown in the “Results” section. Simultaneous application of the stimulant and the $\text{Ca}^{2+}$ chelator avoids cell damage (Klauke et al., 2000).

### 2.4.3 Fluorometry and image analysis

Most of the experiments have been done under the conventional double wave length microscope ($2\lambda$) and only few trials have been performed by the confocal laser scanning microscope (CLSM/$1\lambda$). This was because the double mutant pwA-nd12 cells showed a strong photoactivation phenomenon due to excitation of the fluo-3 by the laser beam in CLSM that produced an increase in the fluorescence activity of fluo-3, the injected fluorochrome. This photoactivation peak may interfere with the peak of the calcium transients during triggering. While, by the conventional ($2\lambda$) microscope, it is possible to get a ratio analysis of two excitation wavelength fluorochrome (fura-red was used in this analysis). In addition, there is no photoactivation observed by fura-red. The pilot data obtained by CLSM only served to guarantee that no fast signals were overlooked. The fluorometry and image analysis technology involves many advanced tools, on which a short account is given on the next few pages.
2.4.3.1 Structural and technical idea of the video-assisted conventional fluorescence microscopy

The fura-red experiments were performed under the inverted conventional microscope, which has a rotating filter wheel and an automatic microprocessor control unit. The filter-wheel was fixed between the mercury lamp housing chamber and the back of the microscope chassis. The control unit could be programmed to change the position of the filter wheel in certain time intervals, keeping it mechanically separated from the microscope. The excitation light was selected from a 50 W mercury lamp by 10 nm bandpass interference filters were mounted on the filter wheel. The rotation exchange cycle of the filters remained 0.5 s i.e., each excitation filter remained 0.5 s in the fluorescence light beam and a ~0.4 s is needed for the exchange of the filters, allowing video recording during the experiment and the production of a ratio picture every ~1.4 s. The excitation light was reflected into the objective (63/1.3 oil) using a dichroic mirror. Emission was collected by the objective and passed the dichroic mirror and barrier filter (590 nm) for fura-red. The microscope and the micromanipulators were placed on a home made vibration free table (Fig. 9).
Alternative phase contrast and fluorescence modes

In order to achieve the different technical steps of the experiment, it was necessary to change between the fluorescence and phase contrast modes in a fast manner. This is performed automatically in harmony after programming the microprocessor. i) During the phase contrast mode, the shutter in front of the halogen lamp is opened allowing passage of the normal transmitted light. The filter-wheel is in closed position preventing passage of the light beam of the mercury lamp needed for fluorescence detection. At the same time, the reflector slider carrying the barrier filter is out of the normal light beam and the dichroic mirror is in erect position i.e., does not reflect the transmitted light beam and
allows it to pass directly into the CCD camera, then to the monitor for controlling the microinjection steps. ii) In order to switch into the fluorescence mode, the shutter of the halogen lamp is closed i.e., no transmitted light passes. The filter-wheel moves into the filter position, allowing passage of UV light coming from the mercury lamp. At the same time, the reflector slider moves internally and the barrier filter comes in the UV beam to select the emission wave length of the used fluorescence camera when the dichroic reflecting mirror was tilted to 45° interposing the UV beam (Fig. 10).

![Fig. 10. A schematic drawing, to show the changes (arrows) required to alternate between the transmitted light mode (left) and the fluorescence mode (right) of the inverted (2λ) microscope. The permutation of the excitation filters, as well as reflector slider movement and shutter position were controlled by a microprocessor and could be synchronised. Each excitation filter usually remained 0.5 s in the fluorescence light beam allowing the production of a ratio picture ~ every 1.4 s. This scheme was designed according to Blanchard (1998).](image-url)
2.4.3.2 Video recording

During the fluorescence mode of the microscope, the fluorescent light rays reach an image intensifier of a moon light black-and-white fluorescent camera (VW-1900). The image intensifier and the incorporated highly sensitive Newvicon tubes of the camera provide high quality video pictures under extremely low light level (in the red and infrared spectrum part) with reduced blooming and fading effects. The light sensitivity gain of the camera is ranging between 0 to 100 times, which could be programmed by an attached control unit. The phase-contrast images were acquired by a CCD (charge-coupled device) camera, which ensures lower geometric distortion and better photometric properties than a conventional video camera. The light sensitivity of the camera could be manually adjusted. The video signals generated by both cameras were directly viewed on two black-and-white TV monitors and recorded as video format using U-matic and S-VHS video tape recorders, respectively. A time-data generated or a time-code generator was connected to the video recorders that could superimpose date and time to any recording. These additional data were recorded on one of the sound tracks of the video-tape and they allow precise analysis of the obtained video frames.

2.4.3.3 Image processing

a) Digitization

Digitization is needed to convert the analog video frames into digital pictures. The pixel analysis for the fluorescence activity could be precisely measured by means of a computer later on. This process has been performed by using a video overlay board (fast video machine), LTC time-code which allowed precise choice of the sequences of frames. These were digitized on “a Pentium III PC” with 20 GB memory. A fast video frame capture card “AV master 2000” has been used with the software program “Ulead Media Studio pro 5.2”, whose installation principle is illustrated in Fig. 11. Each digitized image was 1.26 MB
in a TV size (in pixels, 768 wide x 576 height) i.e., they could be displayed on a control TV monitor and coded as gray level BMB file.

Fig. 11. A diagram showing the computer tools used in digitising the frames obtained from the conventional double wavelength microscope upon different experiments. This diagram is obtained from the manual of AV Master; Fast Multimedia AG (1997).
b) Transferring digital frames to the Unix-work station

In order to analyze the digital frames, they should be exported via “a FTP 95 session” (file transfer protocol) through the Internet to the Unix-workstation. The images were temporarily stored on the Unix hard disc until further image analysis has been completed.

c) Fluorometry

Measuring the fluorescence intensities of the digitized BMB images at both excitation wavelengths was performed by “a Unix-workstation” as specified previously by (Klauke, 1995; Erxleben et al., 1997; Klauke and Plattner, 1997; Blanchard, 1998). First, the BMB file images were transformed into Tiff file images by using xv-editor software program. Second, with a file convertor program, the tiff images were converted into a movie, which could be substacked into serial frames by the intervision 2D analysis software. Then, a box of ~10 x 5 µm was drawn manually in the area of interest of the cortical region (at the triggering region where the stimulus has been applied). The box was copied from the first image then pasted and readjusted manually on all the other frames. The reason for the manual propagating of the analysis box on the other frames is due to the movement of the cell during the triggering experiment. Therefore, this process was one of the most time consuming steps. This step was applied on each series of the frames for the double excitation wavelengths of the fura-red (440 nm and 490 nm) series. As a result of determining the analysis area, automatically the 2D analysis program measures precisely the mean of fluorescence intensity per pixel emitted at this area. The resulting absolute numerical values of the fluorescence intensity were stored as text files format for each wavelength. In case of using the fast CLSM analysis, combined with fluo3/1λ the same above-mentioned procedure was applied to measure the intensity of the fluorescence activity at time (0) and during the stimulation of the cells. Since fluo-3 has only one excitation wavelength (~488 nm), therefore, only one series of frames was obtained.
d) Graphs design

In order to express the results obtained (in the case of fura-red analysis), a ratio of pixel intensities was calculated using “Sigma plot 4.0”. The ratio analysis (490/440) then was transformed into f/f₀ values which means that any fluorescence readings during stimulation (f) are referred to the reading at rest (f₀) at time (t = 0), i.e., just before stimulation. It was preferred not to plot the curves directly as f/f₀ ratios because the increase in [Ca²⁺]ᵢ causes a decrease in the fluorescence emission obtained at 490 nm excitation, thus decrease in f/f₀ ratios can occur as well, that may confuse the reading of the curve. Therefore, the f/f₀ ratios were referred to [Ca²⁺]ᵢ increase values, starting from 1 as a representative [Ca²⁺]ᵢ resting value. On the other hand, f/f₀ ratios (also, referred to [Ca²⁺]ᵢ increase values) were directly obtained in the case of fluo-3/1λ analysis under CLSM, since only one excitation wavelength (488 nm) is used for fluo-3. The graphs shown in the present work are examples of the majority of the reactions of cells seen in all of the experiments. Mostly, only 3 randomly selected cases were chosen, out of 5 to 7 cases, to be completely presented in this work.

e) Preparing false color images

To visualize the change in fluorescence in the images obtained, the gray scale images were displayed in pseudocolor pictures in the Unix-workstation. First, the frames required were selected from the two movie substacks by “2D analysis”. The selection of frames was based upon time course of the experiments (i.e., before and after triggering the cell). Then, application of ratio analysis, which divides pixel by each pixel of every two corresponding frames provided by the dual-wave length (2λ) recording. The resulting ratio images (which are representative of [Ca²⁺]ᵢ change) were then displayed as false-color pictures after definition and loading of “256 pseudocolor map” of the 2D analysis software. The pseudocolor scale was related to the [Ca²⁺]ᵢ representative values used in the sigma plot curves. Finally, image processing and arrangement
was performed by image work, show-case and snapshot software programs. Printing the false color images was done by a color laser printer.

f) Archiving images and Unix data

All data obtained in Unix-workstation were transferred by “FTP 95” session into Pentium III PC, then saved on “650 MB” CD discs.

2.4.3.4 Principle of the confocal laser-scanning microscope

As reported by McCormack and Cobbold (1991), confocal microscopes suitable for imaging fluorescence in living cells are a relatively recent innovation. The optics of these instruments allow improved resolution, particularly in the z-axis, where out-of-focus information can be rejected almost completely. This is achieved by illumination the sample with the image of a pinhole and viewing the fluorescence through a second pinhole at an identical image plane. An image is built up by scanning the confocal pinholes in two dimensions over the sample. The lasers used in confocal microscopes emit at only a limited number of wavelengths, while it is relatively easy and cheap to divide the emitted light according to wavelength and send it to multiple detectors (Rizzuto and Fasolato, 1999). Fig. 12 shows the basic idea of the light path in a confocal laser microscope.

In the present study, semi-quantitative \([\text{Ca}^{2+}]\), measurements were done using CLSM which ensured high temporal and spatial resolution for imaging calcium transients. An inverted microscope, ZEISS Axiovert 35T, is equipped with a confocal imaging system (Odyssey – Noran Instruments) able to acquire pictures at video rate (33 frame/s) because of the installation of an acousto-optical deflector. This deflector provides a high frequency horizontal scan of the laser beam or line scan. After the diffracted beam is reflected by a primary dichroic mirror, it strikes a galvanometer mirror oscillating at a lower frequency, thus providing the vertical or frame scan. The fluorescent light emitted by a sample exposed to the laser beam is shifted to a longer wavelength
than the excitation one, and largely transmitted by the primary dichroic mirror to the fluorescence detection path. Then, the emission fluorescent light passes through the confocal split, before reaching finally the detector.

Though a slit aperture provides a somewhat decreased axial resolution compared to a pinhole aperture, it ensures a better signal-to-noise ratio and favours a higher time resolution. An advantage of the Odyssey system is the possibility to achieve alternating acquisition of fluorescent images and transmitted light images, thus allowing \([\text{Ca}^{2+}]_i\) variations to be correlated with any exocytosis event. The digital pictures were stored and processed on a Unix-workstation. The time course of \(\text{Ca}^{2+}\)-indicators emission was documented using a time-series protocol. Images were acquired with a variable frequency at the same depth in the sample. The maximal frequency that could be used for capturing a series of 620 x 480 pixels large pictures was 30 frames per second (video rate).

**Fig. 12.** The basic ray path of a confocal microscope. Diagram courtesy of Leica Lasertechnik, according to Bolsover (1999).
2.5 \textbf{Ca}^{2+} \textbf{chelators}

2.5.1 \textbf{EGTA}

The ethyleneglycol-bis(\beta-aminooethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) has been used in most of the recent experiments in which a low and controlled free Ca$^{2+}$ was needed (McCormack and Cobbold, 1991). Therefore, EGTA is widely used Ca$^{2+}$-selective chelator, which has a selectivity for Ca$^{2+}$ over Mg$^{2+}$, 5 to 6 orders of magnitude. EGTA is well suited for buffering Ca$^{2+}$ in the physiological range of 10$^{-8}$ – 10$^{-6}$ M in the presence of physiological Mg$^{2+}$ concentrations of 10$^{-4}$ – 10$^{-3}$ M. Fig. 13 shows the chemical structure of EGTA.

2.5.2 \textbf{BAPTA}

In 1980, Tsien designed and synthesized a new family of high-affinity buffer for Ca$^{2+}$. The parent compound was 1,2-bis(o-aminophenoxy)ethane-N, N,N',N'-tetraacetic acid (BAPTA), a relative of the EGTA chelator. BAPTA and its derivatives share the high selectivity for Ca$^{2+}$ over Mg$^{2+}$ of EGTA, but are very much less affected by pH changes and are faster in taking up and releasing Ca$^{2+}$. The affinity of the parent compound for Ca$^{2+}$ (i.e., dissociation constant) is 1.1 x 10$^{-7}$ M in 0.1 M KCl. It was found that BAPTA is a very rapid Ca$^{2+}$ chelator acting in the \textmu s time range (Kao and Tsien, 1988). Therefore, BAPTA can be used to control the level of both intracellular and extracellular Ca$^{2+}$ (Haugland, 1996). BAPTA, tetrapotassium, salt was used in the present work. It has the chemical structure shown in Fig. 14.
Methods and Materials

Fig. 13. The chemical structure of EGTA, free acid.

Fig. 14. The chemical structure of BAPTA, tetrapotassium salt.
2.6 Fluorochromes

In general, most of the Ca\(^{2+}\) fluorescent indicators are derived from the Ca\(^{2+}\) chelator “BAPTA” and they are membrane impermeable in their Ca\(^{2+}\) sensitive form. The advent of acetoxy-methyl ester (AM) forms (Tsien, 1981) of many of these dyes made it possible to load indicators into isolated cells or tissue without prior membrane permeabilization. AM-ester groups are cleaved by endogenous esterases, thus releasing the Ca\(^{2+}\) sensitive form of the indicator intracellularly. A multitude of advantages has made calcium indicator dyes the method of choice for the study of intracellular calcium kinetics (Nuccitelli, 1994). In this study, the fluorochromes used were “fura-red” and “fluo-3”. Each was dissolved in Tris-HCl buffer (10 mM, pH 7.2) for microinjection as indicated by Kersken et al. (1986). The final concentrations yielded inside the cell were 50 µM and 100 µM for fura-red and fluo-3, respectively. In the following few lines, a short idea is introduced about both dyes.

2.6.1 Fura-red

Recently, the use of fura-red as an intracellular calcium indicator in a variety of cells was significantly propagated by many researchers (Kurebayashi et al., 1993; Klauke and Plattner, 1997; 1998; Klauke et al., 2000; Thomas et al., 2000; Bolsover et al., 2001). Fura-red is used for ratiometric measurements of \([\text{Ca}^{2+}]_i\) in living cells. It has major absorbance and fluorescence-excitation bands at visible wavelengths (Fig. 15). The maximum emission of fura-red upon excitation is 650 nm. The wavelengths 440 nm and 490 nm are used for excitation of the dye and the ratio of emission at both wavelengths is calculated. Thus, the two visible-wavelengths excitation and long-wavelength emission of fura-red eliminate interference from autofluorescence and effects from shape change (Haugland, 1996). The fluorescence emission is detected by using a 510 nm split mirror and a 560 nm barrier filter. The ratio method allows measurements of \([\text{Ca}^{2+}]_i\) independently from the fluorochrome concentration in the cell that could be influenced by cell contraction or cyclosis. Increase in
[Ca\(^{2+}\)] results in a significant decrease of emission intensity at 490 nm wavelength and in a smaller increase of fluorescence intensity at 440 nm. The dissociation constant (\(k_d\)) of fura-red under ionic conditions closely resembling *Paramecium* cells is 130 nM (Lumpert et al., 1990). Calibration of fura-red revealed suitability for *Paramecium* cells is in a linear range between \(\geq 20\) nM and \(\leq 5\) µM i.e., close to the manufacturer’s specifications (Klauke and Plattner, 1997). The chemical structure of fura-red is shown in Fig. 16.

**Fig. 15.** Fluorescence emission spectrum of fura-red upon different concentration of [Ca\(^{2+}\)]. (Haugland, 1996).

**Fig. 16.** The chemical structure of fura-red, tetrapotassium salt.
2.6.2 **Fluo-3**

Fluo-3 is a suitable dye for recording Ca$^{2+}$ signals of physiological magnitude because it displays low compartmentalization and an appropriate apparent Ca$^{2+}$ binding affinity. It has a large dynamic range making observation of changes in fluorescence (and therefore Ca$^{2+}$) easy to visualize (Thomas et al., 2000). In fluo-3 injected cells a rise of [Ca$^{2+}$]$_i$ was shown by an increase in fluorescence intensity at ≥514 nm, referred to the fluorescence before stimulation (f/f$_0$), with excitation at 488 nm, as used in some CLSM studies (Bacskai et al., 1995; Klauke and Plattner, 1997; 1998). An intense illumination of the fluorophore component of fluo-3 being based on fluorescein resulted in a bleaching phenomenon (photo-oxidation). Therefore, the duration and energy of the excitation was chosen as low as possible. Fluo-3 does not undergo a significant shift in emission or excitation wavelength upon binding Ca$^{2+}$ and therefore, was used in a single wavelength mode, which precluded the possibility of ratiometric measurements. Fluo-3 is essentially non-fluorescent under Ca$^{2+}$-free conditions. However, the fluorescence enhancement on Ca$^{2+}$ binding is ~50-to-100 fold, making this dye particularly useful for documenting the kinetics of calcium transients. This property and its low affinity for Ca$^{2+}$ (k$_d$ ~400 nM) allowed significant signal changes, even for relatively small [Ca$^{2+}$] variations in a micromolar range. Its suitability for *Paramecium* cells is reported for a range between 30 nM and 4 µM (Minta et al., 1989). A drawback of fluo-3 was the rather small separation between excitation and emission wavelengths (Fig. 17). Therefore, fluo-3 is excited at 488 nm by an Argon laser source and the fluorescence emission is detected after passing the 510 nm band of a triple interference filter (470/510/540 nm) and a 520 nm split filter (colorglass filter). The chemical structure of fluo-3 is shown in Fig. 18.
Fig. 17. Fluorescence emission spectrum of fluo-3 upon different concentration of \([\text{Ca}^{2+}]\). (Haugland, 1996).

Fig. 18. The chemical structure of fluo-3, pentaammonium salt.
2.7 Testing the decondensation capacity of isolated trichocysts from pwA.nd12 cells at 35°C

To check the capacity of the trichocyst contents to undergo decondensation, the following protocol was followed. 1000 ml of cultured pwA-nd12 cells cultivated at 35°C (5 days old) were concentrated 3 times at 800 rpm, each for 2 minutes. Then they were washed in Pipes buffer (5 mM, pH 7.0), centrifuged again at 800 rpm for 3 minutes. The supernatant was carefully removed by a pipette. 5 ml of 5 mM Pipes buffer were added before homogenizing the cells manually for more than 5 times (checked by light microscope). When the trichocysts were found in an isolated, condensed form with their intact membranes, centrifugation was done again at 2000 rpm for 3 minutes to get rid of bacteria. 2 mM CaCl₂ solution (1+1) was added to have 1 mM \([\text{Ca}^{2+}]_o\) as a final concentration in the medium. 10 µl of the mixture was put on a glass slide and 10 µl of 0.2% Triton X-100 was added. Triton X-100 is a detergent which causes damage of the cellular membranes. Thus, the condensed trichocyst contents can be decondensed when they meet calcium. This was done to see whether the trichocyst contents of the 35°C pwA-nd12 cells can decondense, as do those of the 25°C pwA-nd12 and wild type cells. Finally, the trichocysts decondensation capacity was examined using a phase-contrast lens and photomicrographs were taken by the attached camera.

2.8 X-ray microanalysis

This method has been used in the variation called “energy-dispersive x-ray microanalysis” (EDX). During bombardment with electrons, in the electron microscope (EM), element-specific x-ray fluorescence signals are generated. Therefore, EDX can be combined with EM imaging. The best way - as the analyses has been done in the present work - is to use semithin sections (0.5 µm) for analysis in the STEM mode (scanning transmission EM). This allows to obtain sufficient x-ray signals from a relatively large excitation...
volume. To record the precise position of the measuring point, a STEM picture is made before or after such a point measurement has been made. The primary beam normally used to scan a sample is positioned, without scanning, on a specimen detail of interest, e.g. on a precisely crosscut alveolar sac. Usually a primary beam diameter (probe size) of 63 nm has been selected, at 10 µA beam current, 80 kV acceleration voltage. In the resulting excitation volume (a cylinder of ~63 nm [radius] x 500 nm [thickness]) about $10^3$ molecules can be recorded in a spectrum, whereby the Ca signal shows up as a peak of 3.690 keV ($= \text{CaK}_\alpha$ signal), superimposed to a continuous (white) Bremsstrahlung-spectrum. The net counts of CaK$_\alpha$ signals over this continuous spectrum background are proportional to the concentration of total Ca in a measuring spot, [Ca]. This [Ca] is composed of bound Ca and free, i.e. ionically dissolved Ca$^{2+}$ [Ca$^{2+}$]. The latter normally is only a small fraction of total calcium, e.g. 0.1% or less, just like in any other Ca stores.

To localize calcium (total calcium) by EDX, it is prerequisite to retain calcium in place during preparation. This is achieved by fast freezing (combined with triggering over different time periods), followed by freeze-substitution in presence of potassium fluoride, KF, which forms an insoluble complex with calcium, CaF$_2$.

In detail, triggering and freezing of the sample was executed as follows. Cells cultured at 25°C and 35°C respectively were washed two times in 5 mM Pipes buffer containing 1 mM Ca$^{2+}$. The number of cells was adjusted to 36.000–38.000 cells/ml. Exocytosis triggering was carried out by mixing the same amount of cell suspension and 0.02% AED in Pipes buffer plus 1 mM Ca$^{2+}$. 10 s after triggering cells were cryofixed in liquid propane by “the Quenched-flow-method” using “the quenched-flow device” previously described by (Knoll et al., 1991; 1993). Excess of liquid propane was removed at –100°C under vacuum in a freeze dryer (GT1). To perform the freeze-substitution, the frozen material was then transferred to –80°C cold medium,
made of absolute methanol, 3% glutaraldehyde, 1% OsO₄ and 1.45% KF, substituted for 72 hours, and then continuously warmed up (5°C/h) and kept at 5°C overnight. In order to check the calcium inside the alveolar sacs of the double mutant cells before and after stimulation, the samples were then centrifuged, washed twice with 5°C cold methanol, embedded in Spurr’s resin, polymerized, and cut into 0.5 µm thick sections. The sections were mounted on nickel grids that were previously coated with a film of 1% Pioloform dissolved in chloroform to stabilize the sections in the electron beam. Then, samples were analyzed in a ZEISS electron-microscope “LEO 912Ω” operating in the STEM mode with 80kV accelerating voltage, 10 µA emission current and 63 nm spot size (Hardt and Plattner, 1999; 2000). X-rays were collected with a ISO 901 Kevex detector system equipped with an atmospheric thin window (Hardt and Plattner, 1999).

2.9 Freeze-fracture technique

Freeze-fracture is a well known established technique for quantitative EM evaluation of the exocytosis sites in Paramecium cells (Knoll et al., 1991b; Plattner et al., 1994; 1997a). Thus, this technique was used in order to check the membrane fusion capability of the trichocyst docking sites and to investigate the ultrastructure of the fusion sites of the trichocysts in the double mutant cells, pwA-nd12 at 35°C. The methodical details included many different technical steps as follows. Cells cultured at 35°C were concentrated at 800 rpm and checked routinely as mentioned previously with AED, picric acid and K⁺ solution for the complete acquisition of the double mutant properties. To trigger exocytosis, 500 ml of concentrated cells were mixed with same amount of either 0.02% AED (2 µM final conc.), or 1 mM 4CmC (500 µM final conc.) in 5 mM Pipes buffer (pH 7.0). For control purposes cells were mixed with Pipes buffer (1+1). In these three different treatments, [Ca²⁺]o was kept at ≥50 µM. Such concentration of calcium ions outside the cells is needed for normal exocytosis.
activity. Then, the cells were fixed after 5 s of triggering in 2.5% ice-cold glutaraldehyde in 0.1 M Pipes buffer (pH 7.0). The use of ice-cold fixation was to prevent exocytosis, which may otherwise occur even in the control cells. Postfixation was carried out in the same fixative for 1.5 hour at room temperature. After a brief wash in 0.1 M Pipes buffer (pH 7.0), cells were cryoprotected in an ascending series of glycerol (10%, 20%, 30%, etc., each for 1 hour). Cells were then pelleted at 2000 rpm in a Mega-centrifuge and quick-frozen between two thin copper sheets (copper sandwich), mounted on tweezers, and vigorously dipped into liquid propane at (-150°C) according to Gulik-Krzywicki and Costello (1978); Flötenmeyer et al. (1999). Then, the sandwiches were inserted into a Balzers freeze-fracture unit equipped with electron beam evaporators, then fractured at -110°C, etched for 1 minute, and finally replicated by 2 nm of platinum-carbon shadowing at an angle of 45°, then with a 25 nm carbon coating applied at an angle of 90°. Fractured samples were transferred to 40% chromosulfuric acid in order to dissolve the copper sheet and the adhesive biological material. Replicas were washed many times in bidistilled water and mounted on conventional copper grids. Routine EM observation and EM graphs were made with a ZEISS EM10 electron microscope, 80 kV.

Stages for classification of ultrastructural changes during exocytosis were as specified previously (Olbricht et al., 1984; Pape and Plattner, 1985; Knoll et al., 1991b). Because of freeze-fracture replicas are containing small and large PF-face portions of cells, all docking sites belonging to the different categories were counted for each cell and the median of each of the respective stages per cell was computed before averaging over all samples from all experiments of any specific type. This accounted for the necessarily unequal sample sizes per cell and allowed for unconditional statistics. Up to 500 exocytosis sites were thus analyzed per experiment. Finally, the percentage median for all the exocytotic ultrastructural elements for all cells was computed and then plotted by using Sigma plot software (Plattner et al., 1997b).
2.10 Preparing ultrathin sections for routine ultrastructure examination

As a confirmatory technique, the routine semithin sections preparation has been done to check the normal ultrastructure of docking sites of the trichocysts in 25°C and 35°C cells without any stimulation. Therefore, concentrated cells for 2 minutes at 900 rpm were fixed at 0°C with 2.5% (v/v) glutaraldehyde in 50 mM cacodylate buffer, pH 7.0 for 30 minutes. After centrifugation and washing with the same buffer (3 x 10 min), the samples were post-fixed in 1% osmium tetroxide in cacodylate buffer at 4°C for 60 minutes. Then the specimens were washed again in the same buffer for 10 minutes. For dehydration, ascending series of ethyl alcohol were used (50%, 70%, 90%, 96%, each for 10 min, then in absolute alcohol 3 x 10 min) at room temperature. Then, the cells were transferred into a mixture of absolute ethanol and Spurr’s resin (1:1) at 4°C, centrifugation for 5 min at 1500 rpm, to be ready for 3 changes in Spurr’s resin 1 hour each and for embedding. Polymerization of the resin has been done at 65°C for 48 hours. Ultrathin sections were cut at 60 nm thickness, then mounted on 300 mesh copper grids which were previously covered with a film of 1% formvar in 1,2-dichloroethan to stabilize the sections against the electron beam. Sections were double stained with 2% aqueous uranyl acetate for 5 min and 0.4 alkaline lead citrate for 5 min, as well. Examination and making electron micrographs have been done by using ZEISS EM 10 electron microscope at 80 kV acceleration voltage.

2.11 Protein electrophoresis technique

In the mid-1960s, a modified version of SDS poly-acrylamide gel electrophoresis (or SDS, PAGE) was developed that has revolutionized the way proteins are routinely analyzed (Alberts et al., 1989). Therefore, to check the total protein content of pwA-nd12 cells (25°C and 35°C) and occurrence of any variation, SDS-PAGE was used as follows.
2.11.1 Cell concentration

For digestion of the food bacterium, *Enterobacter aerogenes*, the resuspended cells were incubated for 3 hours at their culturing temperatures i.e. 25°C and 35°C, respectively. After the incubation, an exocytosis-test was performed to certify the double mutant properties as a routine check up test. Then, the cell suspension was filtered through 6 layers of cellulose tissue. To concentrate the cells and to remove the cultivating medium, the suspension then was centrifuged for 2 min, 118g (corresponding to 800 rpm/min in a Megafuge 1.0R) in oil centrifuge glasses, then sucking off the cultivating medium and resuspension in washing buffer (5 mM Pipes-NaCl, pH 7.0). This step was done twice under the same conditions.

2.11.2 Total cell homogenate

Cells were centrifuged as described, and resuspended in homogenizing medium containing a cocktail of the following protease-inhibitors, added shortly before use: 3.0 µg/ml Aprotinin (reversible; serin-proteases), 10.0 µM E 64 (irreversible; cystein-proteases), 100.0 µM Leupeptin (reversible; serin-, cystein-proteases), 0.6 µM Pefabloc (irreversible; serin-proteases) and 1.0 µM Pepstatin A (reversible; aspartate-proteases). The addition of protease inhibitors is necessary because of proteases liberated by breaking up the cells (Fok and Paeste, 1982). The buffer used consisted of homogenizing medium (pH 7.4), 20 mM TEA (triethanoleamine-HCl) and 15% (v/v) glycerol. Homogenization was done in a precooled glass homogenizer, at 4°C with a Teflon pistil (150 strokes, 170 rpm/min).

2.11.3 Protein-estimation

The Bradford method (Bradford, 1976) was applied for the estimation of protein concentration by mixing 10 µl of the protein solution with 200 µl of the color solution. The absorption depending on the protein concentration was
measured at 595 nm in the ELISA-Reader. A protein standard was made with BSA (Bovine Serum Albumin) solutions of increasing concentrations, ranging from 20 to 150 µg. The measured absorptions were the basis of a calibration scale on which the absolute protein concentration of the sample had been calculated. The solutions used, were 1 volume-part Protein Assay and 4 volume-parts aqua dist.

2.11.4 SDS-PAGE

The sodium dodecyl sulfate-polyacrylamide-gelelectrophoresis is a possibility to separate proteins electrophoretically, and to evaluate their molecular weight. With this method, developed by Shapiro et al. (1967), the proteins are separated according to their molecular size due to the character of the highly anionic detergents SDS to bind to the hydrophobic parts of the protein molecules and neutralizing individual charge, whereby they are stretched and liberated from their association with other proteins and lipids. The addition of the reducing substance DTT (Dithiothreitol) opens all disulphide-bridges, so that proteins loose their secondary structure. SDS/protein-micelles with a constant net charge are formed, so that the proteins are separated in the gel matrix only according to their molecular size. The size of a protein molecule limits his mobility in the electrical field of the gel matrix.

2.11.4.1 Gels and marker proteins

BioRad Criterion Precast SDS Polyacrylamide Gels (SDS PAGE), linear gradient 4-20% were used. BioRad prestained broad range recombinant marker proteins with molecular sizes of 10, 15, 25, 37, 50, 75, 100, 150 and 250 kDa served as references. The protein mixture was applied according to the advices of the producer.
2.11.4.2 Sample treatment and conditions of electrophoresis

Before processing to SDS-PAGE, samples of the total cell homogenates were diluted with the buffer to protein concentrations of 5 µg per lane for silver staining, and 50 µg for Coomassie blue staining, denatured with 0.5% (w/v) DTT by heating to 95°C for 4 min, then cooling. Then, the samples were kept in darkness with 2% (w/v) iodoacetamide for 30 min. Another part of the samples was processed on SDS-PAGE under non-denaturing conditions (without heating, DTT and iodoacetamide). The separation was performed for 2 hours at a continuous voltage of 170 V. Buffer and solutions used, were as follows; 0.4 M Tris-HCl pH 8.0, 1% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 50% (w/v) DTT stock solution in aqua dist. and 20% (w/v) iodoacetamide stock solution in aqua dist.

2.11.4.3 Silver staining of polyacrylamide gels

For staining protein bands in polyacrylamide gels the method of Heukeshoven and Dernick (1985) was applied which allows to detect protein concentrations of 2.5 - 5.0 ng. The protocol is shown in (Table 2).

**Table 2. Silver staining protocol of a Polyacrylamide.**

<table>
<thead>
<tr>
<th>Step No</th>
<th>Solutions</th>
<th>Time (min) at RT ~20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>fixation solution; 30% (v/v) ethanol, 10% (v/v) acetic acid</td>
<td>180 or overnight</td>
</tr>
<tr>
<td>2</td>
<td>reduction solution; 30% (v/v) ethanol, 0.5 M sodium-acetate, 0.5% (v/v)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>glutaraldehyde, 0.2% (w/v) sodium thiosulfate</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>aqua dist.</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>aqua dist.</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>aqua dist.</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>aqua dist.</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>silver nitrate solution; 0.1% (w/v) silver nitrate, 0.02% (v/v) formaldehyde</td>
<td>60</td>
</tr>
<tr>
<td>8</td>
<td>aqua dist.</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>aqua dist.</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>developing solution; 2.5% (w/v) sodium carbonate, 0.01% (v/v) formaldehyde</td>
<td>2-6</td>
</tr>
<tr>
<td>11</td>
<td>stop solution; 5.0% (v/v) acetic acid.</td>
<td>10</td>
</tr>
</tbody>
</table>
2.11.4.4 Coomassie-blue staining

To verify whether or not all proteins have been detected with silver staining, a second gel was stained with Coomassie-blue. The sensitivity reached with this method is 0.1-0.3 µg protein. The used solutions were BioRad Coomassie stain solution and destaining solution in aqua bidist. (50% “v/v” ethanol, 10% “v/v” acetic acid). The gel was allowed to be stained in Coomassie solution at least for 3 hours, and was destained afterwards until the background color was washed out from the gel matrix.

2.11.4.5 Conserving and drying of polyacrylamide gels

After staining the gels were dried for conserving. They were impregnated for 2 hours in the conserving solution, put between 2 dialyzation folios, saturated with conserving solution, and dried for 4 hours in a vacuum gel drier. The solution used, was 2% (w/v) glycerol and 55% (v/v) methanol.
2.12 Materials

2.12.1 Exocytosis and fluorometry

2.12.1.1 Chemicals (Table 3)

Table 3. Chemicals used for exocytosis tests and fluorometry.

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AED (Aminoethyl dextran, 40kDa)</td>
<td>University of Konstanz, Plattner Lab., Germany</td>
</tr>
<tr>
<td>1,2-bis(O-aminophenoxy)ethan-N,N,N’,N’-tetraacetic acid (BAPTA)</td>
<td>Molecular Probes, Eugene, USA</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Cell culture medium; dried Lettuce and Enterobacter aerogenes</td>
<td>University of Konstanz, Plattner Lab., Germany</td>
</tr>
<tr>
<td>4-Chloro-meta-cresol (4CmC)</td>
<td>Fluka, Buchs, Switzerland</td>
</tr>
<tr>
<td>Compressed air for injection and application of triggers</td>
<td>University of Konstanz, Gas Store, Germany</td>
</tr>
<tr>
<td>Dimethyl sulfoxide „DMSO“ (to dissolve the ionophore A23187)</td>
<td>Fluka, Buchs, Switzerland</td>
</tr>
<tr>
<td>Ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N’,N’-tetraacetic acid (EGTA)</td>
<td>Fluka, Buchs, Switzerland</td>
</tr>
<tr>
<td>Fluo-3, Fura-red (fluorochromes)</td>
<td>Molecular Probes, Eugene, USA</td>
</tr>
<tr>
<td>Immersion Oil, 518C</td>
<td>Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>Ionophore A23187</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>RdH Laborchemicals, Seelze, Germany</td>
</tr>
<tr>
<td>Paraffin oil</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Picric acid</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Pipes-Na2 buffer (pH 7.0, 5 mM)</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Silicon solution</td>
<td>Serva, Heidelberg, Germany</td>
</tr>
<tr>
<td>Stigmasterol (additional nutritional fatty acid to the culture medium)</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Tris/HCl buffer (pH 7.2, 10 mM)</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Fluka, Buchs, Switzerland</td>
</tr>
</tbody>
</table>
2.12.1.2 Equipment and special tools (Table 4)

**Table 4.** Equipment for exocytosis tests and fluorometry.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell culture tools:</strong></td>
<td></td>
</tr>
<tr>
<td>Memmert incubators types: (BK 40, U 60) for cell incubation</td>
<td>Memmert, Schwabach, Germany</td>
</tr>
<tr>
<td>Sorval Heraeus safety cabinets, class 2</td>
<td>Kendro Lab, Hanau, Germany</td>
</tr>
<tr>
<td>Cover slides 24 x 60 mm</td>
<td>Bender and Hobein, Ulm, Germany</td>
</tr>
<tr>
<td>Labofuge M centrifuge</td>
<td>Heraeus, Osterode, Germany</td>
</tr>
<tr>
<td>Memmert oven “U300” to dry slides</td>
<td>Memmert, Schwabach, Germany</td>
</tr>
<tr>
<td><strong>Microinjection tools:</strong></td>
<td></td>
</tr>
<tr>
<td>Chrome-nickel wire ( \varnothing = 0.5 \text{ mm} )</td>
<td>Bulton-Kanther, Waldorff, Germany</td>
</tr>
<tr>
<td>Microloaders tubes ( \varnothing = 5 \text{ mm} )</td>
<td>Dr. Teichmann, Geretsried, Germany</td>
</tr>
<tr>
<td>Micromanipulator “D10/S10”</td>
<td>Brindi, Lörrach, Germany</td>
</tr>
<tr>
<td>Micromanipulator “De Fonbrune”</td>
<td>Bachhofer, Reutlingen, Germany</td>
</tr>
<tr>
<td>Micromanipulator “Leitz”</td>
<td>Leitz, Wetzlar, Germany</td>
</tr>
<tr>
<td>Micromanipulator “M3301”</td>
<td>WPI, Mauer, Germany</td>
</tr>
<tr>
<td>Micropipette puller “livingdtion”</td>
<td>Bachhofer, Reutlingen, Germany</td>
</tr>
<tr>
<td>Soda glass capillaries with or without filament, length =100 mm, outer ( \varnothing = 1.2 \text{ mm} ), inner ( \varnothing = 0.96 \text{ mm} )</td>
<td>Hilgenberg, Malsfeld, Germany</td>
</tr>
<tr>
<td><strong>Microscopy:</strong></td>
<td></td>
</tr>
<tr>
<td>Axiovert “35T” inverted microscope</td>
<td>Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>Confocal laser-scanning microscope</td>
<td>Noran Instruments, Bruchsal, Germany</td>
</tr>
<tr>
<td>“Odyssey”</td>
<td>Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>Inverted microscope “ICM 405”</td>
<td>L.O.T., Darmstadt, Germany</td>
</tr>
<tr>
<td>Interference filter</td>
<td>Carl Zeiss, Jena, Germany</td>
</tr>
<tr>
<td>Standard “25” light microscope</td>
<td>Home made, University of Konstanz, Germany</td>
</tr>
<tr>
<td>Units attached to ICM405 microscope</td>
<td></td>
</tr>
<tr>
<td>(Filterwheel, control units)</td>
<td></td>
</tr>
<tr>
<td><strong>Recording tools:</strong></td>
<td></td>
</tr>
<tr>
<td>Camera BC-2 “CCD” Black and white</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Camera Moon light “Panasonic WV-1900”</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Monitor “AVT-1220” black and white</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Monitor “Sony KX-14CP1” colored</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Monitor “Sony PVM-122CE” b/w</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Time-Date coder “Panasonic WJ-810”</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Videorecorder “PanasonicAG-7330”</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Video recorder “Sony VO-5800PS”</td>
<td>AVT-Horn, Aalen, Germany</td>
</tr>
<tr>
<td>Video tapes (S-VHS, U-matic)</td>
<td>Video Department, Konstanz Univ.</td>
</tr>
</tbody>
</table>
### 2.12.1.3 Software and computer (Table 5)

**Table 5.** Software and computer used for image analysis.

<table>
<thead>
<tr>
<th>Tool</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Personal computer (Intel-Pentium III), Siemens, Fugitsu, 750 MH, 20G mem.</td>
<td>Media Market, Konstanz, Germany</td>
</tr>
<tr>
<td>Fast video frame capture card (AV Master 2000)</td>
<td>Fast multimedia, Munich, Germany</td>
</tr>
<tr>
<td>AV Master software</td>
<td>Fast multimedia, Munich, Germany</td>
</tr>
<tr>
<td>Ulead media studio (pro 5.2) additional software for digitizing the video frames</td>
<td>Additional Ulead systems package from Fast multimedia, Munich, Germany</td>
</tr>
<tr>
<td>File transfer protocol (FTP 95 session)</td>
<td>University of Konstanz, Germany</td>
</tr>
<tr>
<td>Windows 98</td>
<td>Microsoft, Redmont, USA</td>
</tr>
<tr>
<td>Sigma plot 4.0 for statistics and graphs</td>
<td>Jandel Scientific, Erkelenz</td>
</tr>
<tr>
<td>SGI-Indy Unix workstation attached to the CLSM, with software package for image work and fluorochrome analysis</td>
<td>SGI, Mountain View, USA</td>
</tr>
<tr>
<td>Colored laser printer (Tektronix Phaser 750DX)</td>
<td>Kodak, Rochester, USA</td>
</tr>
<tr>
<td>Colored Hp 950C printer</td>
<td>Hewlett Packard, Stuttgart, Germany</td>
</tr>
</tbody>
</table>
2.12.2  Ultrastructural techniques

2.12.2.1  Chemicals used in ultrastructure investigation (Table 6)

Table 6. Chemicals used in ultrastructure techniques.

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cacodylate buffer (pH 7.0, 50 mM)</td>
<td>Serva, Heidelberg, Germany</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Chromosulfuric acid</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Formvar (to coat the copper grids)</td>
<td>Serva, Heidelberg, Germany</td>
</tr>
<tr>
<td>Glutaraldehyde (25%,70% EM-grade)</td>
<td>Polyscience, Warrington, USA</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Lead Citrate</td>
<td>Serva, Heidelberg, Germany</td>
</tr>
<tr>
<td>Methanol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Osmium tetroxide</td>
<td>Polyscience, Warrington, USA</td>
</tr>
<tr>
<td>Pioloform (to coat the nickel grids)</td>
<td>Plano, Wetzlar, Germany</td>
</tr>
<tr>
<td>Potassium Fluoride</td>
<td>RdH Laborchemicals, Seelze, Germany</td>
</tr>
<tr>
<td>Propane</td>
<td>Acid Stuff Fabric, Friedrichshafen, Germany</td>
</tr>
<tr>
<td>Spurr’s resin</td>
<td>Plano, Wetzlar, Germany</td>
</tr>
<tr>
<td>Uranyl acetate</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
</tbody>
</table>

2.12.2.2  Equipment used in ultrastructure investigation (Table 7)

Table 7. Tools used in ultrastructure techniques.

<table>
<thead>
<tr>
<th>Tool</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAF 300 (freeze-fracture device)</td>
<td>Balzer, Leichtenstein</td>
</tr>
<tr>
<td>Copper grids (mesh 300)</td>
<td>Plano, Wetzlar, Germany</td>
</tr>
<tr>
<td>Copper Sandwich (for freeze fracture)</td>
<td>Bal-Tec, Leichtenstein</td>
</tr>
<tr>
<td>Electron microscope “EM10”</td>
<td>Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>Electron microscope “Leo 912Ω”</td>
<td>Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>Freeze dryer</td>
<td>GT1, Leybold Heraeus, Köln, Germany</td>
</tr>
<tr>
<td>ISO 901 detection system (for EDX-microanalysis)</td>
<td>Oxford Instruments, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Mega-Fuge 1.R (centrifuge)</td>
<td>Heraeus, Fellbach, Germany</td>
</tr>
<tr>
<td>Nickel grids (for EDX-microanalysis)</td>
<td>Plano, Wetzlar, Germany</td>
</tr>
<tr>
<td>Ultratome (Ultracut E)</td>
<td>Reichert-Jung, Vienna, Austria</td>
</tr>
</tbody>
</table>
### 2.12.3 Protein electrophoresis

#### 2.12.3.1 Chemicals used (Table 8)

**Table 8.** Chemicals used for protein electrophoresis

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Bovine serum albumine</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>BioRad, München, Germany</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Coomassie stain solution</td>
<td>BioRad, München, Germany</td>
</tr>
<tr>
<td>Criterion precast gels</td>
<td>Bio Rad, München, Germany</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Serva Heidelberg, Germany</td>
</tr>
<tr>
<td>E 64</td>
<td>BioMol, Hamburg, Germany</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>BioMol, Hamburg, Germany</td>
</tr>
<tr>
<td>Methanol</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Pefabloc</td>
<td>BioMol, Hamburg, Germany</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>Serva, Heidelberg, Germany</td>
</tr>
<tr>
<td>Pipes-Na-salt</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Prestained precision protein standards</td>
<td>BioRad, München, Germany</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>BioRad, München, Germany</td>
</tr>
<tr>
<td>Sodium thiosulfate</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Trethanolamine-HCl</td>
<td>Roche, Mannheim, Germany</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
</tbody>
</table>
### 2.12.3.2 Apparatus and special tools (Table 9)

**Table 9.** Apparatus and special tools used in electrophoresis

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrode Inlab 423</td>
<td>Mettler, Steinbach, Germany</td>
</tr>
<tr>
<td>ELISA-Reader Titerk Multiscan Mcc/430MKII Flow</td>
<td>Laboratories, Meckenheim, Germany</td>
</tr>
<tr>
<td><strong>Gelelectrophoresis:</strong></td>
<td></td>
</tr>
<tr>
<td>BioRad Criterion chamber</td>
<td>BioRad, München, Germany</td>
</tr>
<tr>
<td>Gel-dryer 224</td>
<td>BioRad, München, Germany</td>
</tr>
<tr>
<td>Power supply: LKB 2103</td>
<td>Pharmacia LKB, Freiburg, Germany</td>
</tr>
<tr>
<td>Sample boiler, Dry Bloc DB-3</td>
<td>Techne, Duxford Cambridge, UK</td>
</tr>
<tr>
<td>Glasshomogenisator with teflonpistill</td>
<td>Braun, Melsungen, Germany</td>
</tr>
<tr>
<td>Megafuge 1.0 R (centrifuge)</td>
<td>Heraeus, Fellbach, Germany</td>
</tr>
<tr>
<td>Oil centrifuge glass</td>
<td>Brand, Wertheim, Germany</td>
</tr>
<tr>
<td>pH-meter, PHM 82</td>
<td>Radiometer, Copenhagen, Denmark</td>
</tr>
</tbody>
</table>
3. Results

3.1 Testing the growth rate of pwA-nd12 cells

It was desirable to ascertain the growth rate of the double mutant Paramecium (pwA-nd12) cells under both conditions; the permissive and non-permissive conditions, i.e., at 25°C and 35°C respectively. According to the growth curve shown in Fig. 19, the pwA-nd12 cells have grown in a normal rate in the lettuce medium, when incubated at 25°C. At the fourth day of incubation, the cells number reached over than 2000 cells/ml which is an acceptable number – in comparison with the wildtype cells – to perform further experiments. The size and shape of the 25°C cells were observed under the light microscope and revealed a normal appearance. When the cultured cells were transferred into a 35°C incubator (non-permissive conditions), the cell density decreased every day gradually to reach almost zero at the seventh day of incubation. Some of the remaining pwA-nd12 cells at 35°C (3 days old), when examined under phase contrast light microscope, showed darker cytoplasm than the cytoplasm of pwA-nd12 cells cultured at 25°C.

3.2 Testing the exocytosis capacity

3.2.1 Exocytosis and reversibility

AED (2 µM final conc.) was mixed with cells in suspension daily after transfer of cells from 25°C into 35°C to check the exocytosis capacity of pwA-nd12 cells at 35°C. Fig. 20, shows that the 35°C cells have performed 100% exocytosis at day 0 till the fourth day. One consider that, at day 0, the cells are still 25°C one. But, at the end of the fifth day the exocytosis capacity started to decrease and finally there was no exocytosis at the end of the sixth day. The [Ca²⁺]o concentration was kept constant at 50 µM in the experiment shown in Fig. 20a. The development of 35°C cells characteristics was not affected.
Results

**Fig. 19.** Decrease of the density (the rate of growth) of the pwA-n12 cells upon transfer from 25°C to 35°C. (Abciss: minus values are for growth at 25°C).

**Fig. 20.** Change of exocytosis capacity of cells in response to 2 μM AED upon transfer from 25°C to 35°C at t₀ and its reversibility after transfer to 25°C. [Ca²⁺]₀ was kept constant at 50 μM in (a). No difference was observed when [Ca²⁺]₀ was raised to 1 mM at day 4 in (b). Notice at day 0 the cells are still 25°C one.
when $[\text{Ca}^{2+}]_o$ was increased by addition of 1 mM Ca$^{2+}$ at the fourth day of incubation at 35°C. i.e., there was no difference observed in the exocytosis capacity of the 35°C cells (Fig. 20b vs. 20a).

Since the cell density decreases with time at 35°C (Fig. 19), the viability of 35°C cells was assured when cells have been set back to 25°C (Fig. 20), in both cases with either $[\text{Ca}^{2+}]_o$ was 50 µM or when 1 mM Ca$^{2+}$ was added. The characteristics of pwA-nd12 cells at 25°C of exocytosis capacity were re-obtained within ~6 hours (reversibility), while the time required to develop 35°C cells characteristics required (3-5) days.

### 3.2.2 Exocytosis capacity upon different triggering agents

Since the quick picric acid test is routinely used in genetic studies to check the exocytosis capacity, a saturated aqueous picric acid solution was applied to a 35°C cell suspension (1+1) daily, starting from the day 0 at 35°C incubation. The exocytotic capacity was checked under the light microscope and the percentage of the cells undergoing exocytosis was recorded and documented graphically in (Fig. 21a). It was noticed that the exocytosis capacity decreases with every day at 35°C incubation till it reached a 0% level at the fourth day. Parallel experiments have been done by applying AED (2 µM final conc.) to the 35°C cells suspension (1+1) daily. It was found that the exocytosis response upon transfer from 25°C to 35°C decreases in a slower manner with AED than with picric acid (Fig. 21a).

There was a highly significant difference in the exocytotic activity of 35°C cells when tested with 2 µM AED + 1 mm EGTA (Fig. 21b) in comparison to 2 µM AED only. EGTA chelates the external $[\text{Ca}^{2+}]$, reducing its level to ~30 to 50 nM. Thus, the exocytotic activity was reduced to 0% in 35°C cells, starting from the first day. The 25°C cells did also not perform exocytosis upon testing them with AED + EGTA (considering the day 0 is representative for the 25°C cells). This is a strong evidence of the requirement of exocytosis...
Results

for external Ca\(^{2+}\) which should not be less than 50 µM, as it was in the case without application of EGTA.

**Fig. 21.** Change of exocytosis capacity, after transfer from 25°C to 35°C at t₀, upon stimulation (a) with 2 µM AED in comparison to picric acid, at [Ca\(^{2+}\)]₀ = 50 µM, (b) with AED at [Ca\(^{2+}\)]₀ = 50 µM or with EGTA added, (c) with 50 mM caffeine plus 1 mM Ca\(^{2+}\) or 1 mM EGTA, (d) with 0.5 mM 4CmC plus 1 mM Ca\(^{2+}\) or 1 mM EGTA. Notice at day 0 the cells are still 25°C one.
Caffeine is considered one of the most important agents used to trigger exocytosis in *Paramecium* cells (Klauke and Plattner, 1998). Therefore, 50 mM caffeine + 1 mM Ca\(^{2+}\) were applied daily to test the exocytotic capacity of 35°C cells. Increasing Ca\(^{2+}\) outside the cell was to ensure the Ca\(^{2+}\) influx into the cell, due to the difference in the Ca\(^{2+}\) gradient between the extracellular and intracellular Ca\(^{2+}\) concentrations. Also, 50 mM caffeine + 1 mM EGTA were applied in parallel experiment to the 35°C cells suspension (i.e., \([\text{Ca}^{2+}]_o = 30\) to 50 nM). The results shown in (Fig. 21c) proved that, with high \([\text{Ca}^{2+}]_o\), no significant difference in the exocytotic capacity, just as obtained in the previous experiment with AED (Fig. 21b). The decrease in the exocytosis percentage of the 35°C cells started at the fourth day, and it reached 0 level at the seventh day. More intriguing was the occurrence of some exocytotic activity with caffeine in presence of EGTA (Fig. 21c). This contradictory result will be discussed in detail in the “discussion” section.

As to be expected, the effect of the calcium store mobilizing agent 4-chloro-meta-cresol (4CmC) on the exocytotic capacity of 35°C cells was similar to that obtained by AED in both cases (i.e., with 1 mM Ca\(^{2+}\) or with 1 mM EGTA) as documented in (Fig. 21d). It is interesting to mention that the swimming activity of the 35°C cells became slow upon application of 4CmC (0.5 mM final conc.), especially after the 3\(^{rd}\) day of incubation at 35°C. Some other trials have been done using different concentrations of 4-CmC. It was found that application of 250 µM 4CmC did not trigger more than 5% of exocytosis at day 0, while 1 mM 4CmC caused death of the majority of the 35°C cells during one minute after application. Thus, using 0.5 mM 4CmC as a final concentration for further triggering experiments and fluorometry was established.

Therefore, based upon the previous results, 35°C cells finally used for any further experiments were regularly tested for viability as well as for exocytosis performance by picric acid and AED. In addition to the application
of high K⁺ solution to cell suspension (1+1) to ensure the incapability of ciliary reversal movement of the 35°C cells as an important phenotype character of the double mutant cells.

3.3 **Cell stimulation and microfluorometric calcium dynamics analysis**

Generally in all the fluorometric calcium dynamics analysis experiments, it was ensured that the fluorescence intensity inside the fluorochrome injected cells revealed a steady state before starting at t₀ any further application of the triggers. This steady state is plotted in the curves as minus (s) time. This steady fluorescence intensity is equivalent to the resting intracellular \([\text{Ca}^{2+}]\) level (= 1 of the \([\text{Ca}^{2+}]\), scale). The triggering agents were applied to the anterior region of individual single cells which were previously injected with one of the fluorochromes (fura-red/2λ or occasionally, fluo-3/1λ analyses). The \([\text{Ca}^{2+}]\), was kept at 50 µM during the experiments or occasionally chelated to ~30 nM with BAPTA. See the “Methods and Materials” section. Then, \([\text{Ca}^{2+}]\), transient analysis has always been performed in the local cortical region (at the application area) that is represented by a frame in Fig. 22. The exocytotic activity of cells during the experiments was recorded by using the transmission light mode (Fig. 23).

3.3.1 **Stimulation of pwA-nd12 cells with AED**

The aminoethylidextran (AED) was derived from dextran T40 with a molecular weight 40 kDa (Plattner et al., 1984). AED is a non-permeable polycationic secretagogue. When it is applied to different strains of *Paramecium*, it causes exocytosis of trichocysts accompanied with cortical \([\text{Ca}^{2+}]\), increase (Plattner et al., 1984; Klauke and Plattner, 1997).

It was important to find out if the \(\text{Ca}^{2+}/(\text{polyvalent cation})\)-sensing receptors function normally in the double mutant cells pwA-nd12 under both permissive and non-permissive conditions (i.e., 25°C and 35°C) upon triggering with different concentration of AED.
3.3.1.1 \([\text{Ca}^{2+}]_i\) transients due to 2 µM AED trigger

1- Using \(2\lambda\) analysis

a) **Triggering of 25°C cells with 2 µM AED**

Fig. 24a documents the \([\text{Ca}^{2+}]_i\) increase in the 25°C cells due to 2 µM AED stimulation, \([\text{Ca}^{2+}]_o = 50 \mu\text{M}\). The AED was applied at \(t_0\), then the \([\text{Ca}^{2+}]_i\) began to increase drastically to reach its maximum evoke at \(\sim 1.4\) s. The \([\text{Ca}^{2+}]_i\) changes were variable between cells, thus indicating variable individual reactions of the different cells. Some cells showed about 80% \([\text{Ca}^{2+}]_i\) increase above the resting intracellular \([\text{Ca}^{2+}]\) level. Other cells showed an increase of \([\text{Ca}^{2+}]_i\) ranging between 37% to 40% above the basal \([\text{Ca}^{2+}]_i\) level. Then, the intracellular \([\text{Ca}^{2+}]_i\) level remained significantly high for a few seconds, varying from one cell to another, but most of the cells showed more than 2 seconds time with the highest increase of the cortical \([\text{Ca}^{2+}]_i\) level. Later on, the \([\text{Ca}^{2+}]_i\) started to decrease gradually in the majority of cells. In some cells, it reached again the basal level within 5 to 6 s. Other cells required more than 20 s to return close to the resting state. Therefore, the plateau in Fig. 24a, proves that there was a local \([\text{Ca}^{2+}]_i\) increase in the 25°C pwA-nd12 cells accompanied to exocytosis of trichocysts during AED stimulation.

b) **Triggering of 35°C cells with 2 µM AED**

Stimulation of the 35°C cells with 2 µM AED at \([\text{Ca}^{2+}]_o = 50 \mu\text{M}\), caused weaker local cortical \([\text{Ca}^{2+}]_i\) increase than that obtained with 25°C cells upon the same triggering agent and under the same conditions. It was found that the \([\text{Ca}^{2+}]_i\) increase in 35°C cells due to AED stimulation is very short and a peak is obtained after 1.4 s triggering in most of the cells. Sometimes, it has a delay of 6 s after stimulation. This peak was ranging between 14% and 25% above the resting level. Many cells started re-establishment of the resting \([\text{Ca}^{2+}]_i\) level at the 8 s after triggering. The basal \([\text{Ca}^{2+}]_i\) level was completely re-obtained 20 s after AED application. There was no exocytosis upon AED
stimulation of 35°C cells although the trichocysts occurred normally of their docking sites.

**Visualizing the changes in fluorescence of cells and their exocytosis upon 2 µM AED stimulation**

In the present work, Fig. 22 provides an example of a false color imaging upon 2 µM AED stimulation of fura-red injected 25°C and 35°C cells, respectively. The effect on 25°C cells is much more pronounced than that on 35°C. The series in the Fig. 22 from “a to d” shows the reaction of 25°C cells at \([Ca^{2+}]_o = 50 \mu\text{M}\). The resting cortical \([Ca^{2+}]_i\) level is shown in Fig. 22a at time (0), while Fig. 22b shows a strong increase in the cortical \([Ca^{2+}]_i\) signal at the site of local triggering after 1.4 s of AED application. The red color indicates the highest \([Ca^{2+}]_i\) increase in the local cortical area, while the blue color corresponds to the resting level according to the calibrated color bar provided in the upper part of Fig. 22. It was also noticed that there is a global \([Ca^{2+}]_i\) increase in the cell indicated by the change of the blue color into greenish yellow. After 8.4 s (Fig. 22c) of stimulation the \([Ca^{2+}]_i\) transients started to be re-established to the resting state which is completely re-gained after 21.0 s as shown in Fig. 22d when the cell returned to the global blue color again.

On the other hand, Fig. 22 a` to d` shows a 35°C cell with a weak local cortical \([Ca^{2+}]_i\) 1.4 s after AED application (Fig. 22b` vs. Fig. 22b). The resting \([Ca^{2+}]_i\) level shown in Fig. 22a` is reassumed after 8.4 s (Fig. 22c`) of stimulation, i.e., the resting \([Ca^{2+}]_i\) level is re-obtained in stimulated 35°C faster than that in 25°C cells. The false color fluorescence image for both 25°C and 35°C cells shows a uniform distribution of the fluorochrome injected (fura-red) through out the cell, except in the contractile vacuole which appeared close to the rear blunt end of the 35°C cell (Fig. 22a`-d`) i.e., the fura-red did not fill the contractile vacuole, indicating absence of fluorochrome sequestration into the vacuoles during the experiment.
The local cortical $[Ca^{2+}]_i$ increase in the 25°C cell upon AED stimulation was accompanied by trichocyst exocytosis as indicated by a partial deformation of the cell in the local cortical area (Fig. 22a-d). This deformation is a sign of a massive exocytosis. It can be concluded that the local $[Ca^{2+}]_i$ increase due to AED stimulation induces exocytosis in 25°C cells, while there is only little local $[Ca^{2+}]_i$ increase and no exocytosis in 35°C cells upon the same stimulation.

The transmitted light image (Fig. 23) shows an obvious example of the exocytosis process of the trichocysts by 25°C cells after AED triggering. The trichocysts appear as needle-like structures outside the cell and in a massive amount close to the stimulation site. One can see many trichocysts were exocytosed from the cell cortex and decondensed due to presence of $[Ca^{2+}]_o$, giving a needle-like appearance to the released trichocysts. The exocytosis reaction was not restricted only to the local region of the AED application but it also occurred in the adjacent parts of the cell cortex, though with less drastic action. The cell cortex at the application site showed a little deformation indicating the strong exocytotic action at this area, like it was shown in the false color photo (Fig. 22) as well. Watching 35°C cells by using the transmission light mode did not show any sign for exocytosis upon AED application, under the same conditions like that used in the case of 25°C cells.

c) **Triggering of 25°C cells with 2 µM AED + 1 mM BAPTA**

There was no exocytosis upon stimulation with 2 µM AED + 1 mM BAPTA (i.e., at $[Ca^{2+}]_o \sim 30$ nM). The $[Ca^{2+}]_i$ transient analysis showed only little significant increase due to the trigger (Fig. 24c). Most of the cells showed the maintenance of the $[Ca^{2+}]_i$ level fluctuating above and below the resting $[Ca^{2+}]_i$ level. Only one cell showed a slightly $[Ca^{2+}]_i$ increase not more than 20% above the resting level after 1.4 s stimulation time, but this little increase was short and decreased rapidly below the resting state within 8 s of application. Another cell recorded showed $\sim 5\% [Ca^{2+}]_i$ increase above the basal level, 3 s
after stimulation, then decreased rapidly below the resting level. In some cases, there was a short \( [\text{Ca}^{2+}]_i \) increase (~2%) above the resting level within 3 s of AED + BAPTA application then started to increase again at 13 s to reach its maximum level (~18%) above the basal level, 18 s after stimulation. Finally, this cell started to decrease its \([\text{Ca}^{2+}]_i \) level toward the resting value and about 8% increase at 24 s. One cell showed more or less no change in its transients during the experiment. The decrease of the \([\text{Ca}^{2+}]_i\) level below the resting state may be referred to the effect of the calcium chelator which probably extracts some \( \text{Ca}^{2+} \) from the cell.

d) **Triggering of 35°C cells with 2 µM AED + 1 mM BAPTA**

Application of 2 µM AED + 1 mM BAPTA to the 35°C pwA-nd12 cells caused no exocytosis of the docked trichocysts. The majority of cells showed very short and weak reactions in their \([\text{Ca}^{2+}]_i\), which were considered as non significant increase above the resting state due to stimulation. These peaks may be due to the mechanical change between the filters wheel in the 2λ conventional microscope or due to a weak contraction of the cells during the experiment. The final possibility is that there was a tiny \( \text{Ca}^{2+} \) release from the alveolar sacs upon application. All of these possibilities would need more investigation, as it will be shown in the next few pages. The plotting points were ranging slightly above and below the resting \([\text{Ca}^{2+}]_i\) value after AED + BAPTA application. Some cells recorded a maximum \([\text{Ca}^{2+}]_i\) increase of ~9% to 12% above the resting level, 3 s after application, then a very fast decrease to the resting level within 2 s maximum. One of the cells analyzed showed only 1% \([\text{Ca}^{2+}]_i\) increase after 4 s, then oscillating below and above the resting level.

The results obtained from the four previous experiments are summarised in Table 10.
Fig. 22. False colour images obtained with fura-red injected 25°C (a-d) and 35°C (a'-d') pwA-nd12 cells, respectively, during stimulation with 2µM AED applied at the arrowhead. $[\text{Ca}^{2+}]_o$ was kept at 50 µM. (Note that the false colour imaging presented is in appropriate to resolve the difference in basal $[\text{Ca}^{2+}]_i$ in 25°C and 35°C cells, respectively). The framed area represents the local cortical area of fluorochrome analysis.
Fig. 23. A transmitted light photomicrograph of a 25°C pwA-nd12 *Paramecium* cell (P), showing the exocytotic process of a massive bulk of needle like trichocysts (T) upon 2 µM AED application close to the anterior part of the cell. [Ca$_{\text{o}}^2+$] was kept at 50 µM. Note presence of intact cortex except at the anterior pole (application area) there is a little deformation of the cell cortex at the local releasing site (arrow). The cytostome (C), some vacuoles (V) and the granular cytoplasm are normal aspects of the cell under investigation. Mag. = 630x
Fig. 24. Time course of cortical \([\text{Ca}^{2+}]_i\) signal (fura-red/2λ) close to the site of 2 µM AED application at \(t_0\). In (a) - (d) typical examples are presented, each from several randomly selected cells. (a, b) \([\text{Ca}^{2+}]_o = 50\ \mu\text{M}\), (c, d) 1 mM BAPTA in trigger medium; (a, c) 25°C cells, (b, d) 35°C cells.
Table 10. Summary of the \([\text{Ca}^{2+}]_i\) increase vs. time (s) during application of 2 µM AED, with or without BAPTA, obtained with 25°C and 35°C pwA-nd12 cells. Changes referred to the respective reading value at \(t_0\) (= 1.00). s.e.m. = standard error of the mean.

<table>
<thead>
<tr>
<th>Cells and application</th>
<th>([\text{Ca}^{2+}]_i) increase</th>
<th>Rise time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>25°C cells, 2 µM AED, ([\text{Ca}^{2+}]_o = 50 \mu M) (Fig. 24a):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.34</td>
<td>1.0</td>
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<tr>
<td>cell 2</td>
<td>1.36</td>
<td>1.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.39</td>
<td>1.0</td>
</tr>
<tr>
<td>cell 4</td>
<td>1.79</td>
<td>3.0</td>
</tr>
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<td>mean ± s.e.m.</td>
<td>1.47 ± 0.11</td>
<td>1.50 ± 0.50</td>
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<tr>
<td><strong>35°C cells, 2 µM AED, ([\text{Ca}^{2+}]_o = 50 \mu M) (Fig. 24b):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.25</td>
<td>1.0</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.25</td>
<td>1.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.14</td>
<td>5.6</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.21 ± 0.04</td>
<td>2.53 ± 1.53</td>
</tr>
<tr>
<td><strong>25°C cells, 2 µM AED + 1 mM BAPTA, ([\text{Ca}^{2+}]_o \sim 30 nM) (Fig. 24c):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.21</td>
<td>1.2</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.05</td>
<td>3.0</td>
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<tr>
<td>cell 3</td>
<td>1.02</td>
<td>3.0</td>
</tr>
<tr>
<td>cell 4</td>
<td>1.02</td>
<td>4.4</td>
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<tr>
<td>mean ± s.e.m.</td>
<td>1.08 ± 0.05</td>
<td>2.90 ± 0.53</td>
</tr>
<tr>
<td><strong>35°C cells, 2 µM AED + 1 mM BAPTA, ([\text{Ca}^{2+}]_o \sim 30 nM) (Fig. 24d):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.12</td>
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<tr>
<td>cell 2</td>
<td>1.09</td>
<td>2.4</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.01</td>
<td>4.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.07 ± 0.03</td>
<td>2.93 ± 0.53</td>
</tr>
</tbody>
</table>
II- CLSM (1λ) analysis

Triggering of 35°C cells with 2 µM AED

Analysis of local cortical [Ca\(^{2+}\)]\(_i\), by CLSM enables one to record very fast [Ca\(^{2+}\)]\(_i\) evoked during triggering of the cells within a millisecond time range. Therefore, single 35°C cells were injected with fluo-3 and prepared for stimulation by 2 µM AED under the CLSM as explained in detail in the “Methods and Materials” section. The curve obtained (Fig. 25) shows a very fast and short duration evoke of the cortical [Ca\(^{2+}\)]\(_i\), due to AED application. This [Ca\(^{2+}\)]\(_i\) increase reached its maximum ~47% above the resting level within 1 s after starting stimulation. Then, the [Ca\(^{2+}\)]\(_i\) level decreased to reach the resting level within ~6 s. There was no exocytosis of trichocysts observed due to stimulation. The purpose of this experiment, as mentioned before in “Methods and Materials” was to guarantee that any fast signals were not overlooked.

![Graph showing [Ca\(^{2+}\)]\(_i\) increase over time](image)

**Fig. 25.** Very weak and short cortical [Ca\(^{2+}\)]\(_i\) response (recorded by CLSM (1λ) after fluo-3 loading) in a pwA-nd12 (35°C) cell upon stimulation with AED = 2 µM at [Ca\(^{2+}\)]\(_o\) = 50 µM. Stimulation remained without induction of any exocytosis.
3.3.1.2 [Ca\(^{2+}\)]\(_i\) transients due to 10 µM AED trigger

a) Triggering of 25°C cells

Increasing the concentration of the triggering agent AED to 10 µM caused exocytosis of a massive number of trichocysts as expected. Analysis of the intracellular [Ca\(^{2+}\)]\(_i\) changes showed a drastic increase in the [Ca\(^{2+}\)]\(_i\) level within 1.4 s after the beginning (Fig. 26a). The maximum increase of the cortical [Ca\(^{2+}\)]\(_i\) for most of the 25°C cells was 8 fold above the resting [Ca\(^{2+}\)]\(_i\) level. Few cells showed a short delay, to reach a peak between 6 to 8 s. Sometimes, the increase in the [Ca\(^{2+}\)]\(_i\) level remains few seconds (~3 s) at its maximum, then it decreases gradually. Other cells showed a sharp peak of the [Ca\(^{2+}\)]\(_i\) increase, i.e., the decrease started quickly within a time range less than one second. However, all cells showed a decrease in the [Ca\(^{2+}\)]\(_i\) level within 22 s, then [Ca\(^{2+}\)]\(_i\) remained slightly higher than the resting value. Generally, the increase in the cortical [Ca\(^{2+}\)]\(_i\) in the 25°C pwA-nd12 cells triggered with 10 µM AED was not much higher than that obtained in the 25°C cells triggered with 2 µM AED (Fig. 24a).

b) Triggering of 35°C cells

Application of 10 µM AED to the single 35°C cells caused no exocytosis of the docked trichocysts. On the other hand, the cortical [Ca\(^{2+}\)]\(_i\) transients showed a slight increase, ~2 fold above the resting [Ca\(^{2+}\)]\(_i\) level (Fig. 26b). It was noticed that the increase of [Ca\(^{2+}\)]\(_i\) was obtained with a time delay in some cells, ranging between 4 to 6 s, in other cells the [Ca\(^{2+}\)]\(_i\) increase was observed 1.4 s after stimulation. Then, most of the cells showed decay of the [Ca\(^{2+}\)]\(_i\) increase within 20 s after starting. The increase in the cortical [Ca\(^{2+}\)]\(_i\) due to 10 µM AED showed no great difference in comparison to that obtained in the 35°C cells stimulated with 2 µM AED (Fig. 24b). Later on, in the “Discussion” section, there will be an explanation of aim and of these results of the application of 10 µM AED.
The increase of $[\text{Ca}^{2+}]_i$ obtained in 25°C and 35°C cells upon the 10 µM AED stimulation are shown in (Table 11).

**Fig. 26.** Time course of cortical $[\text{Ca}^{2+}]_i$ signal (fura-red/2λ) close to the site of 10 µM AED stimulation at $t_0$, $[\text{Ca}^{2+}]_o = 50$ µM. (a) 25°C cells, (b) 35°C cells.
Table 11. Summary of the $[\text{Ca}^{2+}]_i$ increase vs. time (s) during application of 10 µM AED, obtained with 25°C and 35°C pwA-nd12 cells. Changes referred to the respective reading value at $t_0$ (= 1.00). s.e.m. = standard error of the mean.

<table>
<thead>
<tr>
<th>Cells and application</th>
<th>$[\text{Ca}^{2+}]_i$ increase</th>
<th>Rise time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C cells, 10 µM AED, $[\text{Ca}^{2+}]_o = 50$ µM (Fig. 26a):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.68</td>
<td>3.0</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.65</td>
<td>3.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.66</td>
<td>8.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.66 ± 0.01</td>
<td>4.67 ± 1.67</td>
</tr>
<tr>
<td>35°C cells, 10 µM AED, $[\text{Ca}^{2+}]_o = 50$ µM (Fig. 26b):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.14</td>
<td>2.8</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.20</td>
<td>3.8</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.21</td>
<td>7.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.18 ± 0.02</td>
<td>4.53 ± 1.27</td>
</tr>
</tbody>
</table>
3.3.2 Stimulation of pwA-nd12 cells with ryanodine receptor triggers

3.3.2.1 Triggering with 50 mM caffeine

   a) Triggering 25°C cells with 50 mM caffeine

   The investigation started with 25°C cells, since they are considered as control. In 25°C cells (Fig. 27a) bathed in the usual [Ca\textsuperscript{2+}]\textsubscript{o} of 50 µM, caffeine induced a moderate fura-red signal which was 133% in some cells. The recorded peak was only ~1 s after stimulation, indicating a quick effect of the caffeine. There was a decay in the intracellular [Ca\textsuperscript{2+}] to reach a level slightly above the resting level within 24 s. A second cell showed a cortical [Ca\textsuperscript{2+}]\textsubscript{i} increase ~131% after 2 s, then it decreased within 3 s to swing between 122% and 124% [Ca\textsuperscript{2+}]\textsubscript{i} increase for the following 8 s. Finally, the cell showed a recovery state slightly above the resting level after 24 s. The third cell in the plateau recorded in Fig. 27a had [Ca\textsuperscript{2+}]\textsubscript{i} increase of 134% after 5 s of caffeine application, indicating a longer time to reach its maximum activity, but the cell gradually re-obtained the resting level within 24 s, similar to the first cell. All cells exocytosed their trichocysts in parallel to the intracellular [Ca\textsuperscript{2+}]\textsubscript{i} increase due to caffeine stimulation.

   b) Triggering 35°C cells with 50 mM caffeine

   The stimulated 35°C cells (Fig. 27b) showed weak [Ca\textsuperscript{2+}]\textsubscript{i} increase in comparison to 25°C cells when triggered with 50 mM caffeine in presence of 50 µM [Ca\textsuperscript{2+}]\textsubscript{o}. In addition, the time recorded to reach the first maximum [Ca\textsuperscript{2+}]\textsubscript{i} peak for each cell showed a longer delay, generally. In more detail, the fastest cell in its reaction showed only 102% [Ca\textsuperscript{2+}]\textsubscript{i} increase within 1.5 s, then a gradual increase – which is oscillating sometimes – to reach a maximum of 115% increase after 16 s of starting the experiment. The following [Ca\textsuperscript{2+}]\textsubscript{i} decay reached ~107% after 24 s. Another cell had 5 s time delay to react upon caffeine stimulation (i.e., it began the [Ca\textsuperscript{2+}]\textsubscript{i} increase 7 s after stimulation), whereas the normal reaction time is 2 s according to (Klauke and Plattner, 1998). In other words, the cortical [Ca\textsuperscript{2+}]\textsubscript{i} increase was 107% within 7 s after stimulation, then, there was a [Ca\textsuperscript{2+}]\textsubscript{i} fluctuation between 101% and 107% during the whole
experiment. The longest time of 35°C cells to start the reaction upon caffeine triggering was 11 s. The local cortical \([\text{Ca}^{2+}]_i\) peak did not exceed 104%. Decay of the \([\text{Ca}^{2+}]_i\) required 24 s. Watching the cells by the transmission light mode proved that there was no exocytosis after triggering with caffeine.

c) **Triggering 25°C cells with 50 mM caffeine + 1 mM BAPTA**

In 25°C cells, chelation of \([\text{Ca}^{2+}]_o\) by BAPTA strongly attenuated the local \([\text{Ca}^{2+}]_i\) increase and there was no exocytosis. Fig. 27c shows that only one cell had \(~104\%\) \([\text{Ca}^{2+}]_i\) increase after 4 s of caffeine + BAPTA application, with a slight time delay \(~2\) s. There was a steady state at this 104% level for \(~4\) s, after that time there was a recovery state. In the other two cells the \([\text{Ca}^{2+}]_i\) increase was too weak, and it did not exceed 101%. In one of these cells, this 101% \([\text{Ca}^{2+}]_i\) increase recorded after 4 s, then it reached 104% after 14 s and it remained at this level till the end of the experiment. While in the other cell, the 101% \([\text{Ca}^{2+}]_i\) increase occurred after \(~8\) s, then the resting level was re-obtained normally within 24 s.

d) **Triggering 35°C cells with 50 mM caffeine + 1 mM BAPTA**

All 35°C cells showed no exocytosis nor a significant increase in \([\text{Ca}^{2+}]_i\), as documented in Fig. 27d, upon triggering with caffeine + BAPTA. Only, one cell recorded 104% \([\text{Ca}^{2+}]_i\) increase 1.5 s after stimulation and it kept this level till 8 s. Later on, gradually it decayed to the resting state at the end of the experiment. One of the investigated cells showed a steady state of the \([\text{Ca}^{2+}]_i\) near to the resting level, only after 20 s there was 103% increase in the local cortical \([\text{Ca}^{2+}]_i\), then re-obtained the resting \([\text{Ca}^{2+}]_i\) level soon. The third cell showed \(~104\%\) \([\text{Ca}^{2+}]_i\) decrease 7 s after stimulation, which could be referred to extending the effect of the \([\text{Ca}^{2+}]_i\) chelator, BAPTA, into the cell itself. Then, after 11 s there was 103% \([\text{Ca}^{2+}]_i\) increase and \([\text{Ca}^{2+}]_i\) changes slightly up and down around the resting level till the end of the experiment.

The results obtained upon the stimulation by caffeine are summarised in Table 12.
Fig. 27. Time course of cortical $[Ca^{2+}]_i$ signal (fura-red/2$\lambda$) close to the site of 50 mM caffeine application at $t_0$. In (a) - (d) typical examples are presented, each from several randomly selected cells. (a, b) $[Ca^{2+}]_o = 50$ $\mu$M, (c, d) 1 mM BAPTA in trigger medium; (a, c) 25°C cells, (b, d) 35°C cells.
Table 12. Summary of the \([\text{Ca}^{2+}]_i\) increase vs. time (s) during application of 50 mM caffeine, with or without BAPTA, obtained with 25°C and 35°C pwA-nd12 cells. Changes referred to the respective reading value at \(t_0 (= 1.00)\). s.e.m. = standard error of the mean.

<table>
<thead>
<tr>
<th>Cells and application</th>
<th>([\text{Ca}^{2+}]_i) increase</th>
<th>Rise time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>25°C cells, 50 mM caffeine, ([\text{Ca}^{2+}]_o= 50 \mu\text{M}) (Fig. 27a):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.33</td>
<td>1.0</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.31</td>
<td>2.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.34</td>
<td>5.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.33 ± 0.09</td>
<td>2.7 ± 1.2</td>
</tr>
<tr>
<td><strong>35°C cells, 50 mM caffeine, ([\text{Ca}^{2+}]_o= 50 \mu\text{M}) (Fig. 27b):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.15</td>
<td>16.0</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.07</td>
<td>7.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.04</td>
<td>11.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.09 ± 0.33</td>
<td>11.3 ± 2.6</td>
</tr>
<tr>
<td><strong>25°C cells, 50 mM caffeine + 1 mM BAPTA, ([\text{Ca}^{2+}]_o \sim 30 \text{nM}) (Fig. 27c):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.04</td>
<td>4.0</td>
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<tr>
<td>cell 2</td>
<td>1.01</td>
<td>4.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.01</td>
<td>4.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.02 ± 0.1</td>
<td>4.0 ± 0.0</td>
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<td><strong>35°C cells, 50 mM caffeine + 1 mM BAPTA, ([\text{Ca}^{2+}]_o \sim 30 \text{nM}) (Fig. 27d):</strong></td>
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<tr>
<td>cell 1</td>
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<tr>
<td>cell 3</td>
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<td>mean ± s.e.m.</td>
<td>1.03 ± 0.03</td>
<td>10.8 ± 5.3</td>
</tr>
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3.3.2.2 Triggering with 0.5 mM 4-chloro-meta-cresol

a) Triggering 25°C cells with 0.5 mM 4CmC

The fluorochrome analysis (Fig. 28a) proved that the 25°C cells reacted positively upon triggering with 4CmC (0.5 mM final conc.), at \([\text{Ca}^{2+}]_o = 50 \, \mu\text{M}\). All cells showed a \([\text{Ca}^{2+}]_i\) increase and trichocyst exocytosis also occurred. One of the cells showed a 46% \([\text{Ca}^{2+}]_i\) increase above the resting level, \(~2 \, \text{s after stimulation}\). This \([\text{Ca}^{2+}]_i\) increase did not remain for long time, since it decreased within 2 s reaching \(~23\%\) above the resting level. Then, the \([\text{Ca}^{2+}]_i\) increased slightly again to 35% above the resting level after 10 s, finally, at 24 s the \([\text{Ca}^{2+}]_i\) decayed to a level very close to the resting \([\text{Ca}^{2+}]_i\) level. In the second cell, the increase in the intracellular \([\text{Ca}^{2+}]_i\) occurred 2 s after application, recording \(~33\%\) above the resting level. Then, this cell showed a gradual and slow decrease in its \([\text{Ca}^{2+}]_i\), reaching 20% above the resting level within 24 s. The third cell revealed \(~35\%\) \([\text{Ca}^{2+}]_i\) increase above the resting level 4 s after stimulation, with a \(~2 \, \text{s delay in its response in comparison to the previous two cells. This third cell re-obtained the resting \([\text{Ca}^{2+}]_i\) level gradually which then was only \(~3\%\) above the resting level after 24 s.

b) Triggering 35°C cells with 0.5 mM 4CmC

Triggering 35°C cells with 4CmC caused no exocytosis, but a slight increase in the intracellular \([\text{Ca}^{2+}]_i\), occurred. Fig. 28b shows that The \([\text{Ca}^{2+}]_i\) increase ranged between 14% to 21% above the resting \([\text{Ca}^{2+}]_i\) level. The rise time was very close in most of the cells \(~1.2 \, \text{s after stimulation}\), only one cell showed a delay of \(~1.2 \, \text{s in comparison to the other cells analyzed. Mostly cells had a sharp intracellular \([\text{Ca}^{2+}]_i\) increase which did not continue for a long period, whereas it started to decrease within 1.5 s. The plateau shows a gradual \([\text{Ca}^{2+}]_i\) decay 10 s after stimulation, recording a steady resting level till the end of the experiment. This steady state was slightly above the resting \([\text{Ca}^{2+}]_i\) level by \(~3\%\) only.
c) **Triggering 25°C cells with 0.5 mM 4CmC + 1 mM BAPTA**

Rapid chelating of the extracellular \([\text{Ca}^{2+}]\) to \(~30\) nM with BAPTA negatively affected the response of the 25°C cells to 4CmC. A long period of \([\text{Ca}^{2+}]_i\) stability at the resting level after triggering is shown in Fig. 29c. However, the recorded \([\text{Ca}^{2+}]_i\) increase was not significant in all cells analyzed. The \([\text{Ca}^{2+}]_i\) peaks ranged between 2% and 4% above the resting intracellular \([\text{Ca}^{2+}]_i\) level. The rise time of \([\text{Ca}^{2+}]_i\) increase after stimulation was 4, 5.8, and 7 s, respectively, for the cells investigated (Fig. 28c). Then, the \([\text{Ca}^{2+}]_i\) peaks continued for \(~11\) s, then started to decrease to the resting level. There was no exocytosis observed in all cells analyzed.

d) **Triggering 35°C cells with 0.5 mM 4CmC + 1 mM BAPTA**

Application of 4CmC + BAPTA to the 35°C cells resulted in neither significant intracellular \([\text{Ca}^{2+}]_i\) increase nor trichocyst exocytosis. Fig. 28d shows that the \([\text{Ca}^{2+}]_i\) increase was very low as well as with a time delay after triggering. The recorded peaks were 2% to 4% above the resting level, resembling the \([\text{Ca}^{2+}]_i\) increase obtained in 25°C cells tested by the same agent and under the same conditions. The time delay ranged between 3 to 6 s after stimulation. The resting \([\text{Ca}^{2+}]_i\) level was nearly re-obtained within 3 s from the increase time till the end of the experiment. Some cells showed a decrease in their intracellular \([\text{Ca}^{2+}]_i\) below the resting level soon after the \([\text{Ca}^{2+}]_i\) increase and also at different intervals. This decrease is referred to the effect of BAPTA which may permeate inside the cell and chelate some of its \text{Ca}^{2+}. Since the decrease is less than 4% below the resting intracellular \([\text{Ca}^{2+}]_i\) level, it was considered as a non-significant decrease, i.e., it is not due to \([\text{Ca}^{2+}]_i\) mobilization in 35°C pwA-nd12 cells.

The results obtained upon the stimulation by 4CmC are summarised in Table 13.
Fig. 28. Time course of cortical \([\text{Ca}^{2+}]_{i}\) (fura-red/2\lambda) signal close to the site of 0.5 mM 4CmC application at \(t_0\). In (a) - (d) are typical examples, each presenting the reaction of different cells. (a, b) \([\text{Ca}^{2+}]_o = 50 \mu\text{M}\), (c, d) 1 mM BAPTA in trigger medium; (a, c) 25°C cells, (b, d) 35°C cells.
Table 13. Summary of the $[\text{Ca}^{2+}]_i$ increase vs. time (s) during application of 0.5 mM 4CmC, with or without BAPTA, obtained with 25°C and 35°C pwA-and12 cells. Changes referred to the respective reading value at $t_0 (= 1.00)$. s.e.m. = standard error of the mean.

<table>
<thead>
<tr>
<th>Cells and application</th>
<th>$[\text{Ca}^{2+}]_i$ increase</th>
<th>Rise time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>25°C cells, 0.5 mM 4CmC, $[\text{Ca}^{2+}]_o= 50 \mu$M (Fig. 29a):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.46</td>
<td>2.0</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.33</td>
<td>2.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.35</td>
<td>4.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.38 ± 0.04</td>
<td>2.7 ± 0.67</td>
</tr>
<tr>
<td><strong>35°C cells, 0.5 mM 4CmC, $[\text{Ca}^{2+}]_o= 50 \mu$M (Fig. 29b):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.14</td>
<td>1.2</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.20</td>
<td>1.2</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.21</td>
<td>2.4</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.18 ± 0.02</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td><strong>25°C cells, 0.5 mM 4CmC + 1 mM BAPTA, $[\text{Ca}^{2+}]_o= \sim30 \text{nM}$ (Fig. 29c):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.04</td>
<td>4.0</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.03</td>
<td>5.8</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.02</td>
<td>7.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.03 ± 0.01</td>
<td>5.6 ± 0.87</td>
</tr>
<tr>
<td><strong>35°C cells, 0.5 mM 4CmC + 1 mM BAPTA, $[\text{Ca}^{2+}]_o= \sim30 \text{nM}$ (Fig. 29d):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.02</td>
<td>2.8</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.03</td>
<td>4.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.04</td>
<td>5.8</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.03 ± 0.01</td>
<td>4.2 ± 0.87</td>
</tr>
</tbody>
</table>
3.3.3 Exposure of pwA-nd12 cells to increased $[\text{Ca}^{2+}]_o$

a) Exposure of 25°C cells to 1 mM extracellular $[\text{Ca}^{2+}]$

Fig. 29a represents 3 cells exposed to 1 mM $[\text{Ca}^{2+}]_o$. All cells reacted quickly and started to increase their $[\text{Ca}^{2+}]_i$ 1 s after exposure. The first cell reached 48% above the resting level 1.5 s after exposure. Then, it took ~6 s to re-obtain the resting $[\text{Ca}^{2+}]_i$ level. The cell did not reach the resting level completely, but it kept its $[\text{Ca}^{2+}]_i$ at 120% till the end of the experiment. The second cell recorded 36% $[\text{Ca}^{2+}]_i$ increase above the resting level, 3 s after exposure; there was ~1.5 s delay in comparison to the first cell. Then, the $[\text{Ca}^{2+}]_i$ decayed soon till reached 2% above the resting $[\text{Ca}^{2+}]_i$ level after 24 s. The third cell reacted gradually till its maximum degree 7 s after exposure, recording 145% $[\text{Ca}^{2+}]_i$ increase with 5.5 s delay. The resting $[\text{Ca}^{2+}]_i$ level was re-obtained gradually after 24 s. Thus, these plots show a variable individual reaction of 25°C cells in response to exposure to 1 mM $[\text{Ca}^{2+}]_o$. It is interesting to mention that there was no exocytotic activity of the cells in spite of the $[\text{Ca}^{2+}]_i$ increase.

b) Exposure of 35°C cells to 1 mM extracellular $[\text{Ca}^{2+}]$

The reaction of 35°C cells in response to exposure to 1 mM $[\text{Ca}^{2+}]_o$ is shown in Fig. 29b. There was a variable reaction between individual cells. One of the three cells showed a semicircular curve after exposure. The maximum increase in this semicircular curve was only 24% above the resting level, ~13.5 s after exposure. This $[\text{Ca}^{2+}]_i$ increase is half of the maximum level recorded in the 25°C cells in the previous plateau (Fig. 29a) under the same experimental conditions. Then, the $[\text{Ca}^{2+}]_i$ decayed to 7% above the resting level. The second cell showed 107% $[\text{Ca}^{2+}]_i$ increase after 11 s. Then, it oscillated down and up between 105% and 113%, respectively for the following 3 s. Then, the cell down-regulated its $[\text{Ca}^{2+}]_i$ to 107% again. The last cell showed gradual $[\text{Ca}^{2+}]_i$ increase till 108%, ~12 s after exposure. Then, it increased to ~111% after the following 3 s. Finally, the cell decreased its $[\text{Ca}^{2+}]_i$ level to ~6% above the
resting level till the end of the experiment. There was no visible trichocyst released upon exposure of the cells to 1 mM \([Ca^{2+}]_o\).

c) Exposure of 25°C cells to 10 mM extracellular \([Ca^{2+}]\)

Fig. 29c shows the \([Ca^{2+}]_i\) increase due to exposure of 25°C cells to 10 mM \([Ca^{2+}]_o\). The first cell showed 119% \([Ca^{2+}]_i\) increase, 1.5 s after exposure. Then, it increased slightly more till 121% for the following 13 s. Finally, the cell re-obtained the \([Ca^{2+}]_i\) resting level after 24 s. The second cell showed a gradual \([Ca^{2+}]_i\) increase till 117%, 9 s after exposure. Then, there was a little increase to 119% within 2 s more. The \([Ca^{2+}]_i\) decay recorded 2% above the resting level after 24 s. The curve obtained by this cell is a semicircular, resembling one of the curves obtained with a 35°C cell upon 1 mM calcium exposure (Fig. 29b). Another cell showed 106% \([Ca^{2+}]_i\) increase, 1.5 s after exposure. Then, its \([Ca^{2+}]_i\) oscillated up and down between 106% and the resting level till 19 s. Interestingly, later on it increased to 120%, 21 s after exposure. Again, it oscillated between 109% and 118%. The last \([Ca^{2+}]_i\) level recorded of this cell was 108%. The \([Ca^{2+}]_i\) increase recorded by the fourth cell was 105%, 4 s after exposure. Then, it showed almost steady state for ~6 s. Gradually, \([Ca^{2+}]_i\) reached a level of 117%, 17 s after exposure. This 117% increase continued for 2 s, then it decreased to the resting level gradually. It was clear that no trichocysts were released from all investigated 25°C cells exposed to 10 mM \([Ca^{2+}]_o\).

d) Exposure of 35°C cells to 10 mM extracellular \([Ca^{2+}]\)

The change in the \([Ca^{2+}]_i\) in 35°C cells was not of any significant degree upon exposure to 10 mM \([Ca^{2+}]_o\) (Fig. 29d). One of the cells analysed showed only 3% \([Ca^{2+}]_i\) increase above the resting level, 1.5 s after exposure. Then, we recorded ~5% \([Ca^{2+}]_i\) increase above the resting level, 7 s after exposure. The second cell did not exceed 2% \([Ca^{2+}]_i\) increase above resting level, 1.5 s after exposure. Then, it reached ~8% \([Ca^{2+}]_i\) increase, 14 s after exposure. The third cell recorded only 105% \([Ca^{2+}]_i\) increase, 4 s after exposure.
All the cells showed complete decay of the $[\text{Ca}^{2+}]_i$ to the resting level at the end of the experiment. There was no sign of any exocytosis observed upon exposure of the 35°C cells to 10 mM $[\text{Ca}^{2+}]_o$.

All the results obtained by the analysis of the $[\text{Ca}^{2+}]_i$ transients upon exposure of the cells to increased $[\text{Ca}^{2+}]_o$ are summarised in (Table 14).
Fig. 29. Time course of cortical $[Ca^{2+}]_i$ signal (fura-red/2λ) close to the site of exposure at $t_0$ to 1 mM $[Ca^{2+}]_o$ (a, b) and 10 mM $[Ca^{2+}]_o$ (c, d), respectively. In (a) - (d) typical examples are presented, each from several randomly selected cells, (a, c) 25°C cells, (b, d) 35°C cells.
Table 14. Summary of the \([\text{Ca}^{2+}]_i\) increase vs. time (s) during exposure of 25°C and 35°C cells to 1 mM or 10 mM \([\text{Ca}^{2+}]_o\). Changes referred to the respective reading value at \(t_0 (= 1.00)\). s.e.m. = standard error of the mean.

<table>
<thead>
<tr>
<th>Cells and exposure</th>
<th>([\text{Ca}^{2+}]_i) increase</th>
<th>Rise time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>25°C cells, 1 mM ([\text{Ca}^{2+}]_o),</strong> (Fig. 30a):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.48</td>
<td>1.5</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.36</td>
<td>3.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.45</td>
<td>7.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.43 ± 0.04</td>
<td>3.83 ± 1.64</td>
</tr>
<tr>
<td><strong>35°C cells, 1 mM ([\text{Ca}^{2+}]_o),</strong> (Fig. 30b):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.24</td>
<td>13.5</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.07</td>
<td>11.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.08</td>
<td>12.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.13 ± 0.06</td>
<td>12.17 ± 0.73</td>
</tr>
<tr>
<td><strong>25°C cells, 10 mM ([\text{Ca}^{2+}]_o),</strong> (Fig. 30c):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.19</td>
<td>1.5</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.17</td>
<td>9.0</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.06</td>
<td>1.5</td>
</tr>
<tr>
<td>cell 4</td>
<td>1.05</td>
<td>4.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.25 ± 0.15</td>
<td>4.0 ± 1.77</td>
</tr>
<tr>
<td><strong>35°C cells, 10 mM ([\text{Ca}^{2+}]_o),</strong> (Fig. 30d):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell 1</td>
<td>1.03</td>
<td>1.5</td>
</tr>
<tr>
<td>cell 2</td>
<td>1.02</td>
<td>1.5</td>
</tr>
<tr>
<td>cell 3</td>
<td>1.05</td>
<td>4.0</td>
</tr>
<tr>
<td>mean ± s.e.m.</td>
<td>1.04 ± 0.01</td>
<td>2.33 ± 0.83</td>
</tr>
</tbody>
</table>
3.4 **Determination of the resting \([\text{Ca}^2+]_{\text{rest}}\) level**

Table 15 shows the statistical results obtained with the fluorometry analysis of \([\text{Ca}^2+]_{\text{rest}}\) level in 35°C cells in comparison to that occurring in 25°C. The \([\text{Ca}^2+]_{\text{rest}}\) level is significantly lower, i.e., ~59%, in 35°C cells, as compared to that in 25°C cells. As it will be discussed later on in the “Discussion” section, this result suggests that it is too difficult for 35°C cells to reach the threshold \([\text{Ca}^2+]_{\text{act}}\) upon different types of stimulation even if any \([\text{Ca}^2+]\) increase would occur by the trigger action.

**Table 15.** Relative values\(^1)\) of \([\text{Ca}^2+]_{\text{rest}}\) determined in pwA-nd12 (25°C) and pwA-nd12 (35°C) cells using fura-red/2λ. Data obtained for 25°C cells are normalized to 100%. N = number of 25°C and 35°C cells, respectively, analysed. s.d. = standard deviation. Data are significantly different, according to t-test, on a 99% level.

<table>
<thead>
<tr>
<th>Relative ([\text{Ca}^2+]) signals</th>
<th>25°C cells</th>
<th>35°C cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>100(^1))</td>
<td>59(^1))</td>
</tr>
<tr>
<td>± s.d.</td>
<td>±8</td>
<td>±7</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Footnote to Table 15**

\(^1)\)The \(f/f_o\) ratios obtained from cortical regions by dual wavelength recordings in randomly taken cells, evaluated as indicated in “Methods and materials” section, yield values independent of cell geometry (thickness). To facilitate comparison, data are normalized to the value obtained for 25°C cells.
3.5 The effect of calcium ionophore A23178 on pwA-nd12 cells

The calcium ionophore A23187 induces massive exocytosis, i.e., membrane fusion, contents release and resealing, in the wild type cells (Plattner, 1974). Thus its effect on pwA-nd12 cells was investigated in order to check the sensitivity of the exocytotic membrane fusion to Ca\(^{2+}\) upon transfer from 25°C to 35°C. The reactivity is increasingly reduced and finally abolished with increasing time after transfer of cells from 25°C to 35°C (Fig. 30). The experiment has been done under two conditions. First, addition of 1 mM Ca\(^{2+}\) to the ionophore A23187 to increase the driving force of the [Ca\(^{2+}\)] influx into the cells. Second, chelating the [Ca\(^{2+}\)]\(_o\) to ~30 nM by using 1 mM EGTA, thus preventing the [Ca\(^{2+}\)] influx into the cell. Remarkably, in the first case the reaction was 100% at the day 0 till the third day after transfer from 25°C to 35°C. Considering that the day 0 is representative for the 25°C cells, the effect of ionophore A23187 on the 25°C cells was to cause complete exocytosis. After the fourth day of incubation at 35°C the exocytotic activity reduced to 80%, then to 0% in the next days. This result was similar to the results obtained by the effect of all other triggering agents in cell suspension (Fig. 21). In the case of chelating [Ca\(^{2+}\)]\(_o\) by EGTA, there was no exocytosis of trichocysts from 25°C (cells at day 0) or from 35°C cells. This result is considered as a confirmation of the normal functioning of the control 25°C cells and it ensured the negative result obtained with the 35°C cells upon ionophore application.
3.6 The decondensation ability of trichocysts of 35°C cells

Fig. 31a shows the condensed state of the trichocysts isolated from 35°C cells. When the trichocyst membrane was permeabilized by using Triron X-100 and the trichocyst content met Ca$^{2+}$ (see the “Methods and Materials” section), the trichocysts decondensed, extending to several times their resting condensed length and became needle-like structures (Fig. 31b). Thus, it is proved that the trichocysts of 35°C cells have a normal capacity to decondense. Therefore, the reason or reasons of disability of 35°C cells to undergo exocytosis upon different stimuli cannot be attributed to any functional disorder of the trichocysts contents to decondense.

![Graph showing change of exocytosis performance in response to ionophore A23187 (+/- Ca$^{2+}$ added) upon transfer of pwA-nd12 cells from 25°C to 35°C.](image)

**Fig. 30.** Change of exocytosis performance in response to ionophore A23187 (+/- Ca$^{2+}$ added) upon transfer of pwA-nd12 cells from 25°C to 35°C.
**Fig. 31.** Phase contrast images of membrane bounded trichocysts isolated from pwA-nd12/35°C cells, showing decondensation capability. (a) Condensed state, (b) decondensation by adding 0.1 % Triton X-100 + 0.5 mM Ca$^{2+}$. Mag = 1,200x in (a) and (b).
3.7 EDX microanalysis

The EDX microanalysis approach has been applied in order to check i) whether 35°C cells would have the same amount of calcium stored in their alveolar sacs as that found in 25°C cells and ii) whether they are also capable of releasing the same amount of Ca\(^{2+}\) from their alveolar sacs upon stimulation, i.e., whether the calcium release channels of the alveolar sacs are normal in their function.

Fig. 32 shows an example of a STEM analysis of a 500 nm thick section of a resting 25°C cell. The alveolar sacs are easily recognized as flat sacs closely apposed to the plasma membrane and adjacent to the docked trichocysts. The section includes also some mitochondria and a part of a cilium indicating a good healthy cell before fixation and further EDX microanalysis. For control purpose, 25°C and 35°C cells were used without AED stimulation to analyze the \([\text{Ca}]_{\text{total}}\) in the alveolar sacs at the resting state. The spectra shown in (Fig. 33, a and b) for 25°C and 35°C cells, respectively, were chosen as typical examples of the large number of samples analyzed in these experiments. In these spectra the spot measurements show clear Ca\(_{\text{kk}}\) signals selectively in the alveolar sacs. Such figures ensure that the Ca\(_{\text{kk}}\) obtained in the alveolar sacs are roughly the same in 25°C and 35°C cells. The 10 s AED stimulation of 25°C and 35°C cells, respectively, (Fig. 33, c and d) shows more or less similar Ca\(_{\text{kk}}\) signals from the alveolar sacs. However, peaks are much smaller than before stimulation, thus indicating the release of \([\text{Ca}^{2+}]\) from the alveolar sacs upon triggering. The other signals appearing in the different spectra are due to fluorescence excitation and/or absorption effects by the heavy metals, such as Os from freeze-substitution medium or Ni from EM support grids (Hardt and Plattner 1999).

In these double mutant pwA-nd12 (25°C and 35°C) cells, EDX microanalysis was done in 5 independent sets of experiments. In each EDX analysis series, 5 cells per sample were evaluated (without and with 10 s AED stimulation). The 10 s stimulation was applied to take into account even slow
mobilization, while normally this requires only fractions of a second (Hardt and Plattner, 2000), whereas, re-uptake during 10 s is negligible (Plattner and Klauke 2001). Data have been collected from many samples to account for any potential uneven distribution of calcium in alveolar sacs and for any possible changes of sensitivity during analysis. By use of statistical evaluation of the net Ca\textsubscript{kat} peaks, the systematic errors could be minimized. As a reference, it has previously been determined, in painstaking calibration work, that [Ca] in alveolar sacs of unstimulated wild type cells is 43 mM (Hardt and Plattner 1999, 2000). Thus, within statistical limits, Ca content in alveolar sacs is only 11% lower in 35°C than in 25°C cells and we show that almost the same percentage is mobilized upon stimulation (Table 16), namely ~46% in either case. The difference is only ~1% and thus appears too small to be a reason of low [Ca\textsuperscript{2+}]\textsubscript{i} increase and failure in exocytosis performance upon AED stimulation. For more detailed explanation see the “Discussion” section.
Fig. 32. STEM image of resting 25°C pwA-nd12 cell showing alveolar sacs (AS). The region of EDX microanalysis is indicated by black spots (arrow) due to contamination upon electron irradiation (standing beam). A docked trichocyst that has a body (TB) and a tip (TT), a cilium (Ci) and some mitochondria (M) appear in the micrograph, too. Section thickness = 500 nm, beam spot size = 63 nm (top), 73 nm (bottom), Acceleration voltage = 80kV, Mag. = 36,000x.
Fig. 33. EDX spectra obtained by spot measurements on alveolar sacs of unstimulated (control) 25°C and 35°C cells (a, b) respectively, or 10 s AED (2 µM) stimulated 25°C and 35°C cells (c, d) respectively. The signal of interest is the CaKα peak of 3.69 keV energy, emitted from alveolar sacs while other peaks are due to the heavy metals used for preparation (Os, Cl) and electron microscopical techniques (Ni, Ti).
Table 16. EDX analysis of pwA-nd12 cells, net Ca_{K_e} counts (normalized to value obtained for unstimulated 25°C cells = 100 %)\(^1\). Stimulated cells were exposed to AED for 10 s, quick frozen and processed as described by Knoll et al. (1993) and for EDX analysis of [Ca] in alveolar sacs as described by Hardt and Plattner (1999, 2000). s.e.m. = standard error of the mean, N = cells analyzed, n = alveolar sacs analyzed. Significance +/- (yes/no) according to paired student's t-test.

<table>
<thead>
<tr>
<th></th>
<th>(1) 25°C, -AED</th>
<th>(2) 35°C, -AED</th>
<th>(3) 25°C, +AED</th>
<th>(4) 35°C, +AED</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>100.0%(^1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s.e.m.</td>
<td>±8.9</td>
<td></td>
<td></td>
<td></td>
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<td>N</td>
<td>15</td>
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<tr>
<td>n</td>
<td>50</td>
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<tr>
<td>Significance 95%</td>
<td>(1)--------(-)-----(2) (3)--------(-)-----(4) (1)----------------(+)-----------------(3) (2)-----------------(+)-----------------(4)</td>
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Footnote to Table 16

\(^1\)All data were obtained under identical preparative and analytical conditions and therefore can be directly compared with the value obtained for total [Ca] in alveolar sacs of unstimulated 25°C cells, normalized to 100% to facilitate comparison. Extensive work with carefully controlled calibration in unstimulated wild type cells has shown in alveolar sacs total [Ca] = 43 mM (Hardt & Plattner, 1999; 2000).
3.8 Freeze-fracture EM analysis

Freeze-fracture microscopy is one of the best techniques for studying the molecular organization of biological membranes. Therefore, this technique is widely used up to recently (Gulik-Krzywicki and Costello, 1978; Pouphile et al., 1986; Knoll et al., 1991b; 1993; Plattner et al., 1974; 1980; 1994; 1997a; 1999; Flötenmeyer et al., 1999). The principle of this methodology is that the weak hydrophobic bonding between the two lipid bilayer sheets of membranes, including bio-membranes, allows splitting them, when they are in a frozen state. This also allows to visualize membrane-integrated proteins, when all their transmembrane domains show up as 10 nm-particles or intramembranous particles, “IMPs”. A double ring of such particles outlines the preformed potential exocytosis sites in Paramecium. Such structures are present in regular patterns called “kinetids” that are alternating in their arrangement. Each kinetid is 1 µm broad and ~1.5 to 2 µm long, and surrounded by long and perpendicular ridges. Cilia and trichocysts are located alternatively in each kinetids. Whenever an exocytosis-competent trichocyst is docked underneath at the cell membrane, a “rosette” of IMPs is assembled.

Recently, the structural changes in the cell membrane of Paramecium cells were summarised using freeze-fracture technique (Plattner et al 1997a) as follows. Upon stimulation with AED in wild type (7S) Paramecium cells, the rosette IMPs decay to smaller IMPs as a focal exocytotic opening is formed. This opening expands, while endocytosis of an empty “ghost” first results in a “filled ring” (with many small loosely scattered IMPs) which then collapses to an “oval ring” and subsequently to a “parenthesis” when the ghost is completely detached from the cell surface by internalization. Hence, a parenthesis indicates a non-occupied potential docking site. Between the exocytotic opening and filled ring stage, “intermediate stages” may appear occasionally.

This is precisely what also occurs in the pwA-nd12/25°C cells. Intriguingly, the double mutant pwA-nd12/35°C cells have all the same
structure at rest, but their trichocyst docking sites do not perform any ultrastructural changes upon stimulation. In 35°C cells, though they cannot release trichocysts by exocytosis, it was required to reassure that no membrane fusion occurs during the stimulation of these cells. Therefore, freeze-fracture analyses have been carried out. Cryofixed 35°C cells either without stimulation (Fig. 34) or 5 s after stimulation with 2 μM AED (Fig. 35) or with 500 μM 4CmC (Fig. 36) at [Ca\textsuperscript{2+}]\textsubscript{o} = 50 μM, showed clearly – using statistical calculations (Fig. 37) – no membrane fusion within 5 s, with very little difference between stimulated and non-stimulated cells. It should be taken into account that any difference within ~5% limit may be considered of little significance at the EM level. Fig. 38 shows that the trichocysts are docked at the cell membrane of a 35°C cell triggered with AED or with 4CmC. These trichocysts are arranged obliquely with their paracrystalline bodies inserted deeply into the cytoplasm, and their tips attached to the cell membrane. Some inorganic crystals are also present in the cytoplasm that are normally occurring in Paramecium cells.
Fig. 34. A freeze-fracture electron micrograph of a non-stimulated pwA-nd12/35°C cell, showing PF-fracture face of the cell membrane. Notice the presence of many central small aggregates of large “rosette” (Ro) particles, encircled by an outer double “ring” (Ri) of IMPs, and the presence of a trichocyst docked underneath, indicative of resting stages. Very few “oval” or “parenthesis” stages (arrow) appear occasionally in some fields, indicative of unoccupied exocytosis sites (i.e., after detachment of ghosts). Cross sections of cilia (Ci) are present in the micrographs. Regular pattern (kinetids) of the whole structure is obvious by the presence of the vertical and horizontal ridges in the micrograph. Mag. = 35,000x
Fig. 35. A freeze-fracture electron micrograph of a pwA-nd12/35°C cell stimulated with AED (2 µM, 10 s), showing a PF-fracture face of the cell membrane without any structural differences, in spite of AED triggering, in comparison to the non-stimulated cell. Notice the presence of (Ro) and (Ri) indicative of the absence of any exocytosis. Also very few “oval” or “parenthesis” stages (arrow) appear occasionally in some fields, indicative of unoccupied exocytosis sites (i.e. after detachment of ghosts). Mag. = 35,000x
Fig. 36. A freeze-fracture electron micrograph of a pwA-nd12/35°C cell stimulated with 4CmC (0.5 mM, 10 s), showing PF-fracture face of the cell membrane without any structural differences, in spite of 4CmC triggering (like the case of AED stimulation), in comparison to the non-stimulated cell shown in Fig. 34. Notice the presence of (Ro), (Ri) indicative no exocytosis. Also very few “oval” or “parenthesis” stages (arrow) appear occasionally in some fields, indicative of unoccupied exocytosis sites (i.e. after detachment of ghosts). Mag. = 35,000x
Fig. 37. Freeze-fracture analysis of pwA-nd12 (35°C) cells, stimulated for 5 s with either 2 µM AED or 0.5 mM 4CmC at [Ca^{2+}]_o = 50 µM, shows no membrane fusion at trichocyst docking sites when compared with unstimulated controls. For statistical analysis of stages indicated, see “Methods and Materials”. Docking sites are distinguished depending on the presence of “rosettes” (membrane-integrated particles [proteins]) indicative of a docked trichocyst without membrane fusion, exocytotic openings, “filled rings” and “parentheses” (indicative or early and late resealing stages, respectively), as well as stages intermediate between openings and resealing. Note that individual events were analyzed and that intermediate stages are ill defined. Also the percentage of docking sites endowed with rosettes may vary from one experiment to another. Number of cells analyzed, N = 14 (controls), 15 (AED), 22 (4CmC); n (docking sites analyzed) = 384 (controls), 262 (AED), 428 (4CmC).
Fig. 38. A cross freeze-fracture electron micrograph of a pwA-nd12/35°C cell stimulated with AED (2 µM, 10 s), ensuring the lack of exocytotic response revealed by the presence of complete, docked trichocysts with their condensed bodies (TB), the continuous cell membrane (CM) without any interrupting exocytotic openings (arrows) above the trichocyst tips (TT). Notice, presence of inorganic crystals (IC) in the cytoplasm. Mag. = 11,000x
3.9 Ultrathin sections examination

In parallel to the freeze-fracture analysis, the routine ultrastructure examination of ultrathin sections of 25°C and 35°C cells, without stimulation, was also carried out. It was noticed in Figs. 39, 40 and 41 that the structure of the docked trichocysts was the same in the 25°C and 35°C cells. They also showed no other significant alterations. In the non-stimulated state, neither membrane fusion nor trichocyst release is seen in these electron micrographs. The trichocyst body appeared as an oval paracrystalline structure, having an elongated tip that is attached to the cell membrane by a connecting dense material, similar to the wild type cells (Klauke et al., 1998). The alveolar sacs are flat cavities for storing calcium, extending underneath the cell membrane and encircling the trichocyst tips. The only difference noticed in the electron micrographs between 25°C and 35°C cells is that the latter have many autophagosomes present in the cytoplasm (Figs. 40 and 41). Some of these contained lamellar structures and some other ones had dark aggregates or both. Irregular dark material was noticed as a common feature in many micrographs (Fig. 41). This material may be lipid from membrane degradation. In addition, some of the autophagosomes included glycogen aggregates, as seen in (Fig. 40).
Fig. 39. An electron micrograph of an ultrathin section taken from a non-stimulated pwA-nd12/25°C cell. Notice, appearance of normal structure of the trichocysts with their massive bodies (TB), slender tips (TT), and connection to the cell membrane (CM) by an electron-dense connecting material (CoM) at the docking sites. Surrounding each trichocyst tip are the flat calcium storing structures, the alveolar sacs (AS). Mitochondria (M) and glycogen contents (G) also occurred. Mag. = 35,000x
Fig. 40. An electron micrograph of an ultrathin section taken from a non-stimulated pwA-nd12/35°C cell. Notice appearance of normal structures of the exocytotic elements, like those in the 25°C cell in the previous EM photo; (TB), (TT), (CoM), (CM), (AS), (M) and (G). Notice bonafide glycogen aggregates are sequestered into the autophagosomes (Ap). The lower autophagosome has a lamellar structure. Mag.= 35,000x
Fig. 41. An electron micrograph of an ultrathin section taken from a non-stimulated pwA-nd12/35°C cell showing dark material (DM) in the cytoplasm of the cell. This dark material is probably lipid from membrane degradation. The autophagosomes (Ap) with lamellar structures and dark aggregates are also present in this micrograph. The “DM”-type structures probably also represent autophagosomes. All other structures are appearing normally. Mag. = 35,000x
3.10 Protein electrophoresis

Using SDS polyacrylamide gel electrophoresis, as explained before in detail in the “Methods and Materials” section, the expression of protein in the double mutant pwA-nd12 (25°C and 35°C) cells, was compared in order to check the possible structural changes occurring upon transfer from 25°C to 35°C. In general, it was found that all proteins of whole cell homogenates detected by Coomassie-blue staining can also be visualized on silver stained gels. As shown in (Fig. 42), separation under non-denaturing conditions revealed a significantly reduced expression of the surface variant antigen of >250kDa, when cells were cultured at 35°C (compare lane 2 with lane 1). On the other hand, a 70 kDa- protein seems to be expressed in a higher amount at this temperature, since this can also be observed under denaturing conditions (compare lane 4 with lane 3). The 70 kDa protein may represent an endogenous heat-shock protein (HSP 70), which is known to occur in Paramecium (Dessen et al., 2001). In addition, some other proteins also occur in higher amount in cells cultured at 35°C (such as a protein of 140 kDa or ~37 kDa) as observed in lane 4. The identities of these latter proteins is still to be elucidated.
**Fig. 42.** Silver-stained SDS polyacrylamide gel electrophoresis of whole cell homogenates from pwA-ndl2 cells at 25°C (lanes 1 and 3) or 35°C (lanes 2 and 4), separated under non-denaturing (lanes 1 and 2) or denaturing (lanes 3 and 4) conditions respectively. Note the higher amount of some protein bands of 140, 70 and 37 kDa (arrows) in the homogenates obtained from 35°C cells (lanes 2 and 4) in comparison to those obtained from 25°C cells (lanes 1 and 3). The >250 kDa surface protein is less expressed in the 35°C than in 25°C cells (this is much more obvious in non-denatured samples, lanes 1 and 2).
4. Discussion

Is the exocytosis capacity of pwA-nd12 cells normal?

Results obtained from checking the exocytosis capacity (Fig. 21) of the pwA-nd12/35°C cells confirmed that these cells (after four days of incubation at 35°C), do not respond positively (i.e., no exocytosis) to the different triggering agents such as picric acid, AED, caffeine, and 4CmC. Increasing [Ca\(^{2+}\)]\(_o\) does not affect development of the 35°C characteristics, as well (Figs. 20b and 21c,d). Absence of exocytosis with AED in 25°C cells in absence of [Ca\(^{2+}\)]\(_o\) (Fig. 21b) is not surprising since [Ca\(^{2+}\)]\(_o\) is required for decondensation (stretching and discharge) of trichocyst contents (Bilinski et al., 1981). As to be expected, a similar reaction occurs with the calcium store mobilizing agents, caffeine and 4CmC (Fig. 21c, d). In all these cases, absence of any visible trichocyst release would not necessarily imply absence of membrane fusion. Therefore, this has been analyzed independently at the end of this study. More intriguing is the occurrence of some exocytotic activity with caffeine in presence of EGTA (Fig. 21c). This could be explained by a certain permeabilization effect of caffeine, simultaneously with mobilization of Ca\(^{2+}\) from stores, so that some Ca\(^{2+}\) gains access to trichocyst contents, just as previously described (Klauke and Plattner, 1998).

What is revealed by the fluorochrome analysis experiments?

The subsequent fluorochrome analysis for the pwA-nd12/ 25°C and 35°C cells stimulated with different stimulators revealed that 35° cells - despite the presence of [Ca\(^{2+}\)]\(_o\) at the level required for the exocytosis (~50 µM) - do not mobilize Ca\(^{2+}\) at the same level as obtained in 25°C cells upon application of the same stimulators. This was proved by the weak fluorescence signals monitored in the case of 35°C cells in comparison to that seen in 25°C cells. In addition, the [Ca\(^{2+}\)]\(_i\) increase as a result of the different trigger agents in the case of low [Ca\(^{2+}\)]\(_o\) (by the addition of BAPTA) revealed a slight difference between 35°C and 25°C cells which could not reach any significant level. As mentioned before
in the “Methods and Materials” section, BAPTA is a very rapid Ca\textsuperscript{2+} chelator acting in the μs time range (Kao and Tsien, 1988). A drawback is that BAPTA application per se reduces to some extent [Ca\textsuperscript{2+}]\textsubscript{i} and, thus, attenuates any signal when applied during stimulation. With any of the trigger agents tested, at [Ca\textsuperscript{2+}]\textsubscript{o} = 50 µM, there was trichocyst exocytosis in 25°C cells, but not in 35°C cells. In the case of [Ca\textsuperscript{2+}]\textsubscript{o} chelation, there was no exocytotic activity in both, 25°C and 35°C cells, which could be referred to the absence of [Ca\textsuperscript{2+}]\textsubscript{o} that is required for the decondensation of trichocysts during exocytosis process.

Table 17. Summary of [Ca\textsuperscript{2+}]\textsubscript{i} changes vs. time (s) in 25°C and 35°C cells, respectively, upon stimulation with AED (2 or 10 µM), caffeine (50 mM), 4CmC (0.5 mM), or upon exposure to increased [Ca\textsuperscript{2+}]\textsubscript{o} (1 mM or 10 mM). The [Ca\textsuperscript{2+}]\textsubscript{i} increase values indicated are relative to the basal values in the respective cells before stimulation, as well as the rise time required to reach the maximal increase. Values derived from Tables 10 to 14.

<table>
<thead>
<tr>
<th>Application or exposure</th>
<th>[Ca\textsuperscript{2+}]\textsubscript{o} = 50 µM</th>
<th>[Ca\textsuperscript{2+}]\textsubscript{o} = 30 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Ca\textsuperscript{2+}]\textsubscript{i} increase</td>
<td>rise time (s)</td>
</tr>
<tr>
<td>AED, 2 µM</td>
<td></td>
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<tr>
<td>25°C cells</td>
<td>1.47 ± 0.11</td>
<td>1.50 ± 0.50</td>
</tr>
<tr>
<td>35°C cells</td>
<td>1.21 ± 0.04</td>
<td>2.53 ± 1.53</td>
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<tr>
<td>AED, 10 µM</td>
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<tr>
<td>25°C cells</td>
<td>1.66 ± 0.01</td>
<td>4.67 ± 1.67</td>
</tr>
<tr>
<td>35°C cells</td>
<td>1.18 ± 0.02</td>
<td>4.53 ± 1.27</td>
</tr>
<tr>
<td>Caffeine, 50 mM</td>
<td></td>
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</tr>
<tr>
<td>25°C cells</td>
<td>1.33 ± 0.09</td>
<td>2.70 ± 1.20</td>
</tr>
<tr>
<td>35°C cells</td>
<td>1.09 ± 0.33</td>
<td>11.30 ± 2.60</td>
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<tr>
<td>4CmC, 0.5 mM</td>
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<tr>
<td>25°C cells</td>
<td>1.38 ± 0.04</td>
<td>2.70 ± 0.67</td>
</tr>
<tr>
<td>35°C cells</td>
<td>1.18 ± 0.02</td>
<td>1.60 ± 0.40</td>
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<tr>
<td>Calcium, 1 mM</td>
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<tr>
<td>25°C cells</td>
<td>1.43 ± 0.04</td>
<td>3.83 ± 1.64</td>
</tr>
<tr>
<td>35°C cells</td>
<td>1.13 ± 0.06</td>
<td>12.17 ± 0.73</td>
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<tr>
<td>Calcium, 10 mM</td>
<td></td>
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</tr>
<tr>
<td>25°C cells</td>
<td>1.25 ± 0.15</td>
<td>4.00 ± 1.77</td>
</tr>
<tr>
<td>35°C cells</td>
<td>1.04 ± 0.01</td>
<td>2.33 ± 0.83</td>
</tr>
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**Comment on triggering of individual cells with AED**

It was ascertained to check the effect of the triggering agent, AED, on mobilization of $[\text{Ca}^{2+}]_i$ in pwA-nd12/35°C cells in comparison to that of 25°C cells, individually by using fluorochrome analysis. According to the data shown in Table 17, one can conclude that in the 35°C cells, a very weak $[\text{Ca}^{2+}]_i$ increase which may come from the cortical stores in the absence of any Ca$^{2+}$ influx (Kerboeuf and Cohen, 1990) and there was no exocytosis, even upon AED application ($[\text{Ca}^{2+}]_o = 50 \mu\text{M}$) at higher concentration (10 µM). On the other hand, 25°C cells showed a highly significant $[\text{Ca}^{2+}]_i$ increase upon AED stimulation ($[\text{Ca}^{2+}]_o = 50 \mu\text{M}$), accompanied with massive exocytosis. Only a weak and rather variable cortical $[\text{Ca}^{2+}]_i$ signal was seen when 25°C and 35°C cells are analyzed with AED supplemented with BAPTA ($[\text{Ca}^{2+}]_o = 30 \text{nM}$), ensuring the importance of the Ca$^{2+}$ influx to cause a maximum $[\text{Ca}^{2+}]_i$ increase (Erxleben et al., 1997). AED is established as a non permeable polyamine secretagogue, causing exocytosis in *Paramecium* cells, by activating the Ca$^{2+}$/polyvalent cation)-sensing receptors (see Fig. 44) causing $[\text{Ca}^{2+}]_i$ increase by release from alveolar sacs (Klauke et al., 2000). In the end, a $[\text{Ca}^{2+}]_i$ increase is needed for trichocyst release (Plattner et al., 1984; 1985; Knoll et al., 1991b; Erxleben et al., 1997; Klauke and Plattner, 1997).

**Comment on triggering of individual cells with permeable ryanodine receptor stimulators (caffeine and 4CmC)**

As mentioned, *Paramecium* cells contain extensive cortical Ca$^{2+}$ stores, the “alveolar sacs” (Stelly et al., 1991; Knoll et al., 1993; Länge et al., 1995) that are tightly attached at the cell membrane (Plattner et al., 1991) and endowed with calsequestrin-like Ca$^{2+}$ binding protein (Plattner et al., 1997b). Since some time already, circumstantial evidence suggests a major role of alveolar sacs in stimulus-secretion coupling (Erxleben and Plattner, 1994; Stelly et al., 1995; Erxleben et al., 1997; Klauke and Plattner, 1997), although they neither show any response to inositol-tris-phosphate ($\text{InsP}_3$), or ryanodine, nor
any indication of Ca\(^{2+}\)-induced Ca\(^{2+}\)-release, “CICR” (Länge et al., 1995; Zhou et al., 1995). On the other hand, caffeine can mobilize Ca\(^{2+}\) from alveolar sacs causing increase in cortical [Ca\(^{2+}\)]\(_i\) within 2 s in wild type Paramecium (7S) cells accompanied by trichocysts exocytosis (Klauke and Plattner, 1998; Klauke et al., 1998; Länge et al., 1996).

Caffeine is frequently used to activate intracellular Ca\(^{2+}\) stores endowed with ryanodine-sensitive Ca\(^{2+}\) channels (Ehrlich et al., 1994), like sarcoplasmic reticulum, SR, (McPherson and Campbell, 1993; Meissner, 1994; Fryer and Stephanson, 1996) or endoplasmic reticulum, ER, in neuronal or in secretory cells (Bezprovanny et al., 1991; McPherson et al., 1991; Friel and Tsien, 1992; Cheek et al., 1993; Simpson et al., 1996). It was also proved that application of caffeine to pheochromocytoma (PC12) cells (Taylor and Peers, 1999) evoked large, transient rises of [Ca\(^{2+}\)]\(_i\), though it did not trigger exocytosis. Caffeine rapidly penetrates into cells (Bianchi, 1962; O’Neill et al., 1990; Toescu et al., 1992) and, thus, can become active within seconds. However, not all of these stores are sensitive to caffeine (Schmid et al., 1990; Berridge, 1993; Cheek and Barry, 1993; Lynn and Gillespie, 1995). Some of the stores proved less or not sensitive to caffeine (Galione et al., 1991; Buck et al., 1992; Verma et al., 1996). It was also reported by Sitsapesan et al. (1995) that at millimolar concentration, caffeine can activate the raynodine receptor (Ry2). Besides, mobilization of intracellular Ca\(^{2+}\) pools, a variety of other effects have been discovered with caffeine, e.g., activation (Steenbergen and fay, 1996) or inhibition of Ca\(^{2+}\) sequestration (Chapman and Tunstall, 1988; Bassani et al., 1994) or Ca\(^{2+}\) release (Missiaen et al., 1992) as well as some other unrelated effects (Gupta et al., 1990; Berridge, 1991; Sawynok and Yaksh, 1993; Tanaka and Tashjian, 1993; Combettes et al., 1994; Islam et al., 1995). Despite these uncertainties, caffeine has remained a popular tool in secretion studies, partly because it is easy to apply and partly because of the lack of physiological alternatives (Klauke and Plattner, 1998).
In the present investigations with caffeine, only 25°C cells in presence of \([\text{Ca}^{2+}]_o\), show a rather clear \([\text{Ca}^{2+}]_i\) signal (Table 17). Under these conditions, 35°C cells display only a slight tendency of a \([\text{Ca}^{2+}]_i\) increase, while no signal occurs with either cell type when caffeine is applied together with BAPTA. In this specific case, the reason may be “extraction” of \(\text{Ca}^{2+}\) due to the membrane permeabilizing effect of caffeine. Though caffeine is frequently applied within the concentration range used in this work, it appears increasingly liable to artifacts. This is supported by several publications, as cited by Kissmehl et al. (1998), up to very recently (Sei et al., 2001). This also concerns *Paramecium*, where caffeine suppresses \(\text{Ca}^{2+}\) re-uptake by alveolar sacs (Kissmehl et al., 1998) and causes some permeabilization of cells (Klauke and Plattner, 1998), just as one may explain some of the effects described here. Therefore, we looked for an alternative to activate ryanodine receptor type \(\text{Ca}^{2+}\) release channels, as follows.

The membrane permeable pharmacologically active compound, “4CmC” is a potent activator of the ryanodine \(\text{Ca}^{2+}\) receptor release channels present in the skeletal muscle SR of rabbits, bovine cerebellar microsomes and a rat pheochromocytoma cell line (PC12) according to Zorzato et al. (1993). They suggested that the chloro and the methyl groups of 4CmC are important for the activation of the ryanodine receptor \(\text{Ca}^{2+}\) release channel. It was speculated – based upon its relatively simple chemical structure (Fig. 43) – that the electronegative chloride interacts with positively charged amino acids in the RyR gating domain. In addition, presence of the electron donor methyl group may produce a more potent RyR agonist. Therefore, 4CmC is an established activator of ryanodine-type \(\text{Ca}^{2+}\) release channels, even in the case of low ryanodine sensitivity (Herrmann-Frank et al., 1996). It was reported that 4CmC appears to be such a drug which is similar to caffeine since it binds to the ryanodine receptor and increases the frequency of channel opening but with an affinity about 10 times higher than caffeine. Thus, 4CmC is used to mobilize
Ca$^{2+}$ from SR in skeletal muscle cells in low concentration without loss of cell function which may be take place as a side effect in the case of using caffeine in high concentration (Westerbald et al., 1998; Kabbara and Allen, 1999).

Concerning our Paramecium system, it was proved that 4CmC mobilizes Ca$^{2+}$ from the alveolar sacs (Klauke et al., 2000) which, like some ryanodine-sensitive type Ca$^{2+}$ release channels, are also sensitive to caffeine (Erxleben and Plattner, 1994; Klauke and Plattner, 1998), initiating exocytosis in the wild type (7S) Paramecium tetraurelia cells (Klauke et al., 2000). While, caffeine has to be applied in ~50 mM concentration to achieve maximal stimulation in Paramecium (Klauke and Plattner, 1998), just like in higher eukaryotic cells (Cheek et al., 1993), only 0.5 mM of 4CmC is required in Paramecium, whereas the Ca$^{2+}$ mobilization in Paramecium was observed even at more than ~100 times lower concentration of 4CmC than caffeine (Klauke et al., 2000).

As it increasingly emerges from the above-mentioned literature and due to more advantages of 4CmC over caffeine it was decided to test the effect of 4CmC on the [Ca$^{2+}$]$_i$ mobilization in pwA-nd12 (25°C and 35°C) cells and compare the results with those obtained with caffeine.

In fact, the cortical [Ca$^{2+}$]$_i$ transients obtained with 4CmC, are more clear than those with caffeine. In presence of [Ca$^{2+}$]$_o$ = 50 µM, the signal is much stronger in 25°C cells than in 35°C cells, while application together with BAPTA yields only a very weak signal in 25°C cells and none in 35°C cells (Table 17). Only 25°C cells with [Ca$^{2+}$]$_o$ = 50 µM performed exocytosis upon 4CmC triggering.

![The chemical structure of 4-chloro-meta-cresol “4CmC”](image)

**Fig. 43.** The chemical structure of 4-chloro-meta-cresol “4CmC”.
Comment on exposure of individual cells to increased $[Ca^{2+}]_o$

Many eukaryotic cells sense changes in their extracellular $[Ca^{2+}]$, which in turn affect their physiological functions such as secretion and many other vital activities (Brown et al., 1995). Examples of these cells include parathyroid hormone-secreting chief cells (Nemeth and Carafoli, 1990; Brown, 1991; Brown et al., 1993), calcitonin-secreting thyroid C cells (Fried and Tashjian, 1986; Garrett et al., 1995), $Ca^{2+}$-absorbing gastric mucosal and intestinal cells (Pazianas et al., 1995; Cima et al., 1997; Gama et al., 1997), neurons (Raut et al., 1995; Quinn et al., 1997), bone-resorbing osteoclasts and bone-forming osteoblasts (Malgaroli et al., 1989; Zaidi et al., 1989; Leis et al., 1994; Honda et al., 1995) and bovine anterior pituitary cells (Shorte and Schofield, 1991; 1996). As generally accepted, exocytosis could be regulated by an increase of free $[Ca^{2+}]_i$ concentration, which may be produced by an influx of $[Ca^{2+}]_o$, by mobilization from the internal pools, or by both mechanisms in concert (Burgoyne and Morgan, 1993; Cheek and Barry, 1993; Putney, 1993; Alvarez et al., 1994; Meissner, 1994; Pozzan et al., 1994; Taylor, 1994).

In *Paramecium* cells, it was concluded that increasing $[Ca^{2+}]_o$ facilitates exocytotic and endocytotic membrane fusion. (Plattner et al., 1997a). It was also found that in different strains of *Paramecium* cells, simply increasing $[Ca^{2+}]_o$ causes a rapid short-lived fluorescence signal indicating $[Ca^{2+}]_i$ increase (Klauke et al., 2000). Therefore, it was of interest to investigate whether exposure of 25°C and 35°C cells to 1 mM or 10 mM $Ca^{2+}$ extracellular, would cause local cortical $[Ca^{2+}]_i$ transients as well as exocytosis or not.

Table 17 shows a significant $[Ca^{2+}]_i$ increase in 25°C upon exposure to increased $[Ca^{2+}]_o$. This $[Ca^{2+}]_i$ increase was stronger and faster with 1 mM $[Ca^{2+}]_o$ than that obtained with 10 mM $[Ca^{2+}]_o$ exposure. There was a difference in the $[Ca^{2+}]_i$ increase signals between the 35°C and 25°C cells, where it was much weaker with both 1 mM or 10 mM $[Ca^{2+}]_o$ in the case of 35°C cells than that occurring with 25°C cells. It is noteworthy, that there was no exocytosis in
all cases. These results obtained with 25°C cells are in agreement with the results obtained upon exposure of the wild type and pawn (d4-500r) *Paramecium* cells to 10 mM [Ca$^{2+}$]$_{o}$. Clearly despite increasing [Ca$^{2+}$], in response to increased [Ca$^{2+}$]$_{o}$, no exocytosis occurred also according to Blanchard (1998). Similarly it was also reported by Erxleben et al. (1997) that increasing [Ca$^{2+}$]$_{o}$ entailed [Ca$^{2+}$]$_{i}$ increase, though without exocytosis induction. Interestingly that the exposure of 25°C and 35°C cells to 10 mM [Ca$^{2+}$]$_{o}$ caused weaker signal (which was non-significant in 35°C cells) than that obtained with 1 mM [Ca$^{2+}$]$_{o}$. Why is there no exocytosis despite an increase of [Ca$^{2+}$]$_{i}$ in response to [Ca$^{2+}$]$_{o}$ = 1 mM and why is there much less [Ca$^{2+}$]$_{i}$ increase at [Ca$^{2+}$]$_{o}$ = 10 mM?

In fact, according to recent fluorochrome analyses, CaSR stimulation is achieved in the positive or negative sense, by increasing or decreasing [Ca$^{2+}$]$_{o}$, to which [Ca$^{2+}$]$_{i}$ then follows (Squires et al., 2001). While, increasing [Ca$^{2+}$]$_{o}$ to 2.5-15 mM caused [Ca$^{2+}$]$_{i}$ increase in Leydig cells (Adebanjo, et al., 1998) and increasing [Ca$^{2+}$]$_{o}$ from 1 to 10 mM caused double secretion of their testosterone content (Meikle et al., 1991).

The lower effect of higher [Ca$^{2+}$]$_{o}$ may be explained in our opinion, upon increasing the membrane rigidity due to increased concentration (10 mM) of extracellular calcium causing blocking of some calcium, channels thus decreasing the calcium influx force and therefore affecting the calcium release from its stores (alveolar sacs), too.

To summarize the answer of the questions asked above may be as follows. (i) [Ca$^{2+}$]$_{i}$ increase has to be at the “strategic” site. Fluorochrome measurements show only a rough distribution of Ca$^{2+}$ and apparently [Ca$^{2+}$]$_{i}$ does not increase just at the trichocyst exocytosis sites. (ii) If [Ca$^{2+}$]$_{o}$ is increased to too high levels, this may even inhibit Ca$^{2+}$ influx, possibly by the notorious rigidifying effect of high [Ca$^{2+}$]$_{o}$. All this has been discussed for *Paramecium* in previous studies (Erxleben et al., 1997; Blanchard et al., 1999).
How can we explain the weak signals obtained with 35°C cells in all fluorochrome experiments?

Accordingly, a question was addressed, whether the weak signals seen in 35°C cells under several conditions, as described above, may be due to short signal duration and/or low sensitivity to the stimulant? Both aspects have to be considered, as shown in the exemplary analysis by fast CLSM. In 35°C cells, the AED evoked cortical Ca\(^{2+}\) signal may rise very rapidly (Fig. 25) and possibly so fast that weak signals may be overlooked by conventional slow 2λ analyses, even when such signals are to be expected.

Further analyses revealed that increasing AED to 10 µM conc. increased the cortical Ca\(^{2+}\) signal not only in 25°C cells (Table 17) but also in 35°C cells, though to a much smaller extent, where the [Ca\(^{2+}\)]\(_i\) signals were longer than that recorded in the case of lower AED (2 µM) concentration. These data support the presence of a CaSR also in 35°C cells, probably of the same sensitivity as in 25°C cells, as it was derived from further EDX analysis.

Taking into consideration the above mentioned fluorometry data it could be concluded that, (i) at [Ca\(^{2+}\)]\(_o\) = 50 µM, stimulation of any kind always causes a smaller [Ca\(^{2+}\)]\(_i\) signal increase in 35°C cells than in 25°C cells, while the rise time is not significantly different. This can be explained by the absence of the influx component in 35°C cells. (ii) At [Ca\(^{2+}\)]\(_o\) = 50 µM, 10 µM AED causes only slightly more signal increase than 2 µM AED in 25°C cells, and this difference is even less pronounced in 35°C cells (though the rise time is increased for unknown reasons). This reaction is similar to that seen in some other Paramecium tetraurelia strains, while some Paramecium caudatum strains displayed much more diverging reactions (Klauke et al., 1998). (iii) With any stimulation, there is only little signal when [Ca\(^{2+}\)]\(_o\) is reduced to 30 nM (with a longer rise time of the signal).

As an interim statement, it was found that the reaction of pwA-nd12/25°C cells to the different stimuli is rather similar to wild type cells (Klauke
and Plattner, 1997; 1998; Erxleben et al. 1997; Klauke et al., 2000). This contrasts with the reaction of 35°C cells which displayed only low Ca²⁺ signals, if any, and no trichocyst exocytosis under any of the conditions described. How is this possible if it is assumed that Ca²⁺ mobilization from alveolar sacs on its own can produce membrane fusion in a significant proportion of docking sites (Knoll et al., 1993; Plattner et al., 1997 a), even though there no occurrence of Ca²⁺ influx in 35°C cells (Kerboeuf and Cohen, 1990)? To elucidate this very intriguing aspect, some other experiments have been performed, as outlined below.

**Are the [Ca] content in the alveolar sacs and its release normal in 35°C cells?**

Since some time, x-ray microanalysis has contributed to development and progress in the field of cell biology (Plattner and Zingsheim, 1987). Such analyses provide a method to identify elements within thin and thick specimens with high sensitivity and precise location (Dykstra, 1992; Flegler et al., 1993). Values for [Ca] could easily be obtained, in principle, by analytical electron microscopic methods, such as energy-dispersive x-ray “EDX” microanalysis (Hall, 1979; Warely, 1997; Reimer, 1998). This technique (see “Methods and Materials” section) was well established for the analysis of calcium dynamics in *Paramecium* cells during exocytosis stimulation (Hardt and Plattner, 1999; 2000).

Generally, during stimulation of exocytosis, cortical Ca²⁺ may increase by release from cortical stores and/or influx from the outside medium into the cytosol (Berridge, 1998; Rutter et al., 1998; Barritt, 1999; Mackrill, 1999). Ca²⁺ influx coupled to store depletion has been designated “capacitative” or “store-operated Ca²⁺ influx” (SOC), respectively, depending on the activation mechanism (Berridge, 1998; Barritt, 1999; Mackrill, 1999).

Alveolar sacs are dynamic cortical Ca stores (Stelly et al., 1991; 1995; Knoll et al., 1993). In their appearance they are similar to subsurface cisternae in
some neuronal systems (Metuzals et al., 1997; Berridge, 1998) and in pituitary gonadotrophs (Tse et al., 1997). The value for absolute total calcium \([\text{Ca}]\), in alveolar sacs of unstimulated wild type (7S) *Paramecium* cells is \(~43\) mM and in the cytosol is 3 mM, as determined by Hardt and Plattner (1999). In the SR of skeletal muscle, Somlyo et al. (1981) determined [Ca] as \(~33\) mM which is close to the value in *Paramecium*. In addition, AED-stimulated *Paramecium* cells show [Ca] decrease in their alveolar sacs (Stelly et al., 1995; Hardt and Plattner, 1999; 2000).

Thus, further EDX microanalysis has been done and it shows that the total calcium content in the alveolar sacs and its release upon stimulation are more or less the same in both 25°C and 35°C cells. In spite of this result obtained, still 35°C cells can not perform exocytosis nor \([\text{Ca}^{2+}]_i\) increase like that obtained in 25°C cells upon stimulation. This may be explained upon absence of additional \(\text{Ca}^{2+}\) influx in 35°C cells which is normally required for full activation. As it was found that in 7S cells, stimulation at \([\text{Ca}^{2+}]_o \leq [\text{Ca}^{2+}]_i^{\text{rest}}\) (i.e. no influx) still activates only \(~40\)% of all exocytosis sites (Erxleben et al., 1997; Plattner et al., 1997a).

**Is the basic \([\text{Ca}^{2+}]_i^{\text{rest}}\) in 35°C cells the same like that in 25°C cells?**

Comparing free, ionic calcium concentrations at rest and after activation, respectively, normally in animal cells a \([\text{Ca}^{2+}]_i^{\text{rest}}\) of \(~50\) to 100 nM is registered, while amounts of \([\text{Ca}^{2+}]_i^{\text{act}}\) of \(~500\) nM to \(\sim 10\) (occasionally \(\sim 100\)) \(\mu\text{M}\) may be required for full activation, depending on the system analyzed (Bootman and Berridge, 1995). Yet local \([\text{Ca}^{2+}]_i\) required to briefly activate “strategic” sites is much higher (Klingauf and Neher, 1997; Neher, 1998).

In different strains of *Paramecium* (7S wild type, as well as a non-discharge and a trichocyst-free mutant, nd9-28°C and tl), the resting \([\text{Ca}^{2+}]_i\) values are between 50 and 70 nM that increases at the site of AED application, up to \(~800\) nM (\(~10\) fold above the basal level) according to fluorochrome analysis (Klauke and Plattner, 1997).
Therefore, it was noteworthy, to look further for other reasons, one of which was that the basic $[Ca^{2+}]_{\text{rest}}$ is significantly lower (i.e. by ~41% in 35°C cells than in 25°C cells) thus, even if any $[Ca^{2+}]_i$ increase would occur upon stimulation, threshold $[Ca^{2+}]_{\text{act}}$ would not be reached, as previously mentioned in the “Results” section. Remarkably such significantly reduced $[Ca^{2+}]_{\text{rest}}$ values also occur in another *Paramecium tetraurelia* mutant, tl (Klauke and Plattner, 1997), even when grown at the same temperature as the wild type. Use of the wild type cells after cultivation at 35°C, for additional controls, was not possible due to fatality.

**Are the trichocysts of 35°C cells capable to decondense upon membrane fusion?**

The data obtained from application of Ca$^{2+}$ ionophore A23187 showed that the 35°C cells do not perform the expected exocytosis like the wild type cells (Plattner, 1974) with longer incubation period at 35°C (four days). This was to check the sensitivity to Ca$^{2+}$ of exocytotic membrane fusion sites which may have taken place but without trichocyst release, just like with “frustrated exocytosis” (Klauke and Plattner, 2000). Therefore, it was necessary to check the decondensation capability of the trichocysts, isolated from 35°C cells, when they meet Ca$^{2+}$, as in the case of membrane fusion (Bilinski et al., 1981). The trichocysts decondensed normally in the 35°C cells (Fig. 31).

**Do the 35°C cells perform membrane fusion leading to exocytosis upon triggering?**

Subsequently, it was confirmed according to the membrane fusion examination under the electron microscope using freeze-fracture technique, that there is no membrane fusion in 35°C cells within 5 s upon AED or 4CmC as impermeable and permeable triggering agents, respectively. Under these conditions, wild type cells would have 99% trichocyst docking sites transformed from “rosette” containing structures at rest to “filled rings” (early resealing
stages) and/or “parentheses” (late resealing stages) i.e., wild type cells undergo a complete membrane fusion leading to exocytosis by using the same stimulation conditions (Plattner et al., 1997a). The reason why fusion does not occur may be manifold, based on specific functions of proteins and/or lipid components which are not yet established in Paramecium, just as in any other secretory system.

**Additional remarks**

The routine ultrastructure investigation showed presence of autophagosomes dispersed in the cytoplasm of 35°C cells, giving a plausible explanation for the dark appearance of 35°C cells under the phase contrast light microscope in comparison to 25°C cells. This may be explained as a phenomenon of aging of the pwA-nd12 cells upon transfer to 35°C. Along these lines, protein electrophoresis of both, 25°C and 35°C cells, revealed the tendency of presence of higher amount of some proteins (including 140, 70 and 37 kDa proteins) in 35°C cells relative to that occurring in 25°C cells. The 70 kDa protein band may be attributed to the heat-shock protein, hsp70, which is known to occur in Paramecium (Dessen et al., 2001) as mentioned before in the “Results” section. It may be overexpressed at 35°C and be responsible for many functional disturbances in the double mutant pwA-nd12/35°C cells upon transfer from 25°C to 35°C. However, this aspect has not been analyzed in any more detail.

As reported, in many cell types, there are many proteins that belong to the heat-shock protein (hsp). Some of these are only expressed under stress conditions (strictly inducible), while some are present in cells under normal growth conditions and are not heat-inducible. Hsp70 proteins can be found in different cellular compartments such as nuclear, cytosolic, mitochondrial, endoplasmic reticulum, etc. They seem to help the cell survive sever insults (Pelham, 1986; 1988; Snutch et al., 1988; Craig, 1989; Alberts et al., 1989; Flaherty et al., 1990; Stryer, 1995). Also, some ciliates such as Euplotes spp. revealed a temperature-dependent divergence in the capacity to respond to
thermal stress with an activation of the transcription of their hsp70 genes (La Terza et al., 2001). Evidence of the role of (hsp70) and its production upon thermal stress in many other eukaryotic cell types is well established (Beere and Green, 2001; Edwards et al., 2001; Garrido et al., 2001; Molina et al., 2001).

**Characteristics of 35°C cells**

Considering the results previously discussed, the characteristics of 35°C cells could be summarized as follows. The 35°C cells have only slightly reduced total [Ca] in alveolar sacs and only slightly reduced Ca\(^{2+}\) mobilization from these stores, when stimulated with AED or with 4CmC. More significant is the considerably lower [Ca\(^{2+}\)\([^\text{rest}\)]\)] in the cytoplasm of 35°C cells which may prevent them - in conjunction with the absence of any Ca\(^{2+}\) influx - from reaching sufficiently high [Ca\(^{2+}\)\([^\text{act}\)]\)] levels required for exocytosis. They also lack membrane fusion capacity, for reasons to be elucidated. The incapability of trichocyst exocytosis is striking since the ultrastructure of trichocyst docking sites is normal, i.e. in freeze-fractures they contain “rosettes” and in ultrathin sections “connecting material” between trichocyst and cell membrane (Pouphile et al., 1986; Vayssié et al., 2000), features normally indicating exocytosis competence. An unusual extent of autophagic vacuoles in 35°C cells, as well as the occurrence of higher amount of some proteins in 35°C cells compared to those found in 25°C cells, may also indicate heat-induced down-regulation of different functions, while cells remain fully viable (Fig. 20). In other words, it could be speculated that the multiple disturbances which were described above may be due to a heat shock reaction and production or increasing amounts of a heat shock protein (hsp70).

**Aspects of Ca\(^{2+}\) dynamics during exocytosis stimulation**

In *Paramecium*, normally Ca\(^{2+}\) mobilization from alveolar sacs (Erxleben and Plattner, 1994; Stelly et al., 1995; Hardt and Plattner, 2000) is superimposed by a strong Ca\(^{2+}\) influx from the medium (Kerboeuf and Cohen,
1990). Only both components in concert can fully activate trichocyst exocytosis (Plattner et al., 1997) and they normally contribute about equally to exo/endocytosis (Plattner and Klauke, 2001). Analysis of pwA-nd12/35°C cells in comparison to 25°C aliquots appeared a feasible way to further dissect the individual steps possibly involved in Ca\(^{2+}\) dynamics during trichocyst exocytosis. Our nd12/d4-500r cells are devoid of any functional ciliary Ca\(^{2+}\) influx channels (Fig. 44), after culturing at 25°C or at 35°C, while somatic Ca\(^{2+}\) influx channels are functional only in 25°C cells (Kerboeuf and Cohen, 1990). Since our EDX data obtained with 35°C cells show Ca\(^{2+}\) mobilization from stores in the absence of any Ca\(^{2+}\) influx this could be considered as stringent evidence of a SOC-type mechanism (Fig. 44), involving (i) activation of a CaSR in the cell membrane (Klauke et al., 2000), (ii) activation of Ca\(^{2+}\) release channels in alveolar sacs, and - in 25° or wild type cells - (iii) Ca\(^{2+}\) influx from the medium as a secondary step. CaSR-type sensors have recently been shown to occur in widely different cell types (Zaidi et al., 1995; Shorte and Schofield, 1996; Quinn et al., 1997; Vassilev et al., 1997, Adebanjo et al., 1998; Yamaguchi et al., 1998; Oda et al., 2000; Brown and MacLeod, 2001) where they can induce \([\text{Ca}^{2+}]_i\) transients by different mechanisms even in cells not participating in the regulation of \([\text{Ca}^{2+}]_o\) homeostasis (Chattopadhyay and Brown, 2000). Involvement of a CaSR can also explain why electrophysiological hyperpolarization does not activate Ca\(^{2+}\)-activated currents in the plasmamembrane of Paramecium (Erxleben and Plattner, 1994). Our EDX data show normal Ca\(^{2+}\) mobilization from alveolar sacs upon AED stimulation and, thus, also imply that the CaSR is not defective in 35°C cells. We therefore also consider the functional state of Ca\(^{2+}\) release channels in alveolar sacs in the 35°C cells normal. Low or apparently absent Ca\(^{2+}\) signals in 35°C cells during stimulation in presence of BAPTA can be explained by the lower basal \([\text{Ca}^{2+}]_i\) in 35°C cells (see “Results”). Along these lines we excluded occurrence of any weaker signals of short duration by fast CLSM analysis.
Fig. 44. Assumed localization of Ca\(^{2+}\) release channels in alveolar sacs (a). Hypothetical diagram (b) showing the structures involved in [Ca\(^{2+}\)]\(_i\) mobilization during exocytosis, notice the soc influx (3) is defective in the double mutant pwA-nd12/35°C cells.

Abbreviations: alveolar sacs (AS), cilium (Ci), cell membrane (CM), inner alveolar sac membrane (IM-AS), outer alveolar sac membrane (OM-AS), subplasmalemmal space (SS), trichocyst (T).
**Possible site of Ca\textsuperscript{2+} influx disturbance**

We explain the absence of \textsuperscript{45}Ca\textsuperscript{2+} influx and of trichocyst exocytosis in 35°C cells as follows. (i) Reduced \( [\text{Ca}\textsuperscript{2+}]_{\text{rest}} \) may be considered one such defect. (ii) Membrane fusion is impaired, as discussed above. (iii) The main defect responsible for the disturbed Ca\textsuperscript{2+} dynamics in 35°C cells most likely concerns the Ca\textsuperscript{2+} influx channels (Fig. 44). Which one of the potential channels (Machemer, 1988; Preston, 1990) may be affected? While a SOC-type mechanism is established for a variety of higher eukaryotic cells (Cheek et al., 1993; Shorte and Schofield, 1996; Berridge, 1997; Barritt, 1998; 1999; Gregory et al., 1999; Mackrill, 1999; Patterson et al., 1999; Taylor and Peers, 1999; Alderton et al., 2000), the type of plasmalemmal Ca\textsuperscript{2+} influx channels involved is still under debate (Elliott, 2001; Putney, 2001; Yue et al., 2001). In some cases, rather unspecific cation channels may participate, as it is assumed for *Paramecium* (Klauke et al., 2000). Some influx channels could operate in register with Ca\textsuperscript{2+} mobilization from stores, which may also hold true for *Paramecium*. Channels under consideration, though not identified as yet, may be rather unspecific for bivalent cations, as it is derived from the previous Mn\textsuperscript{2+}-quenching experiments with fura-2 loaded cells (Klauke et al., 2000). Specificity of direct or indirect channel activation by positively charged compounds via CaSR-coupled Ca\textsuperscript{2+} channel activation can be quite low. For instance, Ca\textsuperscript{2+}-mediated signaling can be activated in vertebrate cells even with positively charged latex beads (Zhu and Peng, 1988) and this can also induce trichocyst exocytosis in *Paramecium* (Plattner et al., 1985). According to electrophysiology such unspecific plasmalemmal Ca\textsuperscript{2+} conducting channels occur in *Paramecium* (Saitow et al., 1997). Hyperpolarization-sensitive Ca\textsuperscript{2+} channels also have been taken into consideration to explain Ca\textsuperscript{2+} influx during AED stimulation (Kerboeuf and Cohen, 1996). In contrast to that, the scrutinized analysis in my thesis, all evidence points toward a SOC-type...
mechanism which does not depend on hyperpolarization-sensitive Ca\(^{2+}\)-influx channels.

**Conclusions**

It could be confirmed that nd12 cells cultivated at 35°C have normal ultrastructure of exocytosis sites (Pouphile et al., 1986; Vayssié et al., 2000). There is a lack of any significant Ca\(^{2+}\) influx into 35°C cells upon AED triggering (Kerboeuf and Cohen, 1990). A new aspect is that calcium storage in alveolar sacs is normal, as is Ca\(^{2+}\) release from stores. The functional state of the CaSR also appears normal.

As previously reported, the different strains of *Paramecium* cells such as 7S, nd9-18°C or nd12-25°C reflect the normal exocytosis-competent situation (i.e., [Ca\(^{2+}\)]\(_i\) signals and exocytotic responses) upon application of different triggering agents (Pouphile et al., 1986; Vayssié et al., 2000). Also, the molecular background of exocytosis incompetence in nd9 (Beisson et al., 1980; Froissard et al., 2001) is different from the one that is deduced for nd12, in this work, but both result in a nd phenotype. Moreover, as analyzed here, the reason of incapability of exocytotic membrane fusion is much more complex in pwA-nd12 (35°C) cells than in nd9-28°C cells. Novel findings in these 35°C double mutants bear on a defective signal transduction mechanism. Specifically Ca\(^{2+}\) influx channels are most likely defective, thus impeding a SOC-type response. **In other words, it could be concluded that the present cellular, molecular, functional and fluorochrome analysis of the exocytotic double mutant (pwA-nd12 cells) yields a new evidence of the store-operated Ca\(^{2+}\) influx mechanism required for exocytosis in *Paramecium*.**
5. Summary

A non-discharge mutant of *Paramecium tetraurelia* (nd12-35°C, lacking exocytotic response upon stimulation with the non-permeable polycationic secretagogue aminoethylxetran, AED), in the pawnA genetic context (d4-500r, lacking ciliary voltage-dependent Ca\(^{2+}\) influx), was shown to lack \(^{45}\text{Ca}^{2+}\) entry from outside upon AED stimulation (Kerboeuf and Cohen, 1990, J. Cell Biol. 111:2527). In contrast, cells grown at 25°C behave like the wildtype. To check the functional properties in more detail, fluorochrome loaded 35°C cells were stimulated, not only with AED (EC\(_{100}\) = 10\(^{-6}\) M in wild type cells), but also with the permeable activators of ryanodine receptor-type Ca\(^{2+}\) release channels, caffeine (50 mM), 4-chloro-meta-cresol, (4CmC, 0.5 mM), usually at extracellular [Ca\(^{2+}\)] of 50 µM, and eventually with a Ca\(^{2+}\) chelator added. We demonstrate that pwA-nd12/35°C cells lack any Ca\(^{2+}\) influx and any exocytosis of trichocysts in response to any stimulus. As we determined by x-ray microanalysis, total calcium content in alveolar sacs (subplasmalemmal stores) known to be mobilized upon exocytosis stimulation in wild type cells, contain about the same total calcium in 35°C as in 25°C cells, and Ca\(^{2+}\) mobilization from alveoli by AED or 4CmC is also nearly the same. Due to the absence of any AED-induced Ca\(^{2+}\) influx in 35°C cells and normal Ca\(^{2+}\) release from stores found by x-ray microanalysis one can exclude a “CICR”-type mechanism (Ca\(^{2+}\)-induced Ca\(^{2+}\) release) and imply that normally a store-operated Ca\(^{2+}\) (“SOC”) influx would occur (as in 25°C cells). Furthermore, 35°C cells display a significantly lower basal intracellular [Ca\(^{2+}\)], so that any increase upon stimulation may be less expressed or even remain undetected. Under these conditions, any mobilization of Ca\(^{2+}\) from stores cannot compensate for the lack of Ca\(^{2+}\) influx, particularly since normally both components have to cooperate to achieve full exocytotic response. Also striking is our finding that 35°C cells are unable to perform membrane fusion, as analyzed with the Ca\(^{2+}\) ionophore, A23187. These findings were corroborated by cryofixation and freeze-fracture analysis of trichocyst docking sites after AED or 4CmC stimulation which also revealed no membrane fusion. In sum, in nd12 cells increased culture temperature entails multiple defects, notably insensitivity to any Ca\(^{2+}\) signal which, moreover, cannot develop properly due to a lower basal [Ca\(^{2+}\)] level and the lack of Ca\(^{2+}\) influx, despite normal store activation.
Zusammenfassung

6. Zusammenfassung

7. References


References


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References


References


8. Appendix

Antibody V₀-ATPase experiments

Experiment 1
Injection of isolated individual 7S Paramecium cells with antibody V₀-ATPase (final conc. = 6 µM in cell) then checking its effect on the exocytotic activity by application of a polycationic secretagogue AED (final conc. = 2 µM).

<table>
<thead>
<tr>
<th>Cells No.</th>
<th>AED application time in minutes (t₀ = injection time)</th>
<th>Effect on exocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Experiment 2
1% AED was applied into 7S cells in a suspension to cause full exocytosis then injection of these cells individually (n = 3 cells) with antibody V₀-ATPase (conc. = 6 µM in cell).

Waiting 4 hours till redocking of the trichocystes was achieved in the cells again, then checking the effect of the antibody on the exocytotic activity by application of a polycationic secretagogue AED (final conc. = 2 µM).

The idea for the first stimulation for the exocytosis to allow the dispersion of the antibody at the docking sites.

Results the exocytosis is normal.
Experiment 3

Checking the effect of antibody V0 ATPase on the membrane fusion of the food vacuoles in wild type Paramecium (7S cells)

A- staining the yeast cells with congo red

1- Dissolve 15 g yeast in 150ml dist. water (use heating).
2- Add 500 mg congo red and wait 30 minutes.
3- Cook for 1 hour then leave it to be cooled at room temperature.
4- Check under the microscope for the red cells, if not leave them overnight in the cooling room.
5- Remove the supernatant then resuspend the pericepitate with dist. Water and centrifuge at 2000 rpm (2min).
6- Repeat step 5 till the color of the supernatant is pale red.

Notice

1- The pH was 9.5 before removing the supernatant then finally the concentrated stained yeast cells had pH around 7.2.
2- The congo red has a red color at pH between (6.3–9). Then it starts to have a dark greenish blue color at pH (5.8-6) and keeps dark blue color with lower pH.

B- Injecting the 7S cells with the antibody V0-ATPase

1- Isolated Individual Paramecia cells (n = 12) were injected with the antibody V0-ATPase (conc. = 6 µM in cell).
2- Taking the cells and incubate them for 10 minutes in fresh salad medium to recover from the effect of the injecting process.

C- Feeding these 7S cells with yeast cells

1- Incubate these injected 7S cells in a salad medium containing yeast cells (10+1) that were stained previously with congo red.
2- By watching the Paramecia cells; the phagocytosis was normally occurring just after 1 minute of incubation.

3- Digestion starts after 5 minutes and was very active in the posterior cytoplasmic region of the cell where the red stained yeast cells were broken into smaller parts and their colour changed into greenish blue due to mixing with the digestive enzymes at pH 6 nearly.

4- Watching the digestive process for 2.5 hours, it was clear that the whole food vacuoles disappeared due to digestion is completed.

5- The cytoproct also was observed during its fusion with the cell membrane then its strong release from the cell itself to be ejected outside.

6- During the whole digestive process the paramecium cells were stagnant or slowly moving then just after the cytoproct released and the yeast cells have been digested completely the cells moved actively and started to phagocytose other new yeast cells.

**Notice** The contractile vacuoles were pulsating normally and the cilia of the Paramecia were working normally too during the whole experiments.

**Result** no significant effect of the injected antibody on the membrane fusion of the food vacuoles and cyclosis as well as vacuolar digestion were normal in comparison with the uninjected control cells. The cytoproct showed also a good and clear fusion process between the membranes.

**Conclusion of the previous experiments**

The injection of the antibody Vo-ATPase did not affect the membrane fusion in 7S Paramecium cells at the following sites:

1- Exocytotic sites.
2- Formation of food vacuole at the entry site of the cytostome (phagocytosis).
3- Formation of the large food vacuoles after fusion of the small ones.
4- Formation of the secondary phagosome due to fusion between the primary phagosome with the food vacuole.
5- Formation of the cytoproct and its release from the cell.
6- Formation of the contractile vacuoles and its radiating small canals.

Recommended References


