Functional and Fluorochrome Analysis of an Exocytotic Mutant Yields Evidence of Store-operated Ca^{2+} Influx in *Paramecium*

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Abstract. A non-discharge mutant of *Paramecium tetraurelia* (nd12–35°C, lacking exocytotic response upon stimulation with the nonpermeable polycationic secretagogue aminoethyldextran, AED), in the pawnA genetic context (d4-500r, lacking ciliary voltage-dependent Ca^{2+} influx), was shown to lack $^{45}Ca^{2+}$ entry from outside upon AED stimulation. In contrast, cells grown at 25°C behave like the wild-type. 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 wildtype cells), but also with 4-chloro-meta-cresol, (4CmC, 0.5 mM), a permeable activator of ryanodine receptor-type Ca^{2+} release channels, usually at extracellular $[Ca^{2+}]$ of 50 μM, and eventually with a Ca^{2+} chelator added. We confirm 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.

Key words: Ca^{2+} — Calcium — Exocytosis — *Paramecium* — Secretion

Introduction

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 & Klauke, 2001). In *Paramecium*, exocytosis of dense-core vesicles ("trichocysts") can be stimulated by the nonpermeable polyamine secretagogue, aminoethyldextran (AED, Plattner et al., 1984; 1985), which is paralleled by an increase in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, according to electrophysiological recordings of Ca^{2+}-activated currents (Erxleben & Plattner, 1994; Erxleben et al., 1997) and fluorochrome analyses (Klauke & Plattner, 1997).

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Ca^{2+}-buffer injections indicate requirement of a free Ca^{2+} concentration, $[Ca^{2+}]_i^{act}$, of ~5 μM for membrane fusion to occur. Ca^{2+} signals are much weaker when extracellular Ca^{2+} is chelated briefly, within <1 sec, e.g., to $[Ca^{2+}]_o = 30$ nM, a value slightly below resting values, $[Ca^{2+}]^{rest}$ ~65 nM (Klauke & Plattner, 1997). These data fit precisely the observation of $^{45}Ca^{2+}$ influx during AED stimulation, which drives exocytosis down to $[Ca^{2+}]_o$ 300 nM (Kerboeuf & 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 & Plattner, 2000).

In *Paramecium*, this store activation can be dissociated from a Ca^{2+} influx component by stimulation with AED at $[Ca^{2+}]_o \sim 30$ nM (Klauke & Plattner, 1997) and by stimulation with permeable activators of these Ca^{2+} stores, like caffeine (Klauke & Plattner, 1998) or 4-chloro-meta-cresol, 4CmC (Klauke, Blanchard & Plattner, 2000), also at low $[Ca^{2+}]_o$. Since *Paramecium* is sensitive to low $[Ca^{2+}]_o$, this is produced by adding a chelator to the stimulant. While caffeine has to be applied in ~50 mM concentration to achieve maximal stimulation in *Paramecium* (Klauke & 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, Andrade & Islam, 1998; Kabbara & Allen, 1999). Therefore, we preferred using 4CmC in the present work.

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, Braun & Hentschel, 1997; Plattner & Klauke, 2001). This could imply different mechanisms, which we now try to differentiate using cells with and without Ca^{2+} influx, respectively. Theoretically Ca^{2+} could enter from outside and cause a "Ca^{2+}-induced Ca^{2+} release" (CICR), but our present study largely excludes this for *Paramecium*. 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. In detail, when cortical stores are depleted of Ca^{2+} in a primary step, this can secondarily entail a "store-operated Ca^{2+} influx" (SOC), as outlined in recent reviews (Barritt, 1998, 1999; Berridge, 1997; Berridge et al., 2000; Elliott, 2001). Previous evidence was in favor of such a mechanism in *Paramecium* (Klauke et al., 2000) and our present work supports this by showing the absence of this component in a temperature-conditional mutant. A SOC-type mechanism can

include activation of a "Ca^{2+}/(polyvalent cation)-sensing receptor", CaSR, in the cell membrane 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). Generally, CaSR can mediate a $[Ca^{2+}]_i$ increase in response to a $[Ca^{2+}]_o$ increase or to extracellular polyamines, where it may not depend on $[Ca^{2+}]_o$, but rather causes mobilization of Ca^{2+} from internal stores. 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 sub-plasmalemmal space of only ~15 nm width (Plattner et al., 1991). Coupling of alveolar sacs to the cell membrane is not known to involve any known second messenger (Länge, Klauke & Plattner, 1995). Although alveolar sacs resemble the sarcoplasmic reticulum (SR) insofar in both cases Ca^{2+} can be released by caffeine- and 4CmC-sensitive channels (Klauke & 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, Nakaoka & Oosawa (1997). Since the molecular identity of the components envisaged is not yet established in *Paramecium*, this scenario is hypothetic and can be summarized as follows: (i) Activation of a CaSR in the cell membrane by AED, (ii) Ca^{2+} depletion of stores, and (iii) SOC-type Ca^{2+}-influx to increase the cortical $[Ca^{2+}]_i$ signal — the defective site in the conditional temperature-sensitive mutant we analyze here.

Many mutations available in *Paramecium* may facilitate a more detailed analysis of Ca^{2+} dynamics during exocytosis. For instance, the non-discharge mutant, nd12, is unable to release trichocysts by exocytosis after cultivation at 35°C (Cohen & Beisson, 1980), in contrast to 25°C, and it has no $^{45}Ca^{2+}$ influx upon AED stimulation (Kerboeuf & Cohen, 1990). Therefore, the nd12 mutant lends itself to analyze selective components of the Ca^{2+}-signaling pathway, e.g., to discriminate between CICR- and SOC-type mechanisms. To avoid any interference of ciliary Ca^{2+} channels, we performed analyses with the double mutant, pwA-nd12, for which Kerboeuf and Cohen (1990) have shown absence of $^{45}Ca^{2+}$ influx when cultivated at 35°C. The d4-500r, a pawnA (pwA) mutant, is devoid of any functional voltage-dependent ciliary Ca^{2+} channels (Satow & Kung, 1980; Haga et al., 1982). In pwA-ndl2(35°C) cells, any Ca^{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 & Cohen, 1990), as in d4-500r cells (Plattner et al., 1984). We now combined fluorochrome analysis, while stimulating

cells with AED and 4CmC, respectively, under conditions of variable [Ca^{2+}]$_o$. We applied Fura Red/double wavelength (2λ) and eventually fast Fluo-3/1λ confocal laser scanning microscopy (CLSM) analysis to monitor changes in [Ca^{2+}]$_i$ in response to the different stimuli at different [Ca^{2+}]$_o$ values. By EDX we analyzed the total (free and bound) calcium concentration, [Ca], in alveolar sacs, and their capacity to release Ca^{2+} in response to AED and 4CmC, respectively. Normally, bound calcium dominates over free calcium over thousandfold in the cytosol as well as in stores, also in *Paramecium* (Plattner & Klauke, 2001). Finally, membrane fusion capacity has been analyzed by fast freezing and quantitative freeze-fracture analysis.

Materials and Methods

Cells and Isolated Trichocysts

Cells with combined pwA-nd12 genotype were produced as described by Kerboeuf and Cohen (1990) and cultivated monoxenically, with *Enterobacter aerogenes* added, either at 25°C or at 35°C, i.e., under permissive and non-permissive conditions, respectively. In strictly monoxenic cultures, as we routinely use, routine measurements by a Ca^{2+}-selective electrode always yield [Ca^{2+}]$_o$ close to 50 μM; these are our "standard conditions", where any deviation can easily be corrected. Lower values are achieved by adding a chelator to the trigger solution (for reasons of this application mode, *see* Results). The resulting [Ca^{2+}]$_o$ is derived from calculation, also as published previously (Knoll et al., 1991). Evidently, it is important to work with these extremes, i.e., with and without a potential Ca^{2+} influx, influx being impossible when chelators reduce [Ca^{2+}]$_o$ to slightly below [Ca^{2+}]$_i^{rest}$. Both, non-discharge and pawn properties (lack of ciliary reversal reaction upon depolarization) of the fused strains were tested before use, (i) by adding AED (40 kDa, 1-NH$_3^+$/kDa; c.f. Plattner et al., 1984; 1985) and (ii) by chemical depolarization, respectively. 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. We found that at least ≥6 divisions were required to acquire non-discharge properties upon transfer from 25°C to 35°C. Before any further use of 35°C cells we regularly ascertained, in the light microscope, absence of ciliary reversal and of any exocytotic response under the trigger conditions specified in the figure legends. With the Ca^{2+}-ionophore, A23187, used at 33 μM in ≤2% DMSO, we tested membrane fusion capacity by providing artificially increased [Ca^{2+}]$_i$. Furthermore, viability of cells and reversibility of 35°C effects were tested.

The decondensation (stretching) capacity of trichocyst contents isolated from 35°C cells was tested in the phase contrast microscope as described previously (Glas-Albrecht & Plattner, 1990).

Ca^{2+} Fluorochrome Experiments and Recording of Exocytosis

Cells loaded by microinjection with Ca^{2+} fluorochromes, Fura Red or Fluo-3, were stimulated and evaluated in a conventional light microscope by 2λ, analysis and by 1λ, confocal laser scanning microscope (CLSM), respectively, as previously described (Erxleben et al., 1997; Klauke & Plattner, 1997; 1998; Klauke et al.,

2000). Pilot data obtained by CLSM are not shown because they only served to guarantee that we did not overlook fast signals. Fluorochrome concentrations injected were ten times above final concentration in the cell, since the volume we inject is 1/10 of the cell volume (Erxleben et al., 1997), as routinely tested by occasional injection of a clearly visible oil droplet. In f/f_0 ratio analyses, any fluorescence readings during stimulation (f) are expressed as a ratio, i.e., referred to the reading at rest, time t_0 (f_0) just before stimulation. This type of internal standardization, as explained in more detail for *Paramecium* cells in our previous work (Klauke & Plattner, 1997, 1998; Klauke et al., 2000), is routinely used in the literature when the aim is merely to monitor relative changes of [Ca^{2+}]$_i$. The area evaluated was the cortical site where a stimulus had been applied, or a central region of the cell, as specified previously (Klauke & Plattner, 1997; Erxleben et al., 1997). Briefly, cells for 1λ-type f/f_0 ratio evaluation, just like for 2λ measurements, were taken randomly. Areas to be evaluated were randomly selected, yet specifically in anterior cortical domains adjacent to the cell surface, as specified previously (Klauke & Plattner, 1997; Erxleben et al., 1997). In practice, a frame of ~3 × 10 μm was adjusted to such an area, again as in our previous work (Klauke & Plattner, 1997; Klauke et al., 2000). For CLSM a fast system (Noran, Bruchsal, Germany) equipped with an opto-acoustic beam deflection system, requiring only 33 msec per image, was available. However, due to the filter-changing mechanism, 1.35 sec were required to collect signals in the 2λ mode. Resting levels of free intracellular [Ca^{2+}], [Ca^{2+}]$_i^{rest}$, in 35°C cells were determined and compared to those of 25°C cells, using Fura Red-injected cells. Cells were stimulated by adding, through a pipette, AED or 4CmC, and occasionally also with caffeine, at the concentrations indicated. Actual concentrations reaching the cell surface were estimated by recording the dilution of a stain added (Klauke & Plattner, 1998). The usual [Ca^{2+}]$_o$ of 50 μM was eventually reduced to a calculated value of ~30 nM by adding BAPTA to the extracellular stimulation medium (Knoll, Braun & Plattner, 1991) as specified above. (Simultaneous application of stimulant and Ca^{2+} chelator, BAPTA free acid, avoids cell damage [Klauke et al., 2000].) Exocytosis was registered in intermittent transmitted-light pictures and rated as indicated in the table.

Electron Microscopy (EM)

Aliquots of double-mutant cells were processed by routine glutaraldehyde/OsO$_4$/Spurr embedding to ultrathin sections for EM analysis.

Some cultures were stimulated by AED or 4CmC, frozen and freeze-fractured for quantitative EM evaluation of their exocytosis sites as described (Knoll et al., 1991; Plattner et al., 1997).

For EDX analysis, cells were stimulated by manual mixing for 10 sec with AED or 4CmC and subsequent cryofixation/quenched-flow analysis according to Knoll et al. (1991). Further processing and analysis was as described in detail by Hardt and Plattner (1999; 2000). Cryofixation was followed by freeze-substitution under conditions of calcium retention. Analysis was carried out in a STEM-operated Zeiss/Leo EM912 Omega equipped with an energy-dispersive x-ray microanalysis unit. Only strictly cross-sectioned alveolar sacs were evaluated in the spot mode. We analyzed on average 3 alveolar sacs per cell, using a primary beam of 63 nm diameter, with spreading to ~75 nm at the bottom of a 0.5 μm thick section, as compared to the average width of an alveolar sac of <100 nm (Hardt & Plattner, 1999; 2000). For total number of cells and of alveolar sacs analyzed, *see* Table 2. Since Ca$_{K\alpha}$ signals vary widely from one alveolus to another, we fixed the following sampling rules. After randomly selecting a strictly cross-cut alveolus,
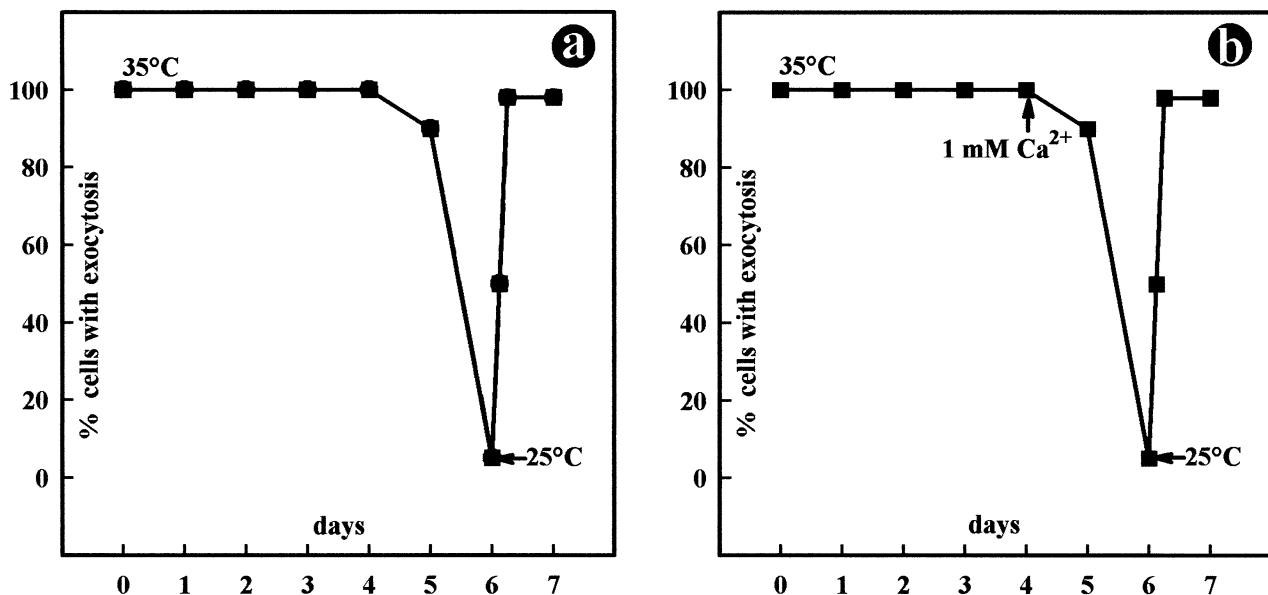
Fig. 1. Change of exocytosis capacity of cells in response to 2 µM AED upon transfer from 25°C to 35°C at t_0 and its reversibility after transfer to 25°C. $[Ca^{2+}]_o$ was kept constant at 50 µM in (*a*). No difference was observed when $[Ca^{2+}]_o$ was raised to 1 mM at day 4 in (*b*).

every spectrum was included in the final evaluation, except for two situations, namely when the beam had shifted out of the structure to be analyzed (recognizable by the contamination spot) or when the section had been torn up (<5% of all cases). To avoid any influence of any possible change in x-ray registration sensitivity of the instrument, data were collected in 5 independent series and in changing sequence of the 4 experimental conditions analyzed, yet this always gave very similar tendencies.

Results

We designate pwA-nd12(25°C)- and pwA-nd12 (35°C)-cells as "25°C cells" and "35°C cells," respectively, with the implication that 35°C cells show no trichocyst exocytosis and no ciliary reversal upon stimulation as typical features.

First we analyzed the change of exocytosis capacity when cells are transferred from 25°C to 35°C. Typical examples of such experiments are shown in Figs. 1 and 2, applying AED stimulation or the "quick picric acid test" routinely used in genetic studies. Increasing $[Ca^{2+}]_o$ does not affect development of 35°C characteristics (compare Fig. 1*b* with 1*a*). Since cell density decreases with time at 35°C, we ascertained the viability of 35°C cells when they were set back to 25°C (Fig. 1). 25°C-characteristics were restored within ~6 hr, while the time required to develop 35°C-characteristics always requires several days, though it may vary. Therefore, 35°C cells used for any further experiments were regularly tested for viability as well as for incapability to perform ciliary reversal and exocytosis. These tests were done with AED, as in subsequent experiments, since the exocytotic response upon transfer from 25°C to 35°C

decreases much faster with picric acid than with AED (Fig. 2*a*).

Absence of exocytosis in 25°C cells upon AED treatment in absence of Ca^{2+}_o (Fig. 2*b*) is not surprising since Ca^{2+}_o is required for decondensation (stretching and discharge) of trichocyst contents (Bilinski, Plattner & Matt, 1981). As expected, a similar reaction occurs with the calcium-store mobilizing agent, 4CmC (Fig. 2*c*). In both 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.

Subsequently we analyzed stimulated cells by fluorochromes, as described in Materials and Methods. An example of false-color imaging upon AED stimulation of a Fura Red-injected cell shows a strong cortical Ca^{2+} signal in a 25°C cell (Fig. 3*a–d*), and a much weaker signal in a 35°C cell (Fig. 3*a'–d'*), both at $[Ca^{2+}]_o = 50$ µM.

Analysis of cortical $[Ca^{2+}]_i$ signals upon stimulation was performed by conventional microscopy, combined with Fura Red/2λ analysis, or by fast CLSM analysis, combined with Fluo-3/1λ and f/f_0 ratio analysis as described previously (Erxleben et al., 1997). Examples of results achieved are documented in Figs. 4 to 6 and summarized in Table 1. CLSM analysis was performed to avoid overlooking fast/ short responses; since this is not the case, such data are not shown. Another corollary was to test AED at usual concentrations, e.g, 2 µM, and at 10 µM because we have realized widely different reactions in work with some other *Paramecium* mutants (Klauke et al., 1998).
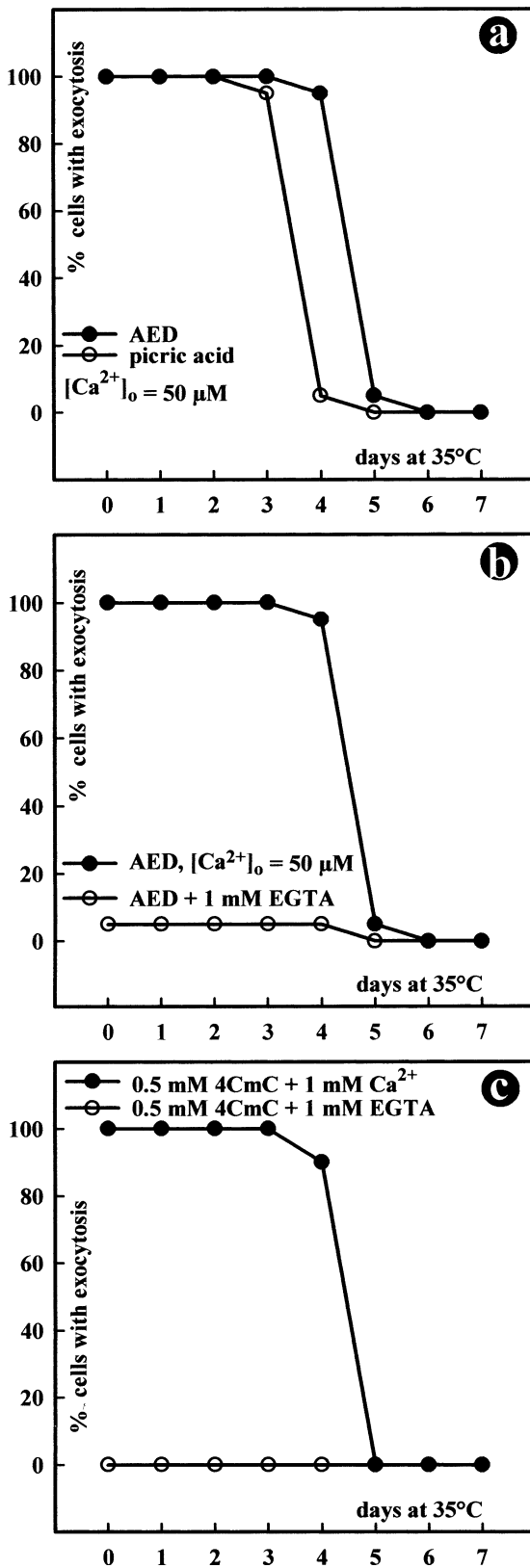
Fig. 2. Change of exocytosis capacity, after transfer from 25°C to 35°C at t_0 upon stimulation (a) with 2 μM AED in comparison to picric acid, at $[Ca^{2+}]_o$ = 50 μM, (b) with AED at $[Ca^{2+}]_o$ = 50 μM or with EGTA added, (c) with 0.5 mM 4CmC plus 1 mM Ca^{2+} or 1 mM EGTA.

Examples of the respective responses of 25°C and 35°C cells to AED (2 μM) or 4CmC (0.5 mM) at different $[Ca^{2+}]_o$ are presented in Figs. 4 and 5. Clearly, Ca^{2+} influx can occur only at $[Ca^{2+}]_o$ = 50 μM, but not at 30 nM since this is slightly below $[Ca^{2+}]_i^{rest}$ (Klauke & Plattner, 1997). For each situation, we present as examples recordings obtained from different cells. For instance, Fig. 4a accounts for some variability of the response to AED in 25°C cells at $[Ca^{2+}]_o$ = 50 μM. Their response is much stronger than that of 35°C cells (Fig. 4b) which, in the absence of any Ca^{2+} influx (Kerboeuf & Cohen, 1990), may come from cortical stores (*see* below). We see only a weak and rather variable cortical Ca^{2+} signal when 25°C and 35°C cells are analyzed with AED supplemented with BAPTA (Fig. 4c, d). BAPTA is a very rapid Ca^{2+} chelator acting in the μsec time range (Kao & Tsien, 1988). A drawback is that BAPTA application per se reduces to some extent $[Ca^{2+}]_i$ and, thus, attenuates any signal when applied during stimulation. With any of the trigger agents tested, at $[Ca^{2+}]_o$ = 50 μM, we see trichocyst exocytosis in 25°C cells, but not in 35°C cells.

With caffeine, Ca^{2+} signals (*not shown*) were similar to those with 4CmC. Only 25°C cells, and only in presence of Ca_o^{2+} show a rather clear signal. Under these conditions, 35°C cells display only a slight tendency of a $[Ca^{2+}]_i$ increase, whereas no signal occurs with either cell type when caffeine is applied together with BAPTA. The reason of this may be "extraction" of Ca^{2+} due to the membrane permeabilizing effect of caffeine (Klauke & Plattner, 1998). Therefore we concentrated on 4CmC. In fact, the cortical $[Ca^{2+}]_i$ transients obtained with this alternative store mobilizing agent (Fig. 5) are more clear than those with caffeine. In presence of $[Ca^{2+}]_o$ = 50 μM, the signal is much stronger in 25°C cells (Fig. 5a) than in 35°C cells (Fig. 5b), while application together with BAPTA yields only a very weak signal in 25°C cells (Fig. 5c) and none in 35°C cells (Fig. 5d).

Further analyses revealed that increasing AED concentrations increase the cortical Ca^{2+} signal not only in 25°C cells (Fig. 6a) but also in 35°C cells, though to a much smaller extent (Fig. 6b). Again CLSM analysis of 25°C cells shows that this cortical $[Ca^{2+}]_i$ increase is very fast, that it may slightly vary in adjacent regions, and that it entails trichocyst exocytosis (*not shown*).

A survey of conventional 2λ-measurements, compiled in Table 1, in conjunction with significance values (P = 0 to 100% for lowest and highest error probability, i.e., highest and lowest significance, respectively) specified in the footnote to Table 1, shows the following main aspects: (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 (P = 3.0%, 0.4%, and 8.6% for data pairs
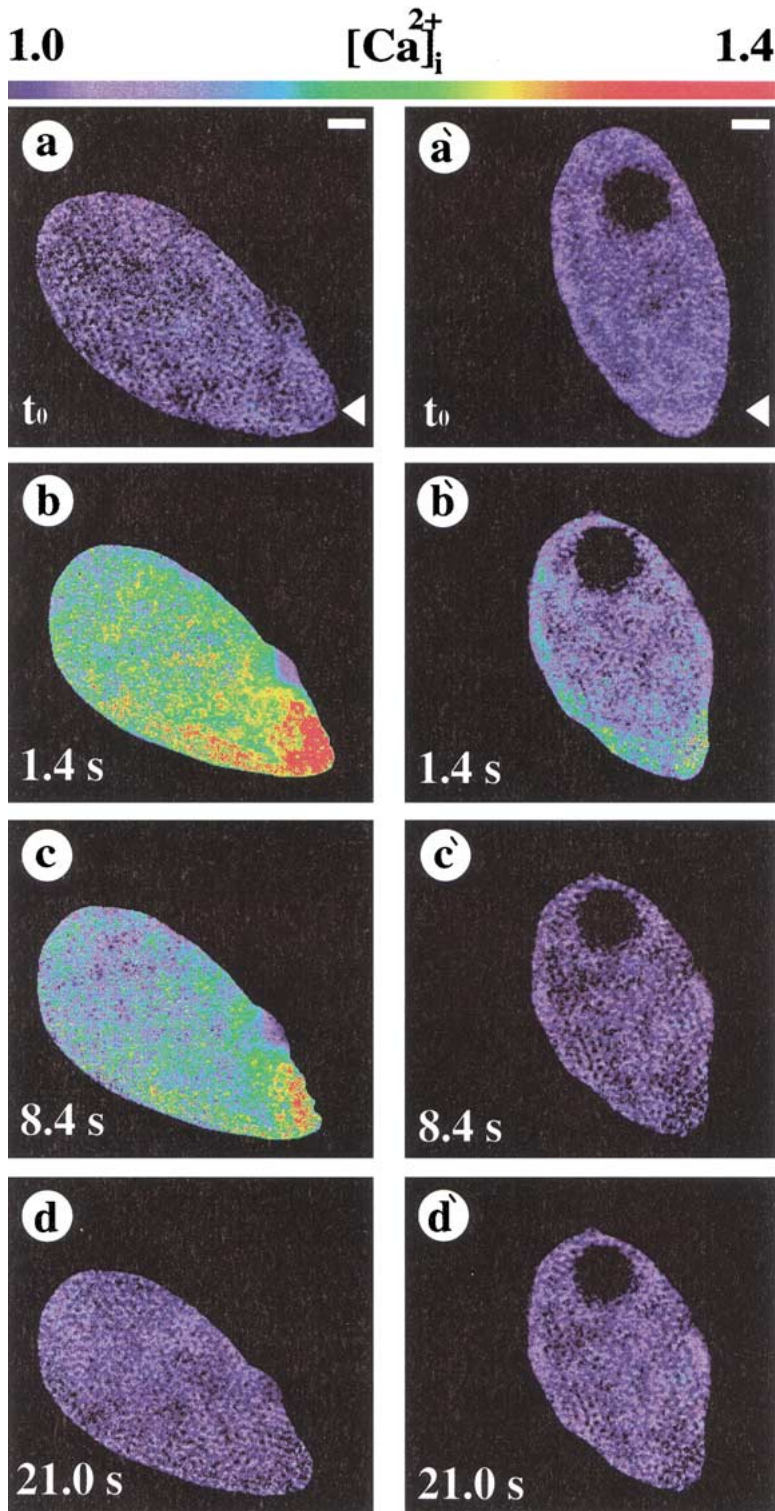
Fig. 3. False-color images obtained with Fura Red-injected 25°C (*a–d*) and 35°C (a′– d′) pwA-nd12 cells, respectively, during stimulation with 2 μM AED, applied at the arrowhead. (Note that the false-color imaging presented is inappropriate to resolve the difference in basal $[Ca^{2+}]_i$ in 25°C- and 35°C-cells, respectively [*see* text].)

1*a*/2*a*, 3*a*/4*a*, and 5*a*/6*a*, respectively, in Table 1), while the rise time is not significantly different (42.3% for pair 1*b*/2*b*, 82.2% for 3*b*/4*b*) except for 4CmC stimulation ($P = 5.7\%$ for pair 5*b*/6*b*). 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 ($P = 0.5\%$), and this difference is even less pronounced and not very significant ($P = 16.1\%$) in 35°C cells, while the rise time is increased for unknown reasons, although with high P-value (85.0%). This reaction is similar to that seen in some other *P. tetraurelia* strains, while some *P. caudatum* strains displayed much more di-
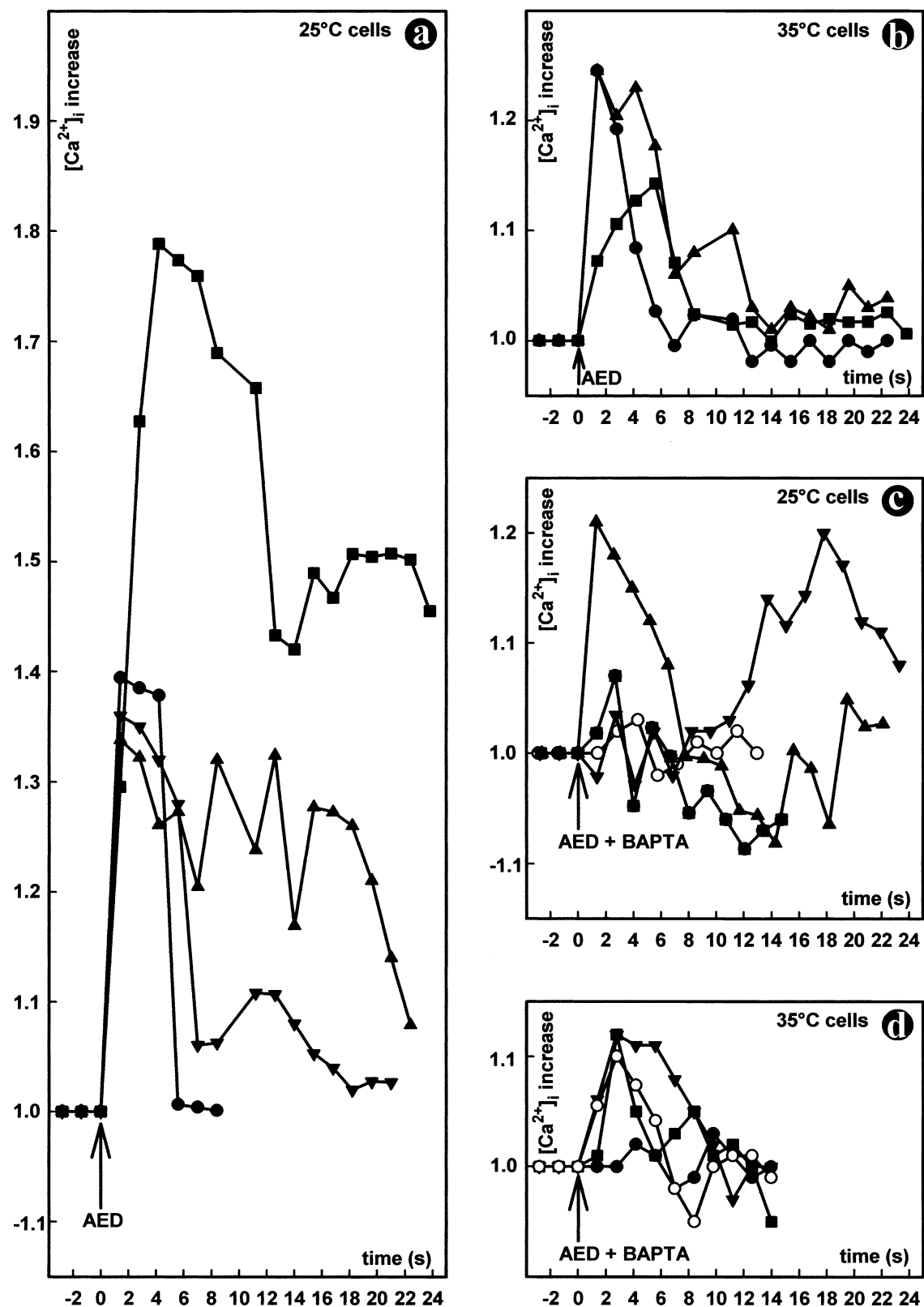
Fig. 4. Time course of cortical [Ca^{2+}]$_i$ signal (Fura Red) close to the site of 2 μM AED application at t_o. In (*a*)–(*d*) we present typical examples, each from several randomly selected cells, (*a, b*) [Ca^{2+}]$_o$ = 50 μM, (*c, d*) 1 mM BAPTA in trigger medium; (*a, c*) 25°C cells, (*b, d*) 35°C cells.
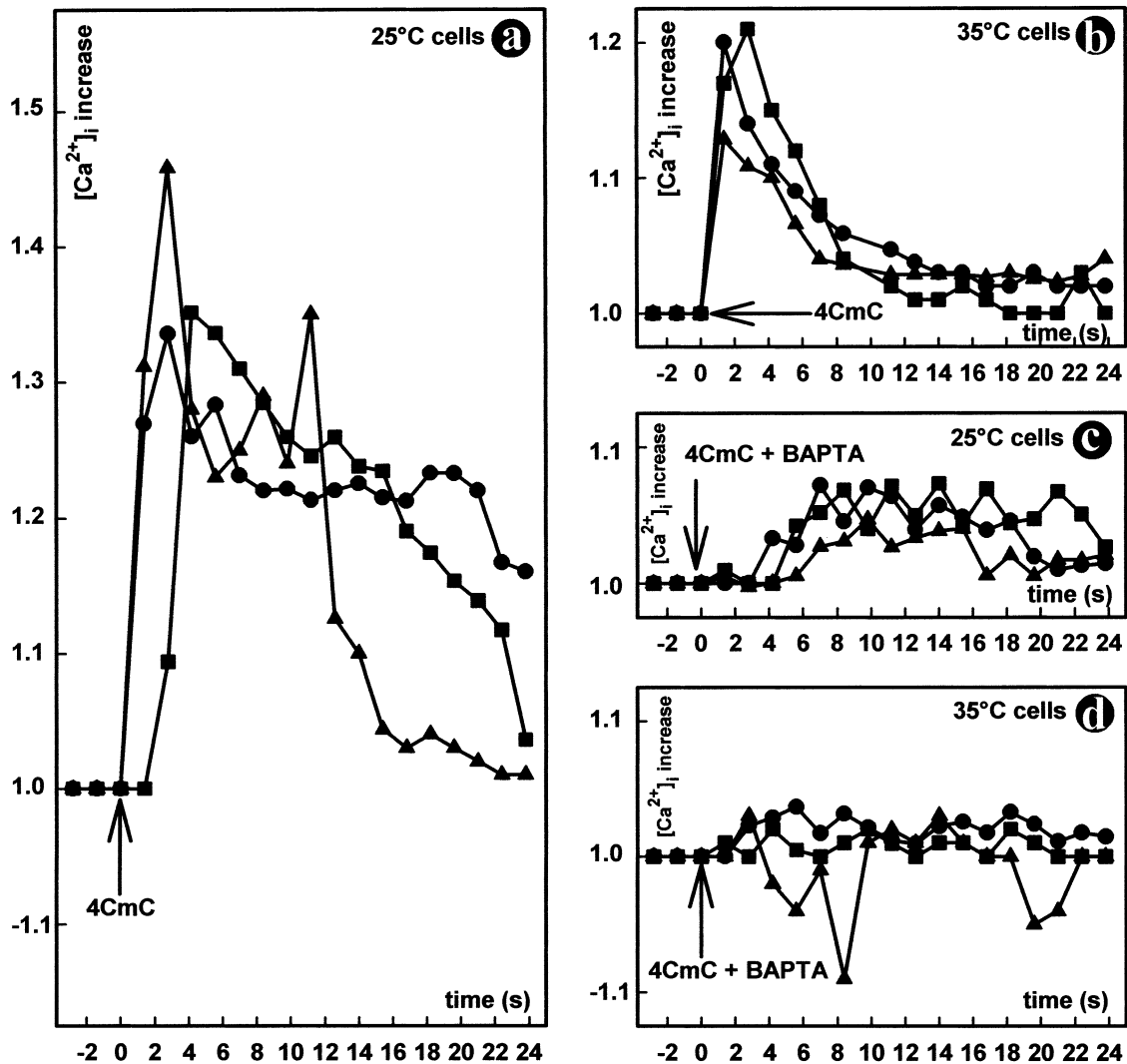
Fig. 5. Time course of cortical $[Ca^{2+}]_i$ signal (Fura Red) close to the site of 0.5 mM 4CmC application at t_0. (*a*)–(*d*) are typical examples, each presenting the reaction of different cells. (*a*, *b*) $[Ca^{2+}]_o = 50$ µM, (*c*, *d*) 1 µM BAPTA in trigger medium; (*a*, *c*) 25°C cells, (*b*, *d*) 35°C cells.

vergent 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), be it with 25°C or 35°C cells. Statistical evaluation contained in the footnote to Table 1 shows *P*-values for peak height differences of 6.1, 16.1, 1.0, and 1.2% for pairs 1*a*/1*c*, 2*a*/2*c*, 5*a*/5*c*, and 6*a*/6*c*, respectively, and 44.8, 84.6, 3.0, and 3.9, respectively, for rise time (pairs 1*b*/1*d*, 2*b*/2*d*, 5*b*/5*d*, and 6*b*/6*d*). The latter data imply wide variation in signal increase with AED, but much less with 4CmC.

As an interim statement, we find that the reaction of pwA-nd12(25°C) cells to the different stimuli is rather similar to wild-type cells (Klauke & Plattner, 1997; 1998; Erxleben et al., 1997; Klauke et al., 2000). This contrasts with the reaction of 35°C cells, which displayed only low Ca^{2+} signals, if any, and no

trichocyst exocytosis under any of the conditions described. How is this possible if one assumes that Ca^{2+} 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), even though there is no occurrence of Ca^{2+} influx in 35°C cells (Kerboeuf & Cohen, 1990)? To elucidate this very intriguing aspect we performed the following experiments.

We analyzed the actual values of total [Ca] in alveolar sacs and the capacity of 25°C and 35°C cells to mobilize Ca^{2+} from alveoli. Cells were rapidly frozen, with or without 10-sec AED stimulation and processed by freeze-substitution under conditions allowing retention of calcium (Hardt & Plattner, 1999; 2000). 10-sec stimulation was applied in order to take into account even slow mobilization, while normally this requires only fractions of a second
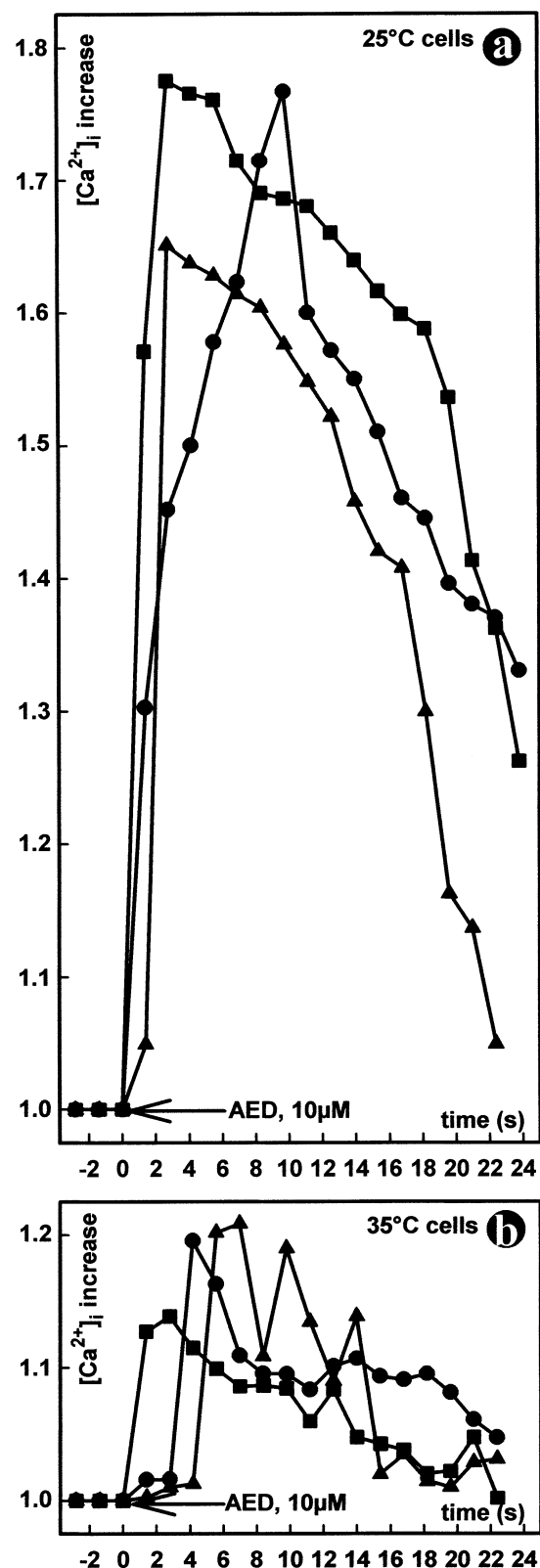
Fig. 6. Time course of $[Ca^{2+}]_i$ transients in Fura Red-loaded double-mutant cells, cultivated at (*a*) 25°C or (*b*) 35°C, upon stimulation with high AED concentration (10 μM) at t_o in presence of $[Ca^{2+}]_o$ = 50 μM. In (*a, b*) typical curves obtained with different cells are presented. Analysis by Fura Red/2λ.

(Hardt & Plattner, 2000), whereas re-uptake during 10 sec is negligible (Plattner & Klauke, 2001). EDX evaluation of net Ca$_{k\alpha}$ peaks revealed that, within statistical limits, Ca content in alveolar sacs is only 11% lower in 35°C than in 25°C cells and that almost the same percentage is mobilized upon stimulation (Table 2), namely ~46% in either case. These differences appear too small to be the only reasons for low $[Ca^{2+}]_i$ increase and failure of exocytosis upon AED stimulation.

Therefore, we looked further for other reasons. We found that basic $[Ca^{2+}]_i^{rest}$ is significantly lower, i.e., by 60%, in 35°C cells than in 25°C cells (Table 3). Even if any $[Ca^{2+}]_i$ increase would occur upon stimulation, threshold $[Ca^{2+}]_i^{act}$ would not be reached. Remarkably, such significantly reduced $[Ca^{2+}]_i^{rest}$ values also occur in another *P. tetraurelia* mutant, tl (Klauke & Plattner, 1997), even when grown at the same temperature as the wildtype. Use of wild-type cells after cultivation at 35°C, for additional controls, is not possible due to their inviability at 35°C.

Finally, we considered sensitivity to $[Ca^{2+}]$ of exocytotic membrane fusion sites. We tested the effects of the Ca^{2+} ionophore, A23187. While this compound induces massive exocytosis, i.e., membrane fusion, contents release and resealing, in wild-type cells (Plattner, 1974), reactivity is increasingly reduced and finally abolished with increasing time after transfer from 25°C to 35°C (Fig. 7). Remarkably, the reaction to the Ca^{2+} ionophore is reduced as with any other trigger agent tested (Figs. 1, 2).

Still, membrane fusion may have occurred, but have remained undetected in the light microscope, just as with "frustrated exocytosis," i.e., membrane fusion without contents release (Klauke & Plattner, 2000). Therefore, we have tested the capacity of trichocyst contents, isolated from 35°C cells, to undergo decondensation (severalfold stretching) when they "see" Ca^{2+}, as is the case after membrane fusion (Bilinski et al., 1981). Decondensation capacity was normal (*data not shown*).

Finally, to monitor membrane fusion capacity directly, we applied cryofixation, either without stimulation or after 5-sec stimulation with AED or with 4CmC (Fig. 8), though 4CmC acts less synchronously than AED. Clearly, no membrane fusion occurs within 5 sec, with very little difference between stimulated and non-stimulated cells. (Any difference within a ~5% limit may be considered of little significance at the EM level.) 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), (Plattner et al., 1997). The reason why fusion does not occur may be manifold, based on specific functions of proteins and/or lipid components that have not yet

Table 1. Summary of $[Ca^{2+}]_i$ changes in 25°C- and 35°C-cells, respectively, upon stimulation with AED or 4CmC

	$[Ca^{2+}]_o = 50\ \mu M$		$[Ca^{2+}]_o = 30\ nM$	
	$[Ca^{2+}]_i$ increase (a)	rise time (sec) (b)	$[Ca^{2+}]_i$ increase (c)	rise time (sec) (d)
AED, 2 μM				
(1) 25°C cells	1.47 ± 0.11	1.50 ± 0.50	1.08 ± 0.05	2.90 ± 0.66
(2) 35°C cells	1.21 ± 0.04	2.53 ± 1.53	1.07 ± 0.03	2.93 ± 0.53
AED, 10 μM				
(3) 25°C cells	1.66 ± 0.01	4.67 ± 1.67	not done	not done
(4) 35°C cells	1.18 ± 0.02	4.53 ± 1.27	not done	not done
4CmC, 0.5 mM				
(5) 25°C cells	1.38 ± 0.04	2.67 ± 0.67	1.03 ± 0.01	5.60 ± 0.09
(6) 35°C cells	1.18 ± 0.02	1.60 ± 0.40	1.03 ± 0.01	4.20 ± 0.87

Indicated are relative f/f_0 increases referred to basal values ($= 1.00$) in the respective cells before stimulation, as well as the rise time (time required to reach maximal f/f_0 values). Values derived from Figs 4–6; $\pm$ SEM; n = 3–4. *P*-values (significance values determined by paired student's *t*-test) were calculated for pairs as specified in the table, like 1a/2a for peak heights (25°C and 35°C cells, respectively, each triggered with 2 μM AED at $[Ca^{2+}]_o = 50\ \mu M$), and like 6b/6d for rise time (35°C cells triggered with 0.5 mM 4CmC at $[Ca^{2+}]_o = 50\ \mu M$ and 30 nM, respectively). $P = 0.0\ \%–100.0\ \%$ are for maximal to minimal significance. *P*-values for peak heights: 1a/2a: 3.0 %; 1a/1c: 6.1 %; 1a/3a: 0.5 %; 1a/5a: 77.8 %; 1c/2c: 65.0 %; 1c/5c: 35.9 %; 2a/2c: 16.1 %; 2a/4a: 16.1 %; 2a/6a: 18.8 %; 2c/6c: 10.1 %; 3a/4a: 0.4 %; 5a/6a: 8.6 %; 5a/5c: 1.0 %; 6a/6c: 1.2 %. *P*-values for rise times: 1b/2b: 42.3 %; 1b/1d: 44.8 %; 1b/3b: 15.9 %; 1b/5b: 13.0 %; 1d/2d: 44.8 %; 1d/5d: 1.5 %; 2b/2d: 84.6 %; 2b/4b; 85.0 %; 2b/6b: 65.0 %; 2d/6d: 10.1 %; 3b/4b: 82.2 %; 5b/6b: 5.7 %; 5b/5d: 3.0 %; 6b/6d: 3.9 %. For any further interpretation, also of *P*-values, *see* text.

Table 2. EDX analysis of [Ca] in alveolar sacs of pwA-nd12 cells, net $Ca_{k\alpha}$ counts (normalized to value obtained for unstimulated 25°C cells $= 100\%$)

	(1)	(2)	(3)	(4)
	25°C, −AED	35°C, −AED	25°C, +AED	35°C, +AED
Mean	100.0 %[1]	88.9 %[1]	53.5 %[1]	54.3 %[1]
SEM	±8.9	±7.8	±7.8	±7.7
N	15	15	15	15
n	50	50	50	50
Significance 95%	(1) --------------- (−) -------------- (2)		(3) --------------- (−) -------------- (4)	
	(1) --------------- (+) -------------- -------------------- -------------------- (3)			
		(2) --------------- (+) -------------- -------------------- -------------------- (4)		

Stimulated cells were exposed to AED 10 sec, quick frozen and processed as described by Knoll et al. (1993) for EDX analysis of Ca in alveolar sacs as described by Hardt and Plattner (1999, 2000). SEM = standard error of the mean, *N* = number of cells analyzed, n = number of alveolar sacs analyzed. Significance +/− (yes/no) according to paired Student's *t*-test.
[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 carfully controlled calibration in unstimulated wild-type cells has shown in alveolar sacs total [Ca] = 43 mM (Hardt & Plattner, 1999, 2000).

Table 3. Relative values of $[Ca^{2+}]_i^{rest}$ determined in pwA-nd12(25°C) and pwA-nd12(35°C) cells using Fura Red

	Relative $[Ca^{2+}]$ Signals	
	25°C-Cells	35°C-Cells
Average	100 %[1]	59 %[1]
SD	±8	±7

[1]The f/f_0 ratios obtained from cortical regions by dual wavelength recordings in randomly taken cells, evaluated as indicated in Methods, yield values independent of cell geometry (thickness). To facilitate comparison, data are normalized to the value obtained for 25°C cells (100 %). Number of 25°C and 35°C cells analyzed = 10 each. SD = standard deviation. Data are significantly different, according to *t*-test, on a 99 % level.

been identified in *Paramecium*, just as in any other secretory system.

A summary of $[Ca^{2+}]_i$ and exocytotic responses in the double mutant, in comparison to wild-type cells and some other *P. tetraurelia* mutants, is contained in Table 4.

Discussion

CHARACTERISTICS OF 35°C CELLS

35°C cells have only slightly reduced total [Ca] in alveolar sacs and only slightly reduced Ca^{2+} mobili-
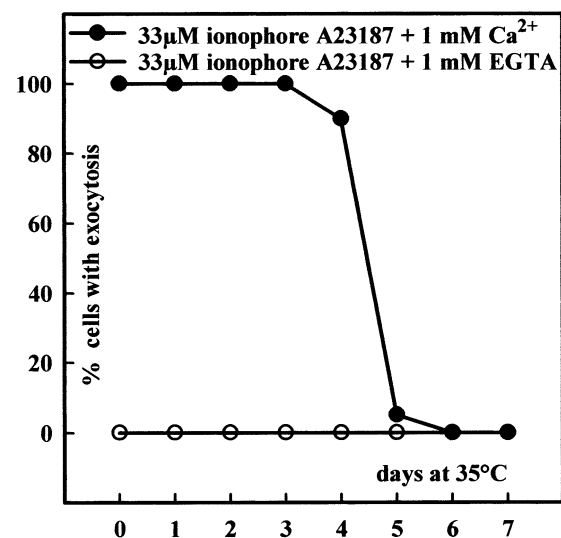
Fig. 7. Change of exocytosis performance in response to iono-phore A23187 ($+/-$ Ca^{2+} added) upon transfer of pwA-nd12 cells from 25°C to 35°C.

Fig. 8. Freeze-fracture analysis of pwA-nd12(35°C) cells, stimulated for 5 sec with either 2 µM AED or 0.5 µM 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* Materials and Methods. 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 of early and late resealing stages, respectively), as well as stages intermediate between openings and resealings. 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).

zation from these stores when stimulated with AED or with 4CmC. More significant is the considerably lower [Ca^{2+}]$_i^{rest}$ in 35°C cells, which may prevent them from reaching [Ca^{2+}]$_i^{act}$ 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 (*data not shown*) may also indicate heat-induced down-regulation of different functions, while cells remain fully viable (Fig. 1).

ASPECTS OF Ca^{2+} DYNAMICS DURING EXOCYTOSIS STIMULATION

In *Paramecium*, Ca^{2+} mobilization from alveolar sacs (Erxleben & Plattner, 1994; Stelly et al., 1995; Hardt & Plattner, 2000) normally is superimposed by a strong Ca^{2+} influx from the medium (Kerboeuf & 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 & Klauke, 2001). Analysis of pwA-nd12(35°C) cells in comparison to 25°C cells appeared a feasible way to further dissect the individual steps possibly involved in Ca^{2+} dynamics during trichocyst exocytosis. Cells are devoid of any functional ciliary Ca^{2+} influx channels after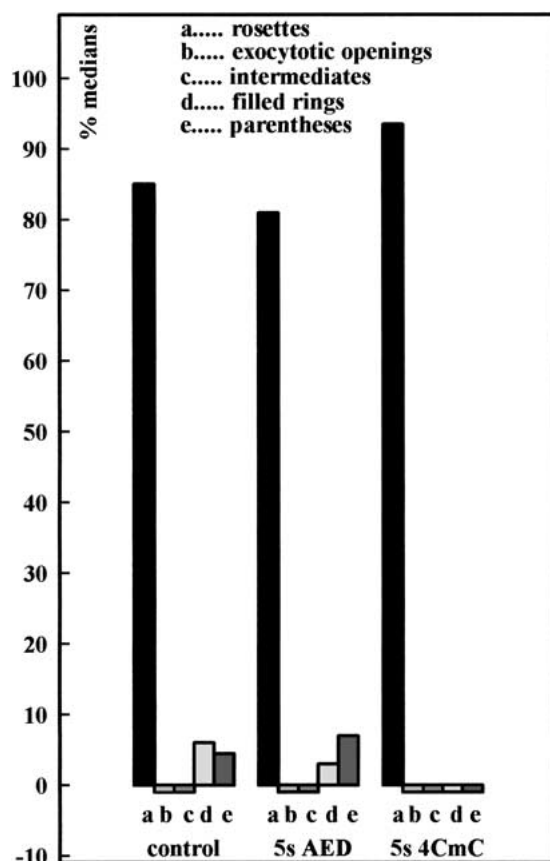 culturing at 25°C or at 35°C, while somatic Ca^{2+} influx channels are functional only in 25°C cells (Kerboeuf & Cohen, 1990). Since EDX data obtained with 35°C cells show Ca^{2+} mobilization from stores in the absence of any Ca^{2+} influx, we consider this as stringent evidence of a SOC-type mechanism involving (i) activation of a CaSR in the cell membrane, (ii) activation of Ca^{2+}-release channels in alveolar sacs, and — in 25°C 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 & Schofield, 1996; Quinn et al., 1997; Vassilev et al., 1997, Adebanjo et al., 1998; Yamaguchi et al., 1998; Oda et al., 2000;

Table 4. Qualitative comparison of parameters relevant for $[Ca^{2+}]_i$ signal generation during trichocyst exocytosis

Stimulation conditions/strain	Cortical $[Ca^{2+}]_i$ signal		Excocytosis	
	$+ Ca_o^{2+}$	$- Ca_o^{2+}$	$+ Ca_o^{2+}$	$- Ca_o^{2+}$
AED				
7S (wildtype situation)	+ + +	+ to + +	+ + +	−
nd9–18°C	+ + +	+ to + +	+ + +	−
nd9–28°C	+ + +	+ to + +	−	−
pwA–nd12(25°C)	+ + +	+ to + +	+ + +	−
pwA–nd12(35°C)	+ to + +	+	−	−
4CmC				
7S wild-type (*see* text)	+ +	+ + +	+ +	−
pwA–nd12(25°C)	+ +	+	+ +	−
pwA–nd12(35°C)	+ to + +	+	−	−

$+/- Ca_o^{2+}$ means standard (50 μM) or reduced (30 nM) conditions. Exocytosis is scaled $+$, $+ +$ and $+ + +$, indicating weak, medium or strong response, based on quantitative work cited for 7S and nd9 cells and on present observations. The minus sign signifies no exocytosis. A similar scale is used for the cortical $[Ca^{2+}]_i$ signal. Data for 7S and nd9–28°C cells are from Klauke and Plattner (1997). Trichocyst contents from all specimens were able to undergo "decondensation" (*see* text).

Brown & MacLeod, 2001) where they can induce $[Ca^{2+}]_i$ transients by different mechanisms even in cells not participating in the regulation of $[Ca^{2+}]_o$ homeostasis (Chattopadhyay & 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 & Plattner, 1994).

Our EDX data also imply that the CaSR is not defective in 35°C cells. We also consider the functional state of Ca^{2+} release channels in alveolar sacs in the 35°C cells normal. 4CmC is an established activator of ryanodine-type Ca^{2+}-release channels even in the case of low ryanodine sensitivity (Zorzato et al., 1993; Herrmann-Frank et al., 1996; Westerblad et al., 1998; Kabbara & Allen, 1999). This is precisely the case with alveolar sacs of *Paramecium* (Länge, Klauke & Plattner, 1995) which, like some ryanodine-sensitive type Ca^{2+}-release channels, are also sensitive to caffeine (Erxleben & Plattner, 1994; Klauke & Plattner, 1998), just as we find here (*not shown*). Low or apparently absent Ca^{2+} signals in 35°C cells during stimulation in presence of BAPTA can be explained by the lower basal $[Ca^{2+}]_i$ in 35°C cells (*see* Results). Along these lines we excluded occurrence of any weaker signals of short duration by fast CLSM analyses (*not shown*).

Possible Site of Ca^{2+} Influx Disturbances

We explain the absence of $^{45}Ca^{2+}$ influx and of trichocyst exocytosis in 35°C cells as follows: (i) Reduced $[Ca^{2+}]_i^{rest}$ may be considered one such defect. (ii) Membrane fusion is impaired, as discussed above. (iii) The main defect responsible for the disturbed Ca^{2+} dynamics in 35°C cells most likely concerns the Ca^{2+} influx channels. Which one of the potential channels (Machemer, 1988; Preston, 1990) may be affected? In a previous paper we already have found

some evidence of a SOC-type mechanism in normal *Paramecium* cells (Klauke et al., 2000), particularly since store depletion at reduced $[Ca^{2+}]_o$, followed by re-addition of Ca^{2+} to the medium, greatly accelerates Ca^{2+} influx. In *Paramecium*, we also assume the intitial involvement of a CaSR, for reasons outlined in the Introduction. We now can exclude more stringently a CICR-type mechanism, based on our EDX data, which show that AED causes a Ca^{2+} release from alveolar sacs also in 35°C cells, which are devoid of any Ca^{2+} influx. While a SOC-type mechanism is established for a variety of higher eukaryotic cells (Cheek et al., 1993; Berridge, 1997; Barritt, 1998; 1999; Mackrill, 1999), the type of plasmalemmal Ca^{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 we assume for *Paramecium* (Klauke et al., 2000). Some influx channels could operate in register with Ca^{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 we derive from our previous Mn^{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^{2+} channel activation can be quite low. For instance, Ca^{2+}-mediated signaling can be activated in vertebrate cells even with positively charged latex beads (Zhu & Peng, 1988), and this can also induce trichocyst exocytosis in *Paramecium* (Plattner et al., 1985). According to electrophysiology, such unspecific plasmalemmal Ca^{2+}-conducting channels do occur in *Paramecium* (Saitow et al., 1997). Hyperpolarization-sensitive Ca^{2+} channels also have been taken into consideration to explain Ca^{2+} influx during AED stimulation (Cohen & Kerboeuf, 1993; Kerboeuf & Cohen, 1996).

Conclusions

First, we confirm that nd12 cells cultivated at 35°C exhibit normal ultrastructure of exocytosis sites (Pouphile et al., 1986; Vayssié et al., 2000), yet lack any significant Ca^{2+} influx upon exposure to AED (Kerboeuf & 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.

Table 4 compares [Ca^{2+}]$_i$ signals and exocytotic responses in different strains, i.e., 7S, nd9–18°C and nd9–28°C as well as pwA-nd12 (25°C) and pwA-nd12 (35°C). 7S and nd9–18°C or nd12–25°C reflect the normal exocytosis-competent situation (Pouphile et al., 1986; Vayssié et al., 2000). The molecular background of exocytosis incompetence in nd9 (Beisson et al., 1980; Froissard et al., 2001) is different from the one that we deduce for nd12 (this paper), but both result in an nd phenotype. 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.

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