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Caffeine-Induced Ca^{2+} Transients and Exocytosis in *Paramecium* Cells. A Correlated Ca^{2+} Imaging and Quenched-Flow/Freeze-Fracture Analysis

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Abstract. Caffeine causes a $[Ca^{2+}]_i$ increase in the cortex of *Paramecium* cells, followed by spillover with considerable attenuation, into central cell regions. From $[Ca^{2+}]_i^{rest}$ ~50 to 80 nM, $[Ca^{2+}]_i^{act}$ rises within ⩽3 sec to 500 (trichocyst-free strain tl) or 220 nM (nondischarge strain nd9–28°C) in the cortex. Rapid confocal analysis of wildtype cells (7S) showed only a 2-fold cortical increase within 2 sec, accompanied by trichocyst exocytosis and a central Ca^{2+} spread during the subsequent ⩾2 sec. Chelation of Ca_o^{2+} considerably attenuated $[Ca^{2+}]_i$ increase. Therefore, caffeine may primarily mobilize cortical Ca^{2+} pools, superimposed by Ca^{2+} influx and spillover (particularly in tl cells with empty trichocyst docking sites). In nd cells, caffeine caused trichocyst contents to decondense internally (Ca^{2+}-dependent stretching, normally occurring only after membrane fusion). With 7S cells this usually occurred only to a small extent, but with increasing frequency as $[Ca^{2+}]_i$ signals were reduced by $[Ca^{2+}]_o$ chelation. In this case, quenched-flow and ultrathin section or freeze-fracture analysis revealed dispersal of membrane components (without fusion) subsequent to internal contents decondensation, opposite to normal membrane fusion when a full $[Ca^{2+}]_i$ signal was generated by caffeine stimulation (with $Ca^{2+}{}_i$ and Ca_o^{2+} available). We conclude the following. (i) Caffeine can mobilize Ca^{2+} from cortical stores independent of the presence of Ca_o^{2+}. (ii) To yield adequate signals for normal exocytosis, Ca^{2+} release and Ca^{2+} influx both have to occur during caffeine stimulation. (iii) Insufficient $[Ca^{2+}]_i$ increase entails caffeine-mediated access of Ca^{2+} to the secretory contents, thus causing their decondensation before membrane fusion can occur. (iv) Trichocyst decondensation in turn gives a signal for an unusual dissociation of docking/fusion components at the cell membrane. These observations imply different threshold $[Ca^{2+}]_i$-values for membrane fusion and contents discharge.

Key words: Caffeine — Calcium — Exocytosis — *Paramecium* — Secretion

Introduction

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 & Campbell, 1993; Meissner, 1994; Fryer & Stephenson, 1996) or endoplasmic reticulum, ER, in neuronal or secretory cells (Bezprovanny, Watras & Ehrlich, 1991; McPherson et al., 1991; Friel & Tsien, 1992; Cheek et al., 1993; Simpson, Nahorski & Challiss, 1996). However, not all of these stores are sensitive to caffeine (Schmid et al., 1990; Berridge, 1993; Cheek & Barry, 1993; Lynn & Gillespie, 1995). After detection of cyclic adenosine-diphosphoribose (cADPR) as a physiological activator of ryanodine-sensitive Ca^{2+} pools in some secretory cells (Galione, 1994; Lee, 1994), some of the stores proved less or not sensitive to caffeine (Galione, Lee & Busa, 1991; Buck, Rakow & Shen, 1992; Verma et al., 1996), while some caffeine-activated stores were insensitive to cADPR (Meissner, 1994). Thus, cADPR may be considered a physiological equivalent of ryanodine (Galione, 1994) and one may have to take into account some variation among caffeine-sensitive Ca^{2+}-release channels.

Strikingly tens of millimolar of caffeine are required to activate most systems (Cheek et al., 1993; Verkhratsky & Shmigol, 1996) even though caffeine rapidly penetrates cells (Bianchi, 1962; O'Neill, Donoso & Eisner, 1990; Toescu et al., 1992) and, thus, can become active within seconds. Besides mobilization of intracel-

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lular Ca^{2+} pools, a variety of other effects have been discovered with caffeine, e.g., activation (Steenbergen & Fay, 1996) or inhibition of Ca^{2+} sequestration (Chapman & Tunstall, 1988; Bassani, Bassani & Beers, 1994) or Ca^{2+} release (Missiaen, Taylor & Berridge, 1992) as well as some other unrelated effects (Gupta et al., 1990; Berridge, 1991; Sawynok & Yaksh, 1993; Tanaka & Tashjian, 1993; Combettes, Berthon & Claret, 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.

Paramecium cells contain extensive cortical Ca^{2+} stores, the ''alveolar sacs'' (Stelly et al., 1991; Knoll et al., 1993; Länge, Klauke & Plattner 1995) which are tightly attached at the cell membrane (Plattner et al., 1991) and endowed with calsequestrin-like Ca^{2+}-binding protein (Plattner et al., 1997*b*). In addition, *Paramecium* contains abundant ER, with calreticulin-like Ca^{2+}-binding protein, throughout the cell body (Plattner et al., 1997*b*). Circumstantial evidence suggests a major role of alveolar sacs in stimulus-secretion coupling (Erxleben & Plattner, 1994; Stelly et al., 1995; Erxleben et al., 1997; Klauke & Plattner, 1997), although they neither show any response to inositol-tris-phosphate ($InsP_3$), cADPR or ryanodine, nor any indication of Ca^{2+}-induced Ca^{2+}-release, CICR (Länge et al., 1995; Zhou et al., 1995).

Using Ca^{2+}-sensitive fluorochromes, we now show that caffeine causes rapid increase of intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, first in the cortex and then, to a smaller extent, also in the cell center. Since a cortical $[Ca^{2+}]_i$ increase also occurs without extracellular Ca^{2+}, $Ca^{2+}{}_o$, caffeine may mobilize Ca^{2+} from cortical stores and, thus, induce a superimposed Ca^{2+} influx. However, we also recognized a caffeine-induced Ca^{2+} influx into secretory organelles (''trichocysts'') in our system, causing their intracellular decondensation (i.e., severalfold stretching of their rod-shaped secretory contents) without membrane fusion. This is due to the fact that trichocyst contents require Ca^{2+} for decondensation during expulsion (Bilinski, Plattner & Matt, 1981), as confirmed independently (Kerboeuf & Cohen, 1990; Chilcoat et al., 1996). This is opposite to most other secretory organelles, like chromaffin granules (Winkler, 1993), whose contents need abundant Ca^{2+} in tightly bound form at rest, i.e., to maintain the condensed state (Nicaise et al., 1992). In contrast, when the trichocyst matrix ''sees'' Ca^{2+}, even in submicromolar concentrations, they undergo explosive decondensation. The basis of this very fast expulsion process is paracrystalline arrangement (Sperling, Tardien & Gulik-Krzywicki, 1987) of ''trichynin'' proteins (Steers, Beisson & Marchesi, 1969), a collection of ~100 gene products of remarkable similarity (Gautier, Sperling & Madeddu, 1996), with the capability of rapid Ca^{2+}-induced rearrangement. This normally occurs only after access of Ca^{2+} through an exocytotic opening. However, as we show, caffeine can induce matrix stretching *in situ*, when cortical $[Ca^{2+}]_i$ increase is too small to induce previous membrane fusion, by mediating Ca^{2+} entry into trichocysts.

Our data reflect the different Ca^{2+} requirements for membrane fusion ($\leqslant$10 μM, Klauke & Plattner, 1997) and trichocyst contents decondensation (sub-μM, this paper). Under normal conditions, caffeine can clearly activate cortical Ca^{2+} stores, cause a superimposed Ca_o^{2+} influx, and normal exocytosis, i.e., membrane fusion and contents extrusion in *Paramecium* cells.

Materials and Methods

Paramecium tetraurelia cells were cultivated and used for Ca^{2+} imaging and quenched-flow analyses as indicated previously (Erxleben et al., 1997; Klauke & Plattner, 1997; Plattner, Braun & Hentschel, 1997*a*). We used wildtype (7S) cells, eventually in axenic cultures for the isolation of trichocysts (*see below*), nondischarge strain nd9 grown at a nonpermissive temperature of 28°C (Beisson et al., 1976), trichocyst-free strain ''trichless,'' tl (Pollack, 1974), and strains tam38 and tam6, with smaller or larger numbers of always nondocked trichocysts floating free in the cytoplasm (Pouphile et al., 1986).

For $[Ca^{2+}]_i$ measurements we injected cells with Ca^{2+}-sensitive fluorochromes from Molecular Probes (Eugene, OR), dissolved in 10 mM Tris-HCl, pH 7.2, according to methods previously described. Briefly, 50 μM Fura Red (final intracellular concentrations), 100 μM Calcium Green-2 or Fluo-3 were injected. After 2 min, cells were stimulated by a flush of caffeine, 40–50 mM, occasionally with 100 μM fluorescein added to visualize contact with a cell, i.e., for precise timing. $[Ca^{2+}]_o$ usually was 50 μM, unless indicated otherwise. For technical details for fluorescence analysis, *see* Erxleben et al. (1997) and Klauke & Plattner (1997). Essentially this was done by conventional double wavelength recordings or by single wavelength recordings in a confocal laser scanning microscope (CLSM), operated with a fast optoacoustic beam deflection system, from Noran (Bruchsal, Germany). The latter was used to analyze 7S cells, contained in a small droplet to reduce mobility, with high time resolution since otherwise their analysis would be impaired by swimming and rapid dislocation during secretion. Though this is possible only in the single wavelength mode, we have shown the feasibility of this approach (Erxleben et al., 1997). In alternating transmitted light or differential interference contrast (DIC, Nomarski) pictures, we monitored exocytosis or artificial internal decondensation (stretching) of trichocyst contents. Before caffeine application, some nd9 cells were injected with $MgCl_2$ or BAPTA (see text), in some we detached intact trichocysts from the cell surface by cytochalasin B treatment as described (Pape & Plattner, 1990).

Quenched-flow and freeze-fracture or freeze-substitution (for ultrathin sectioning) analysis was performed as described previously (Knoll, Braun & Plattner, 1991; Plattner et al., 1997*a*). Samples with caffeine application of $\leqslant$1 sec were produced in the quenched-flow apparatus (Knoll et al., 1991), others by manual mixing, and quenching in melting propane (86K). Some samples were exposed, also in the quenched-flow apparatus, for 0.5 sec to EGTA to produce free $[Ca^{2+}] \leqslant 50$ nM as indicated previously (Plattner et al., 1997*a*), before applying caffeine. On replicas, trichocyst docking sites were classified in different categories, like ''resting'' stages with a ''rosette'' of intramembraneous particles (IMPs, indicative of an extrudable trichocyst), membrane fusions (minifusions or larger openings), resealing stages

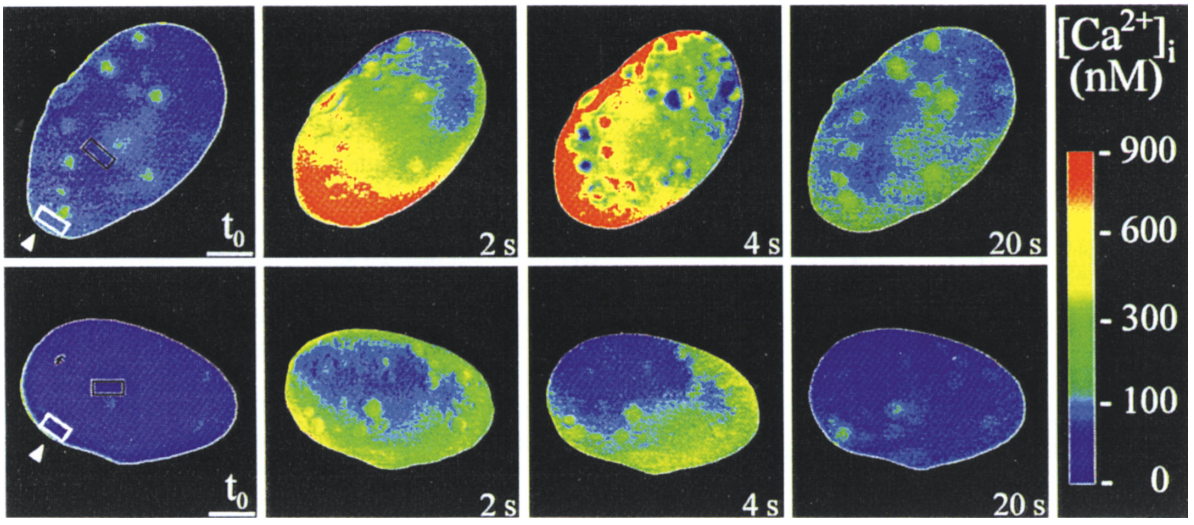

Fig. 1. Fura Red-injected tl cell triggered with caffeine (at arrowhead) at t_o, either in presence of 50 μM Ca_o^{2+} (*top*) or in absence of Ca_o^{2+} (1 mM EGTA for 1 min, *bottom*) using different cells. Framed areas indicate position of fields evaluated in several cells in Fig. 3. Globular structures are vacuoles. Bars = 20 μm.

(''filled rings'') or ''parentheses'' (empty docking sites), as specified by Plattner, Knoll & Pape, 1993; Plattner et al., 1997*a*). We found a new stage, called ''dispersed rosettes,'' indicating internal trichocyst decondensation by caffeine-induced Ca^{2+} entry (*see* Results and summarizing scheme in the Discussion).

Trichocysts with intact membranes were isolated from axenic 7S cells in a medium composed of 5 mM Pipes buffer pH 7.0 plus 0.5 mM $MgCl_2$ and 0.15 mM NaCl according to Glas-Albrecht and Plattner (1990). Different concentrations of EGTA were added to yield an estimated free $[Ca^{2+}]$ of 10, 80 or 330 nM, respectively, as calculated according to Föhr, Warchol & Gratzl (1993) and controlled by Fura Red, before exposure to 40–50 mM caffeine. In vivo this would correspond to values below, close to, or above resting $[Ca^{2+}]_i$ levels, respectively, as occurring in different cell regions during caffeine stimulation (*see* Results). Under these conditions trichocysts were analyzed under phase contrast to follow decondensation reaction in response to caffeine. For different stages of trichocysts, *see* summarizing scheme in the last figure.

Results

Caffeine-induced $[Ca^{2+}]$ Transients

First we again ascertained equal distribution of injected fluorochromes throughout the cells. Since this has been shown previously to occur within ≤2 min (Klauke & Plattner, 1997) we allowed this time to elapse before we continued our experiments.

We started with tl cells (Fig. 1) because they contain no trichocysts whose release can displace a cell and thus impair double wavelength analyses. Furthermore, even small $[Ca^{2+}]_i$ transients would be more easily recognized since free docking sites would facilitate diffusion in tl cells.

In tl cells, bathed in the usual $[Ca^{2+}]_o$ of 50 μM, caffeine induces a strong Fura Red-signal within 2 sec, i.e., the time required for filter changes. $[Ca^{2+}]_i$ first increases at the site of caffeine application, then spreads along the superfused cortex and, with some attenuation, inside the cell (Fig. 1). After 4 sec, the signal spreads further, with the exclusion of larger vacuoles, before it decays within 20 sec. Figure 1 (*top*) also shows some fluorochrome sequestration in large vacuoles. With EGTA added to the medium, to yield $[Ca^{2+}]_o$ ≤50 nM (as measured with Fura Red), a Ca^{2+} signal occurs without delay, but it is weaker and largely restricted to the cortex (Fig. 1, *bottom*). The Fura Red signals of whole cells (Fig. 2) clearly show an overall $[Ca^{2+}]_i$ increase in presence of Ca_o^{2+}, while data scatter obscures any global $[Ca^{2+}]_i$ increase in the absence of Ca_o^{2+} added. When we selectively evaluated cortical regions (at sites of caffeine application) and randomly defined central regions we find that the signal increase with Ca_o^{2+} added is stronger, particularly in cortical regions (Fig. 3*a*), than without Ca_o^{2+} added (Fig. 3*b*), though the signal, up to 230 nM over a ~8 μm broad zone at the site of caffeine application, is not significantly delayed in the latter case. Application of solutions without a trigger agent yields no signal (Erxleben et al., 1997, Klauke & Plattner, 1997), thus excluding mechanical stimulation.

For reasons indicated above we were restricted, in the analysis of wildtype (7S) cells, to single wavelength recordings by fast CLSM after injection of Calcium Green-2. Figure 4 (*top*) shows again that the fluorochrome signal first increases close to the site of caffeine application (0.4 sec), before it spreads all over the cell. This signal increase is weaker without Ca_o^{2+} added (Fig.

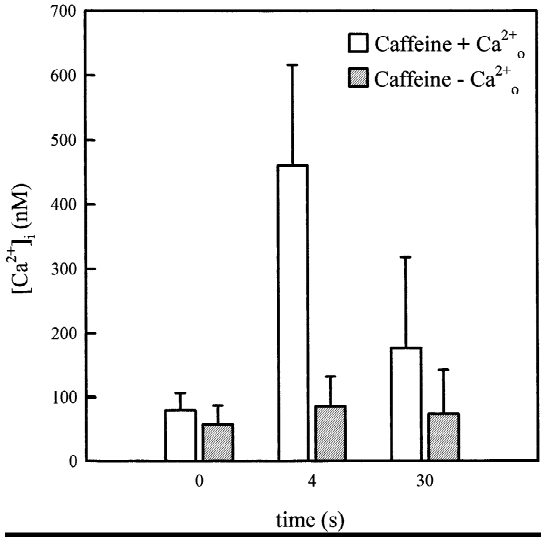

Fig. 2. Global $[Ca^{2+}]_i$ changes measured in tl cells after caffeine triggering in presence of $[Ca^{2+}]_o$ = 50 μM (open columns, n = 5) or in absence of Ca_o^{2+} (hatched columns, n = 4) as in Fig. 1. Bars = standard errors (SE).

4, *bottom*). With or without Ca_o^{2+} added, respectively, whole cell analysis shows, from 2 sec on, a significant increase of the signal measured as f/f_o, i.e., by relating the signal to that at t_o (Fig. 5), as specified previously (Erxleben et al., 1997). In the example shown in Fig. 4 and evaluated in Fig. 6, $[Ca^{2+}]_i$-increase is stronger in the presence of Ca_o^{2+} than in its absence. This difference may be less pronounced in other cells, as one can derive from statistical analysis in Fig. 5. We then compared cortical and central values obtained with or without Ca_o^{2+} added, respectively (*see* Fig. 6*a* and *b* for typical examples). This analysis reveals (i) a rapid signal increase in the cortex in either case, (ii) some delay in central increase and (iii) a reduced increase without Ca_o^{2+} added.

Thus, CLSM data obtained with 7S cells are compatible with double wavelength recordings with strain tl (*see above*) or nd9–28°C (*see below*). They indicate mobilization of a cortical pool, superimposed by Ca^{2+} influx and signal spread throughout the cell, regardless whether a cell is capable of performing exocytosis or not. The occurrence of exocytosis in 7S cells in response to caffeine has been ascertained by DIC microscopy (Fig. 7) showing decondensation of trichocyst contents as they leave the cell as a consequence of Ca^{2+}-mediated membrane fusions.

Next we analyzed Fura Red-injected nd9–28°C cells, i.e., nondischarge cells cultivated at a nonpermissive temperature, by double wavelength recordings (Figs. 8 to 10). Such cells maintain their inability to perform exocytotic membrane fusion for several hours, also during analysis at ambient temperature, although their trichocyst contents are capable of decondensation (Beisson et al., 1976; Pouphile et al., 1986). In principle, Ca^{2+} responses are as described for tl cells, but the signal and its spread is much weaker, with or without Ca_o^{2+} added. One possible explanation may be restricted Ca^{2+} diffusion due to occupation of docking sites by trichocysts. The rise of $[Ca^{2+}]_i$ in nd9–28°C cells could also be more moderate than in tl cells because of Ca^{2+} binding during caffeine-induced internal trichocyst decondensa-

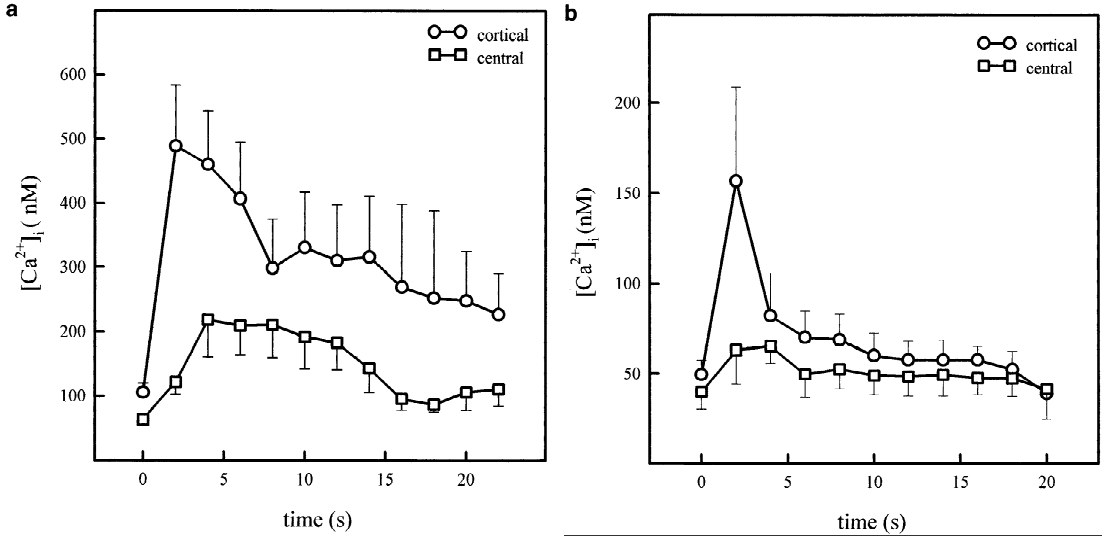

Fig. 3. $[Ca^{2+}]_i$ transients determined in the cortex and in central regions (as indicated in Fig. 1) of tl cells after caffeine stimulation, either in presence of 50 μM Ca^{2+} (*a*) or in absence of Ca^{2+} (1 mM EGTA for 1 min) from the medium (*b*). Bars = standard deviation (SD), n = 5 (*a*) or 4 (*b*).

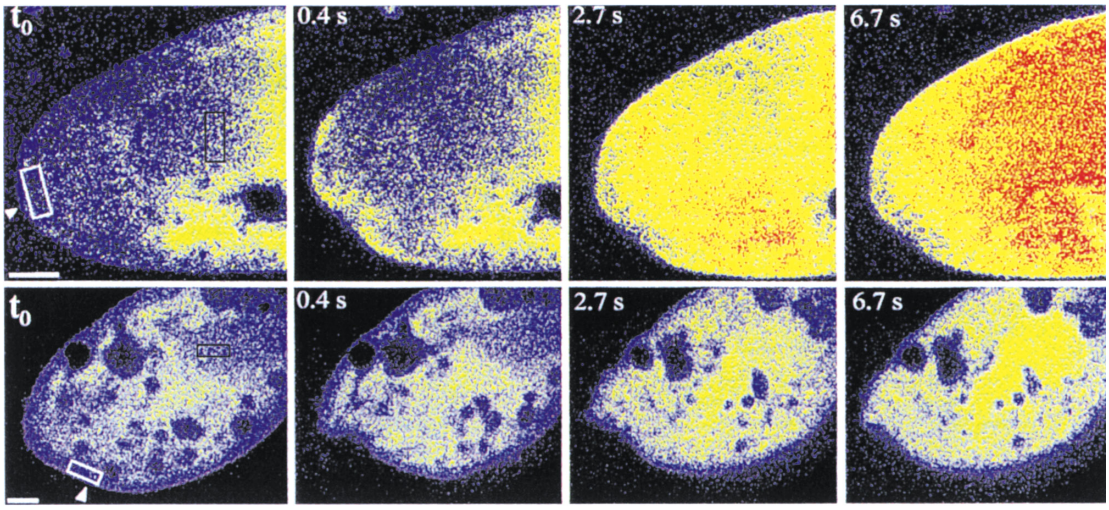

Fig. 4. Calcium Green-2 injected 7S cells stimulated at t_o with caffeine (at arrowhead), one cell in presence of 50 μM Ca^{2+} (*top*) and another cell in presence of EGTA (1 min) in the medium to chelate Ca_o^{2+} below resting $[Ca^{2+}]_i$ level (bottom). Evaluation by CLSM. Frames indicate evaluations in Fig. 6. Globular structures are vacuoles. Bars = 10 μm.

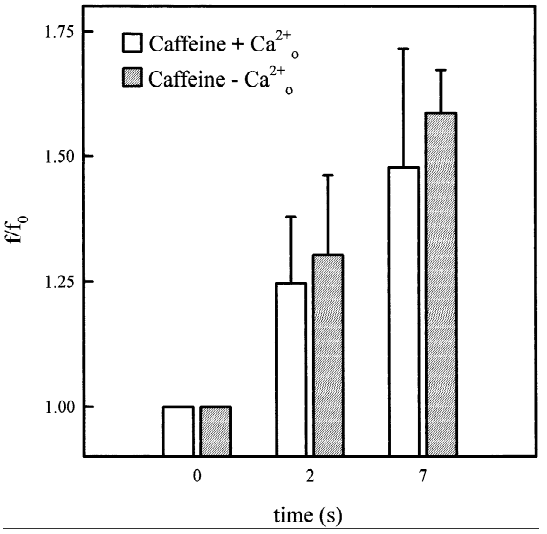

Fig. 5. Overall $[Ca^{2+}]_i$ changes in 7S cells following caffeine application in presence or absence of Ca_o^{2+} as indicated in Fig. 4. Different cells were used for the two sets of experiments. Bars = SD for $n = 3$ ($+Ca_o^{2+}$) or $n = 4$ ($-Ca_o^{2+}$).

tion (*see* Introduction and below). Without Ca_o^{2+} added, the cortical increase is variable and no central $[Ca^{2+}]_i$ rise is recognized (Figs. 8 bottom and Fig. 10*b*).

Normal Exocytosis Vs. Internal Trichocyst Decondensation

During our analyses with nd9–28°C cells we become aware of internal decondensation of trichocysts in response to caffeine (Figs. 11 to 13). Thereby contents explosively stretch as they would physiologically only during exocytosis. This normally implies membrane fusion and release of contents, by vigorous elongation, into the medium. The cell shown in Fig. 11 has been injected with Fluo-3 before stimulation by caffeine and analyzed by CLSM, recording fluorescence and alternating transmitted light pictures. We show internal trichocyst decondensation after $[Ca^{2+}]_i$ increase in response to caffeine. To document the relevance of contact of the cell with caffeine, the caffeine solution has been supplemented with fluorescein in Fig. 12. (This also proves indirectly that the intracellular fluorescence signal recorded in Fig. 11 is not due to caffeine). Evidently $[Ca^{2+}]_i$ increase entails internal trichocyst decondensation in nd9–28°C cells (which are unable to form exocytotic openings as required for external decondensation). Only docked trichocysts are liable to internal decondensation, opposite to ''free'' trichocysts, as documented at higher magnification in Fig. 13 (*top*). When docked trichocysts are experimentally detached from the cell surface in their normal, condensed form (*see* Materials and Methods), they no longer respond to caffeine by internal decondensation (Fig. 13, *bottom*).

Finally we have extended these analyses to different strains at different $[Ca^{2+}]_o$. This again showed that only docked, but not free trichocysts are liable to internal decondensation (Table 1). Microinjected Mg^{2+} can inhibit internal decondensation, as can the Ca^{2+}-chelator BAPTA.

We occasionally observed internal trichocyst decondensation also with 7S cells, depending on the analysis conditions, i.e., depending on concentration and time of

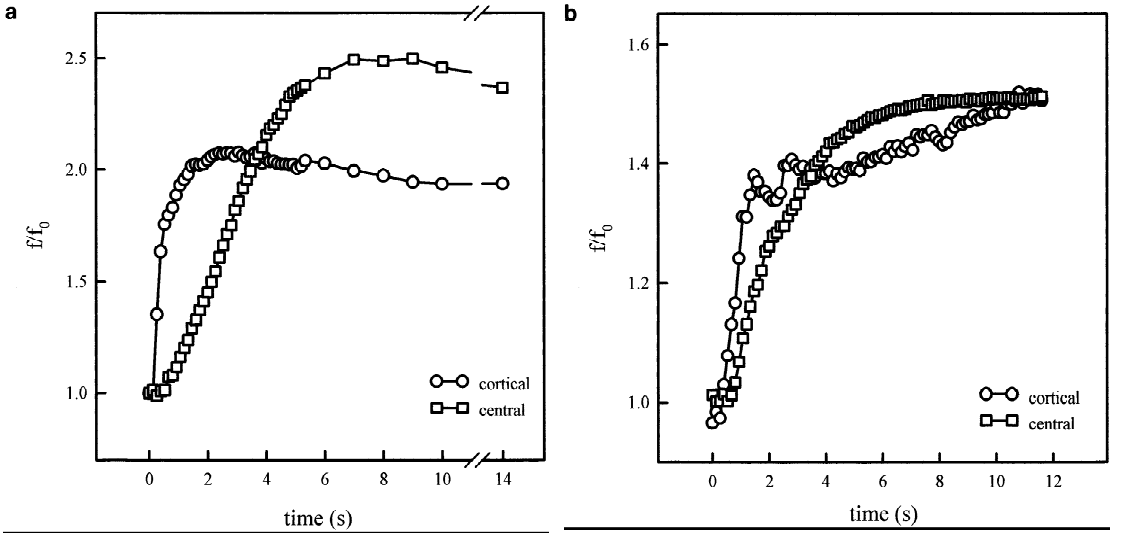

Fig. 6. Typical examples of cortical and central $[Ca^{2+}]_i$ transients in the 7S cells shown in Fig. 5, either in presence (*a*) or absence of Ca_o^{2+} (*b*).

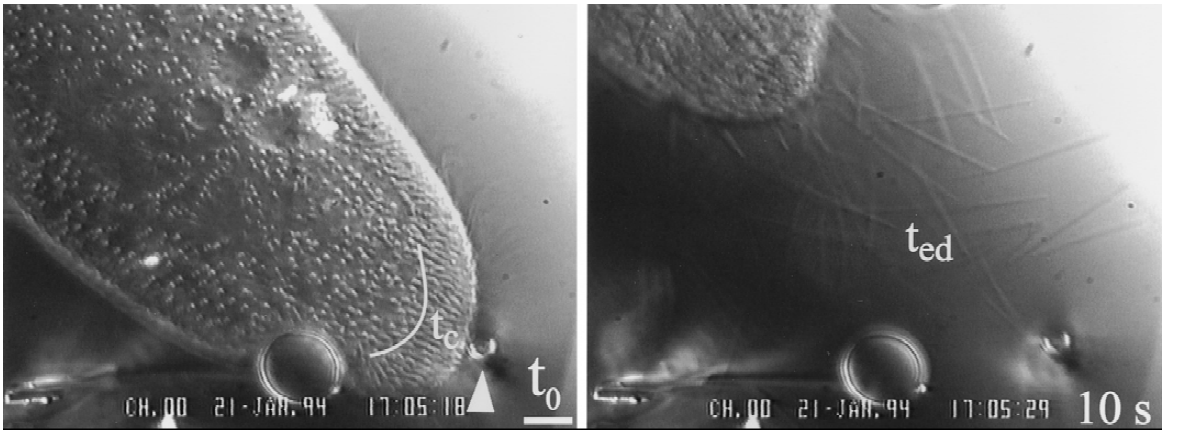

Fig. 7. Caffeine induces exocytosis in 7S cells ($[Ca^{2+}]_o = 50$ μM). Left: Arrowhead points to the capillary (containing caffeine) close to a site with numerous docked condensed trichocysts (t_c, rodlike structures). Right: After caffeine application trichocyst contents are expelled whereby they stretch to long needles (t_{ed} = externally decondensed trichocysts), while the cell is displaced by recoil. Bar = 10 μm.

caffeine application, and whether Ca_o^{2+} was added or not. To ascertain the value of caffeine as a secretagogue, we analyzed, by quenched-flow/freeze-fracture, its capacity to induce normal membrane fusions (Figs. 14 to 16). With this method the entire cell surface is stimulated by rapid mixing with caffeine. Opposite to the other strains used, only exocytosis-competent strains like 7S display rosette IMPs in freeze-fracture replicas (Beisson et al., 1976; Pouphile et al., 1986). This well defined morphology was exploited in these studies. Figure 14 presents trichocyst docking sites at rest (*a*, untriggered), during normal exocytotic membrane fusion (*b*, with dispersing small particles probably representing subunits of decaying rosette IMPs; *see* Knoll et al. [1991] and Discussion) and after formation of large exocytotic openings in response to caffeine (*c*). In addition, we observed dispersal of intact rosette IMPs at some docking sites, also in response to caffeine (Fig. 14*d*). All these stages, as well as unoccupied sites (''parentheses'') or resealing stages from exocytosis-coupled endocytosis (''filled rings'') described previously (Plattner et al., 1993) are quantified 0.5 sec to 3 (or 15) sec after caffeine application, with or without Ca_o^{2+} added, respectively, in Figs. 15 and 16. (Percentage of values indicated in the figures do not add up precisely to 100% because they are median values collected from a large number of cells from different experiments; *see* Knoll et al., 1991; Plattner et al., 1997*a*). In summary, with Ca_o^{2+} added (Fig. 15), the major fraction of ''rosettes'' disappears on account of later stages of the exo-endocytosis cycle as characteristic of normal exocytosis. This includes minifusions (which remain statistically at 0% in Fig. 15 because of their ex-

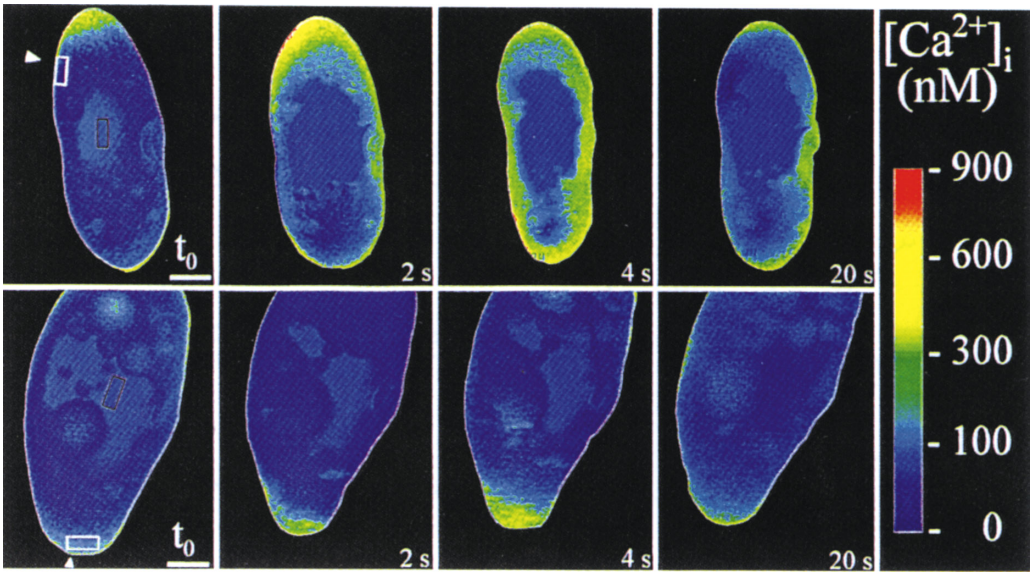

Fig. 8. Fura Red injected nd9–28°C cell stimulated at t_o by caffeine (at arrowhead), either in presence (*top*) or absence (bottom) of Ca_o^{2+}. Note framed fields for further evaluation in Fig. 10. Bars = 20 μm.

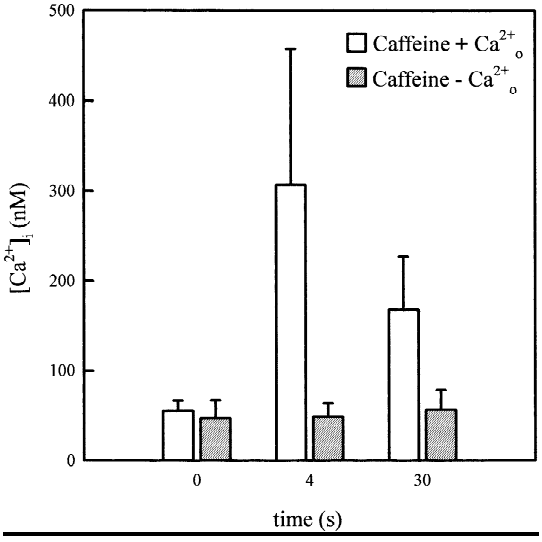

Fig. 9. Global $[Ca^{2+}]_i$ changes in caffeine triggered nd9–28°C cells, with (open columns, $n = 4$) or without (hatched columns, $n = 3$) Ca_o^{2+} as in Fig. 8. Bars = SE.

tremely short [msec] lifetime [Plattner et al., 1993]), dispersal of rosette IMPs as subunits, also indicative of normal membrane fusion (Knoll et al., 1991), and formation of larger exocytotic openings. Only a smaller fraction of rosettes undergoes dispersal without decay into subunits. Hence, in the presence of Ca_o^{2+}, caffeine induces predominantly normal membrane fusion, starting with minifusion accompanied by decay of rosette IMPs and followed by expansion of exocytotic openings. With caffeine this requires several seconds, i.e., much longer than with the secretagogue, aminoethyldextran (AED), (Knoll et al., 1991). Without Ca_o^{2+} added (Fig. 16), caffeine application results in considerably increased formation of ''dispersed rosettes,'' their number increasing with time of caffeine application. This seems to be preceded by internal trichocyst decondensation, as we conclude from a parallel analysis of ultrathin sections and freeze-fracture replicas from the same freeze-substituted samples (Table 2). In such samples, the percentage of internally decondensed trichocysts relative to normal condensed ones increases faster than the percentage of dispersed rosettes. Hence, internal decondensation may precede and possibly cause rosette dispersal.

Ca^{2+} dependency of caffeine-mediated trichocyst decondensation in vitro is shown in Fig. 17. We have mimicked $[Ca^{2+}]$ according to values occurring during simulation in vivo. Only $[Ca^{2+}] \geqslant 300$ nM, as occurring in the cell cortex during caffeine application, permits the drug to induce trichocyst stretching (Fig. 17*c*) in response to Ca^{2+} entering the secretory organelle (Glas-Albrecht & Plattner, 1990). Lower $[Ca^{2+}]$ combined with caffeine causes less or no decondensation of trichocysts in vitro (Fig. 17*a, b*). We conclude that deeper inside the cell, where free trichocysts are encountered, concentration of Ca^{2+} may not suffice to provoke caffeine-induced internal trichocyst decondensation.

Discussion

Methodical Aspects

Methods to immobilize and microinject *Paramecium* cells and availability of appropriate fluorochromes were

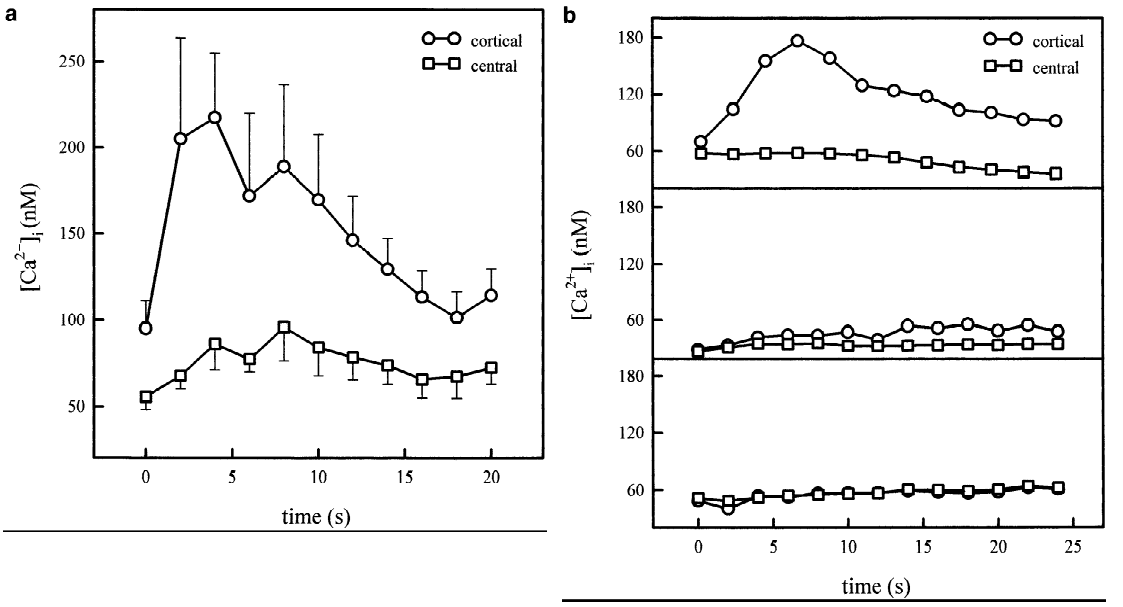

Fig. 10. Cortical and central $[Ca^{2+}]_i$ changes in nd9–28°C cells after caffeine application in presence ([*a*], $n = 4$, bars = SD) or absence of Ca_o^{2+} (*b*). Evaluation as indicated in Fig. 8. Because of widely varying results with $[Ca^{2+}]_o = 0$, three typical examples are shown in (*b*).

prerequisite to our current analysis, particularly since our cells do not take up fluorochrome esters from the medium. Solutions to all these problems have been presented previously (Klauke & Plattner, 1997). Another methodical aspect is that we used different cells for analyzing reactions with or without Ca_o^{2+} added, so we can exclude any potential interference of preceding treatments. Finally, we have to define some terms used in the following discussion. ''Cortical'' Ca^{2+}-signals designate fluorescence signals recognized in the outermost ~2 μm broad cell layer, whereas ''subplasmalemmal'' Ca^{2+}-signals are those recognized by electrophysiological and other methods of higher resolution relative to the cell membrane. Trichocyst stages and the potential influence of Ca^{2+} on them are summarized in Fig. 18.

Does Caffeine Mobilize Subcortical Ca^{2+} Stores?

Mobilization of cortical Ca^{2+} stores in *Paramecium* is poorly understood, though it can be safely assumed to occur during stimulus-secretion coupling (Plattner et al., 1991). This is based on electrophysiological (Erxleben & Plattner, 1994) and Ca^{2+} imaging methods by electron energy loss spectroscopic imaging (EELS/ESI) (Knoll et al., 1993), secondary ion mass spectroscopic (SIMS) (Stelly et al., 1995) and fluorochrome studies (Erxleben et al., 1997; Klauke & Plattner, 1997), as well as on analysis of isolated alveolar sacs using $^{45}Ca^{2+}$ (Länge et al., 1995).

Nevertheless, up to now we found no second messenger or any other signal that could account for activation of alveolar sacs. For instance, depolarization or hyperpolarization gave no exocytotic response (Erxleben & Plattner, 1994). $InsP_3$, ryanodine, cADPR and some other potential agonists also remained without any effect and CICR could not be induced (Länge et al., 1995). Our findings are compatible with patch-clamp analysis of reconstituted putative Ca^{2+} release channels from *Paramecium* (Zhou et al., 1995). These may be insensitive to ryanodine because of the low evolutionary level of *Paramecium.* Concomitantly, in another ciliate, *Vorticella,* stores can be mobilized by caffeine, but not by ryanodine or $InsP_3$ (Katoh & Naitoh, 1994). Alternatively, in some higher eukaryotic systems not all ryanodine receptors may be sensitive to caffeine (Giannini et al., 1992). Different binding sites may occur for cADPR, ryanodine and caffeine (Sitsapesan, McGarry & Williams, 1995). May the Ca^{2+} signals we obtained indicate caffeine-sensitivity of the subplasmalemmal pool and superposition of its mobilization by a Ca^{2+} influx from outside?

Our assumption of Ca^{2+} mobilization from subplasmalemmal stores is based on the following aspects. (i) Isolated alveolar sacs showed only slow $^{45}Ca^{2+}$ leakage in response to caffeine, i.e., ~1% within 3 sec of caffeine application (as derived from Table 2 in Länge et al. [1995]), whereas caffeine mediates a much more efficient cortical $[Ca^{2+}]_i$ increase in vivo even in the absence of Ca_o^{2+} (*see below*). This difference may be due to the assembly of components *in situ* and their loss during isolation, respectively. (ii) After Ca^{2+}-chelation, caffeine causes in vivo a cortical $[Ca^{2+}]_i$ signal increase to ~230 nM in an ~8 μm broad zone (Figs. 1 and 3). As previously discussed (Plattner et al., 1997*a*) one may reasonably assume $[Ca^{2+}]_{total} = 3$ to 5 mM (average 4

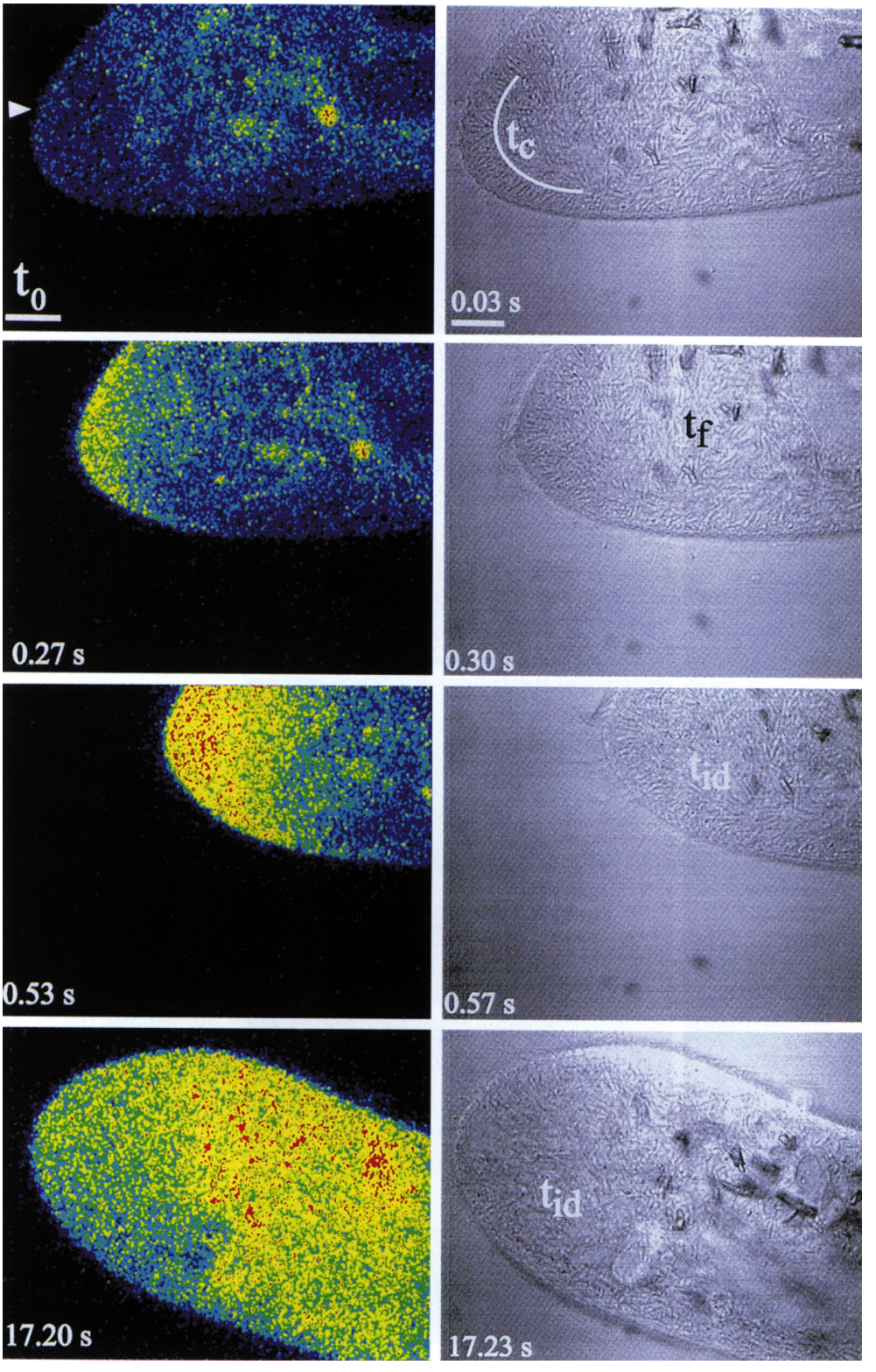

Fig. 11. Fluo-3 loaded nd9–28°C cell triggered with caffeine at arrowhead (t_o). Alternating fluorescence (CLSM, *left*) and transmitted light (*right*) images reveal cortical and then central $[Ca^{2+}]_i$ increase (e.g., between 0.53 and 17.20 sec). Numerous rodlike structures are trichocysts, partly docked at the cell periphery (t_c), partly free in the cytoplasm (t_f), both in condensed form. The docked subpopulation is liable to internal decondensation (from 0.57 to 17.23 sec) as recognized by disappearance of compact docked trichocyst and simultaneous occurrence of more elongated decondensed trichocyst states (t_{id} = internally decondensed trichocysts) in the cell apex. This aspect is analyzed in more detail in Figs. 12 and 13. Bars = 10 μm.

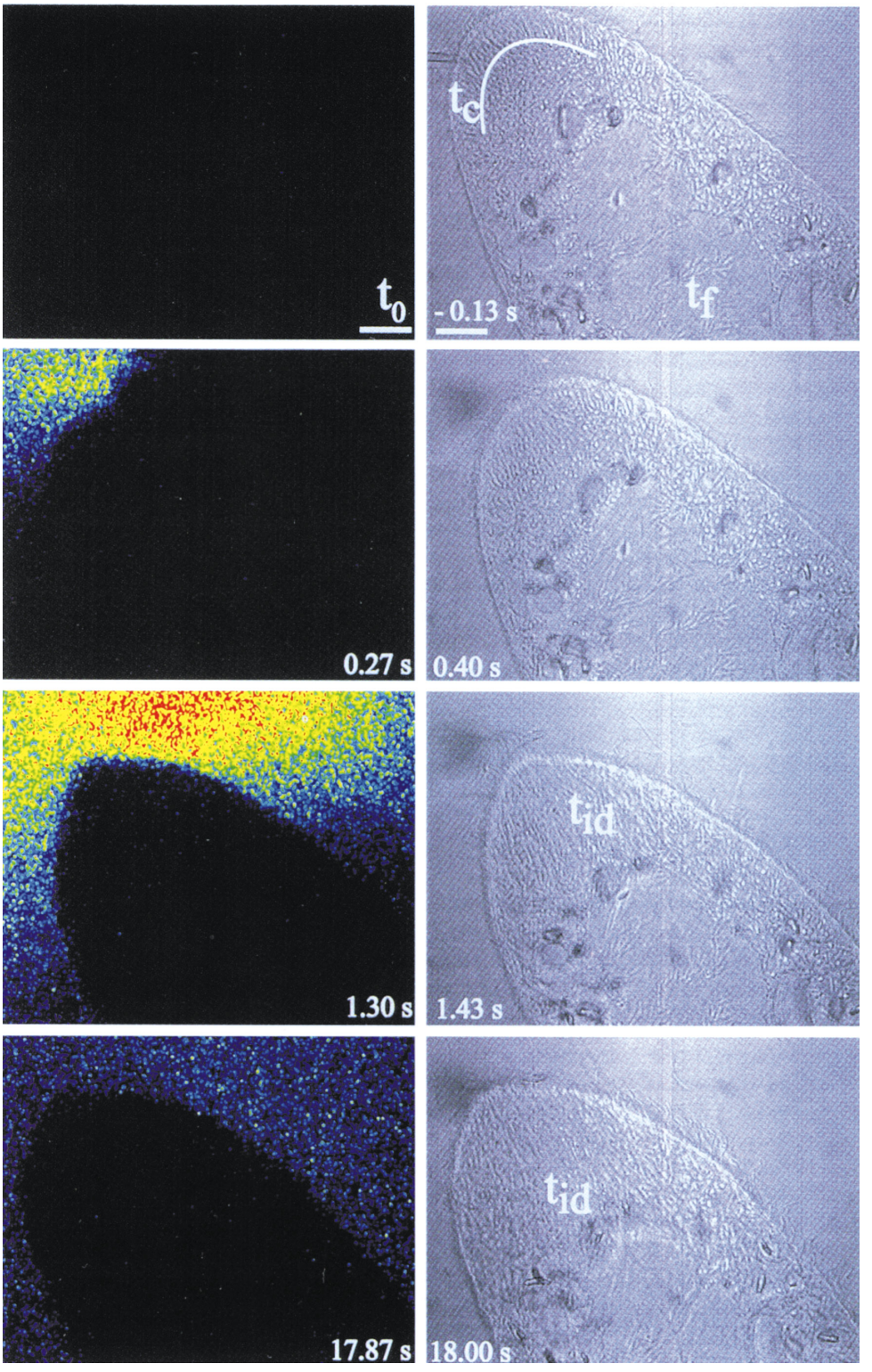

Fig. 12. Stimulation of a nd9–28°C cell at t_o with caffeine supplemented with fluorescein for CLSM analysis by alternating fluorescence and transmitted light. Note free (t_f) and docked condensed trichocysts (t_c) as rodlike structures (shown in more detail in Fig. 13) and internal decondensation of docked trichocysts (t_{id}) after massive contact of the cell with caffeine (from 1.43 sec on). Bars = 10 μm.

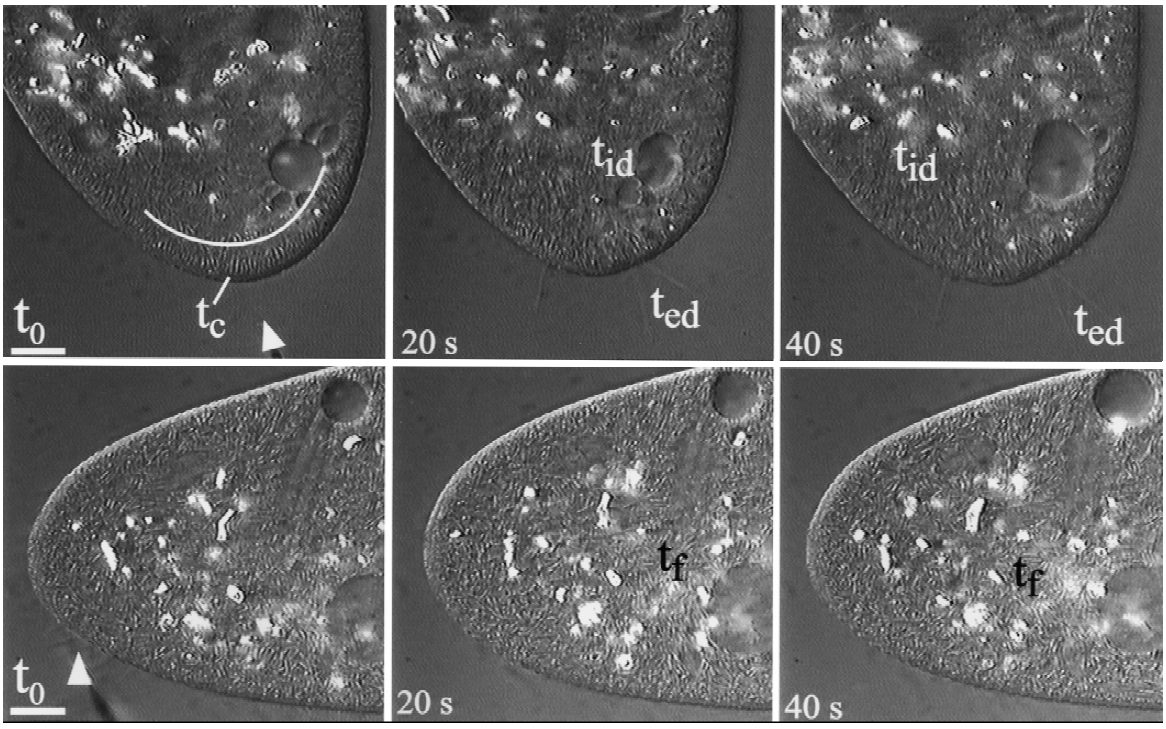

Fig. 13. Caffeine (applied at arrowhead, t_o) causes internal decondensation selectively of docked trichocysts in nd9–28°C cells. *Top:* Untreated cell with numerous docked condensed trichocysts (t_c) in parallel alignment at the cell surface (t_o). Docked trichocysts undergo internal decondensation (t_{id}, in this cell mainly between 20 and 40 sec), while only a very few trichocysts are partly extruded (t_{ed} = externally decondensed during exocytosis, *see* text). Bottom: Trichocysts removed from the cell surface (t_f) by cytochalasin B treatment do not decondense in response to caffeine. Bars = 10 μm.

mM) in alveolar sacs, which follow the cell surface as a ~0.1 μm wide compartment. Furthermore, we have previously shown that, due to the cytoplasmic binding of ~99% of Ca^{2+} by endogenous buffers during stimulated increase, only ~1% would be available for fluorochrome signals (Klauke & Plattner, 1997). Dilution in the cortical area would then be 80-fold and binding would reduce the signal 100-fold, thus resulting in a 8000-fold attenuation. The cortical $[Ca^{2+}]$ increase by ~150 nM above basal values, as observed with caffeine at $[Ca^{2+}]_o \leqslant [Ca^{2+}]_i^{rest}$ would then imply that ~8% of Ca^{2+} stored in alveolar sacs could be mobilized within ~2 sec. This is one order of magnitude more than releasable by leakage (*see above*). Moreover, the real value of specific Ca^{2+} release by caffeine would be much higher, considering simultaneous Ca^{2+}-pumping activity in a stimulated cell. Since a $[Ca^{2+}]_i$ = 230 nM achieved in the absence of Ca_o^{2+} is well below that required to induce membrane fusion (Klauke & Plattner, 1997) this also explains absence of fusion profiles in freeze-fracture replicas under such conditions (Fig. 16). (iii) Ca^{2+} release from alveolar sacs (Länge et al., 1995) shares some other properties with Ca^{2+} channels in the SR (Rousseau & Meissner, 1989), like stimulation by ATP and inhibition by Mg^{2+}.

Table 1. Only docked trichocysts are liable to internal decondensation in response to 50 mM caffeine

Strain	$[Ca^{2+}]_o$	Manipulation	State of trichocysts	Caffeine response	*n*
7S	50 μM	None	Many docked	−/+	10
			Few free	−	10
	30 nM	None	Many docked	+	10
			Few free	−	10
tam6	50 μM	None	Few docked	−/+	23
tam38	50 μM	None	Only free	−	3
nd9–28°C	50 μM	None	Many docked	++	11
			Few free	−	11
		Cyt. B[a]	Only free	−	11
		Mg^{2+} inj.[b]	Docked	−	9
		BAPTA inj.[c]	Docked	−/+	12
	30 nM	None	Many docked	+	10
			Few free	−	10
	1 mM	None	Many docked	++	15
			Few free	−	15

Rating "−, + and ++" indicates "no, weak to medium and strong" internal decondensation response. *n* = number of cells analyzed.
[a] Cytochalasin B incubation to detach docked trichocysts from the cell surface (*see* Materials and Methods)
[b] $MgCl_2$ injected to yield an intracellular concentration of 10 mM.
[c] BAPTA injected to yield 0.1 mM.

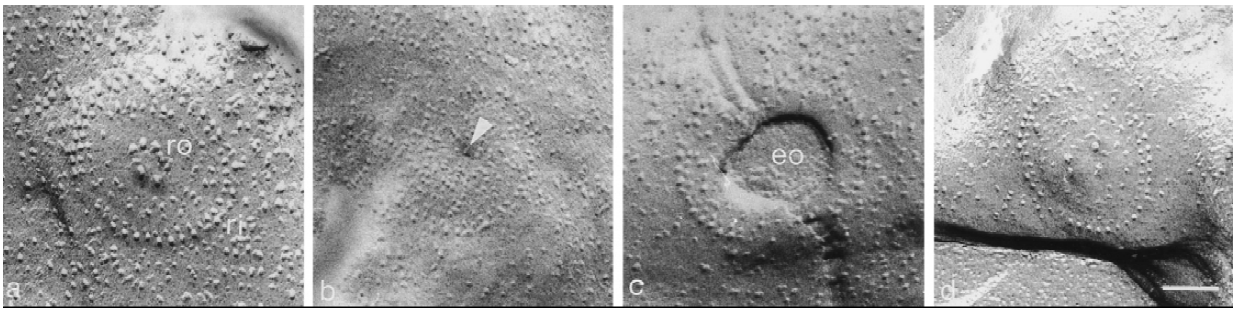

Fig. 14. Freeze-fracture appearance of trichocyst docking sites. (*a*) ''Resting'' stage with an IMP ''rosette'' (ro) in the center of an IMP ''ring'' (ri) indicative of an extrudable trichocyst underneath. (*b)* Early stage of normal exocytotic membrane fusion (with ''minifusion'' as the earliest, though short-lived, rare stage) upon caffeine stimulation (focal fusion [arrowhead] surrounded by dispersing rosette IMP subunits; *see* text). (*c*) Normal expanded exocytotic opening (eo). (*d*) Dispersal of intact rosette IMPs (without decay into subunits) as a nonfusogenic effect of caffeine. Bar = 0.1 μm.

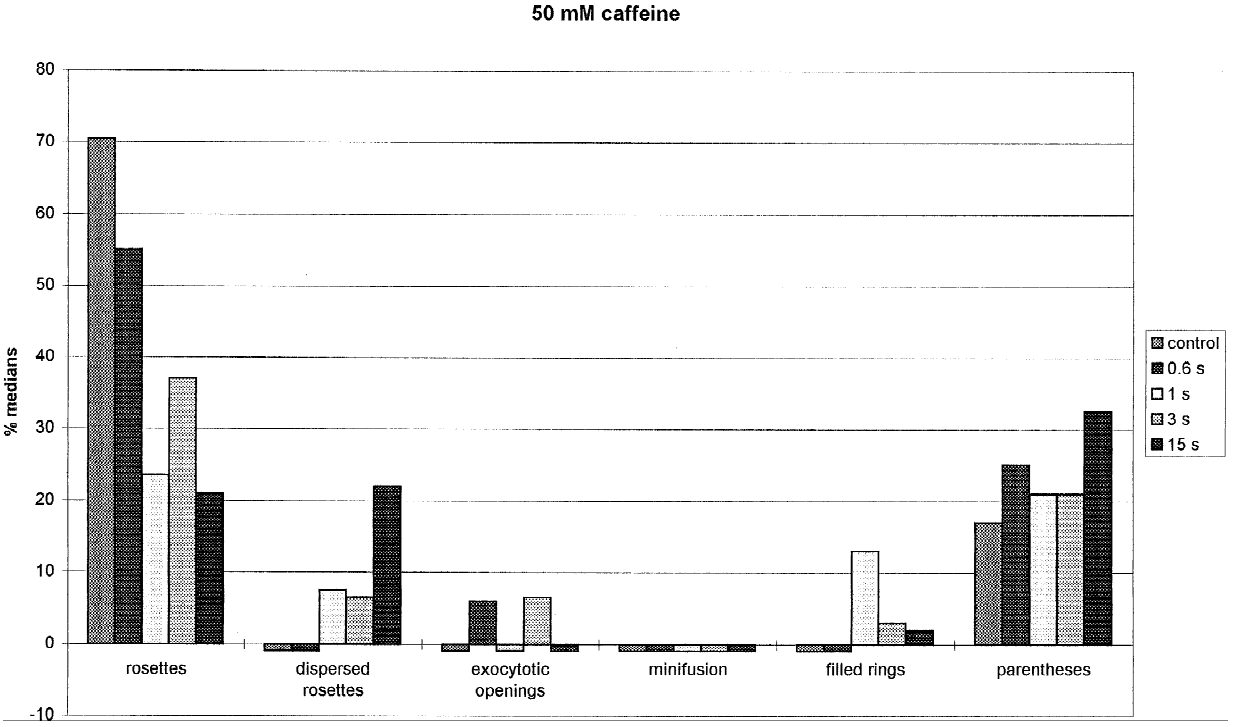

Fig. 15. Quenched-flow/freeze-fracture analysis of caffeine effects on ultrastructure of trichocyst docking sites in 7S cells in presence of Ca^{2+} (50 μM). Rosette IMPs (indicative of a docked exocytosis-competent trichocyst available in 70% of all docking sites) occur in addition to normal stages of the exo-endocytotic cycle (*see* Fig. 14 and text). Caffeine causes reduction of rosettes on account of other stages, e.g., exocytotic openings, ''filled rings'' and ''parentheses,'' thus indicating a normal exocytotic cycle. However, some rosette IMPs can be dispersed without forming membrane fusions and openings. This novel IMP rearrangement increases with time, affecting ~10 (1 sec) to 30% (15 sec) of docking sites, while ~30% of rosettes originally present remain unaltered over 15 sec. Overall induction of exocytosis (decay of rosette IMPs into subunits, rather than dispersal of intact IMPs) and of resealing stages (filled rings, parentheses) proceeds rather slowly. For samples taken 0 sec (controls), 0.6, 1, 3, and 15 sec after caffeine application the number of cells analyzed was 21, 21, 14, 14 and 26, containing 374, 462, 218, 227 and 502 docking sites, respectively. Note that mean values do not add up precisely to 100% (*see* text). Data from two independent experiments.

(iv) In cells with only diffusely distributed ER, the signal produced by caffeine is diffuse (Burgoyne et al., 1989), i.e., quite different from what we see. Concomitantly, restriction of the Ca^{2+} signal in time and space, as we have documented, is in contradiction to what would be expected from diffuse activation. Trichocyst release occurs quite promptly, i.e., within seconds, in response to caffeine. This is quite comparable to systems with established caffeine effects, e.g., exocytosis in chromaffin cells (Guo et al., 1996) or $[Ca^{2+}]_i$ increase in smooth

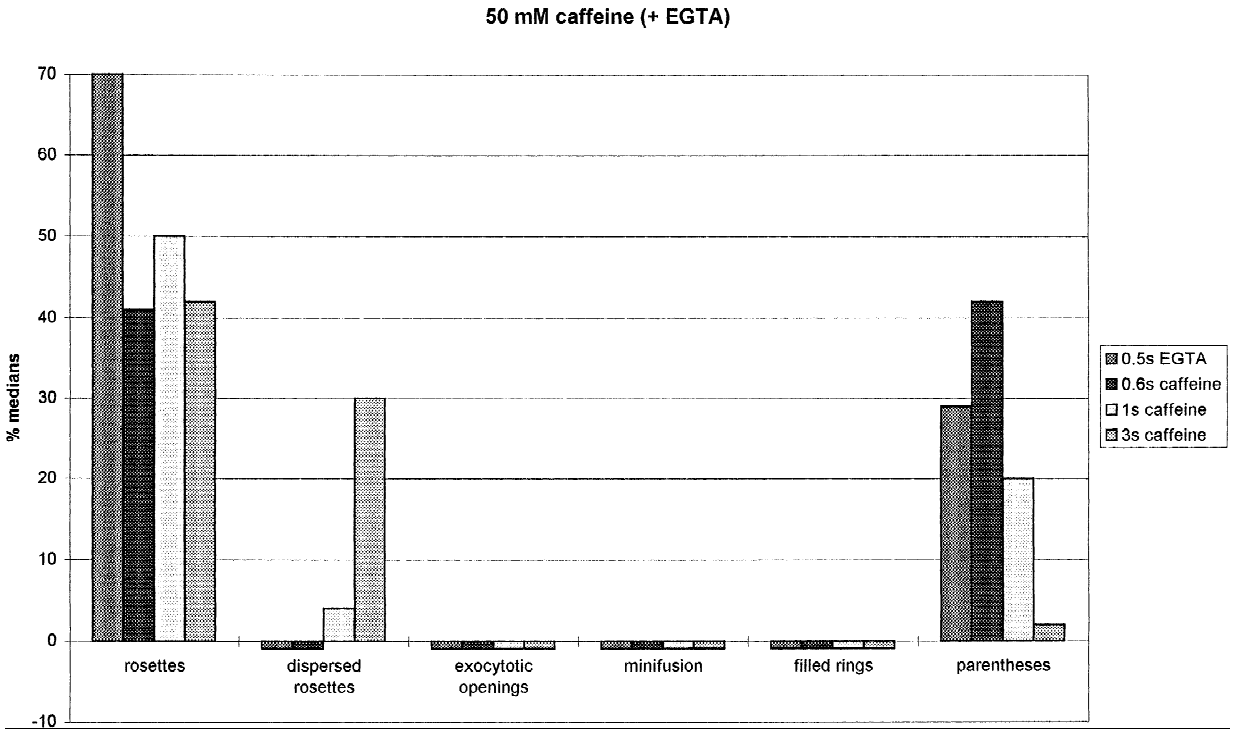

Fig. 16. Same type of experiment as in Fig. 15 but without Ca_o^{2+}. Rosettes are reduced by caffeine within brief times, dispersal of intact rosette IMPs increases with time, parentheses initially increase and are reduced subsequently, while other stages are missing. For 0 sec (controls), 0.6, 1 and 3 sec after caffeine application the number of cells analyzed was 12, 13, 17 and 10, containing 258, 213, 368 and 177 docking sites, respectively. Data from four independent experiments.

Table 2. In response to caffeine, internal trichocyst decondensation occurs preferentially without Ca_o^{2+} and causes dispersal of intact rosette IMPs in 7S cells

Time after caffeine	Quenched-flow/ freeze-fracture	Freeze-substitution/ ultrathin sections
	Relationship assembled:dispersed rosettes	Relationship condensed:decondensed trichocysts
(A) + Ca_o^{2+}		
0 sec	100:0 (n = 21)	100:0 (n > 30)
3 sec	5:1 (n = 14)	2:3 (n = 55)
15 sec	3:4 (n = 26)	1:5 (n = 26)
(B) − Ca_o^{2+}		
0 sec	100:0 (n = 12)	100:0 (n > 30)
1 sec	10:1 (n = 17)	3:2 (n = 55)
3 sec	2:1 (n = 10)	1:2 (n = 30)

n = number of cells analyzed.

muscle cells (Iino et al., 1993). (v) In the presence of Ca_o^{2+}, this is superimposed by a Ca^{2+} influx, possibly via store-operated Ca^{2+} channels as described in other systems (Fasolato, Innocenti & Pozzan, 1994). Superposition of Ca^{2+} mobilization from stores and Ca^{2+} influx also occurs in some neuroendocrine cells during caffeine stimulation (Barry & Cheek, 1994), just as we observe.

According to the present study, caffeine is much less effective as an agonist for trichocyst exocytosis than the polyamino-secretagogue, AED (Plattner et al., 1985; Knoll et al., 1991). Already ⩾1 μM AED induces a similar Ca^{2+} transient (Erxleben & Plattner, 1994; Klauke & Plattner, 1997) as 50 mM caffeine, a concentration required for maximal activation also in other systems (*see* Introduction).

In spite of store mobilization caffeine does not induce membrane fusion in the absence of Ca_o^{2+} (Fig. 16), in contrast to AED (Knoll et al., 1991; Plattner et al., 1997*a*). Membrane fusion might be regulated by a Ca^{2+} sensor whereby signaling to its target depends on a Ca^{2+} threshold (Burgoyne & Morgan, 1995). As caffeine and AED induce a similar rise of cortical $[Ca^{2+}]_i$ in fluorochrome imaging, but clearly with different efficiency, other mechanisms like site-directed activation or domain regulation might be relevant for membrane fusion in *Paramecium* (Erxleben & Plattner, 1994; Erxleben et al., 1997). Along these lines we noted that membrane fusion was accelerated with $[Ca^{2+}]_o$ increasing beyond 100 μM only with AED (Plattner et al., 1997*a*) but not with caffeine (*data not shown*). This again makes induction of unspecific leakage by caffeine quite unlikely.

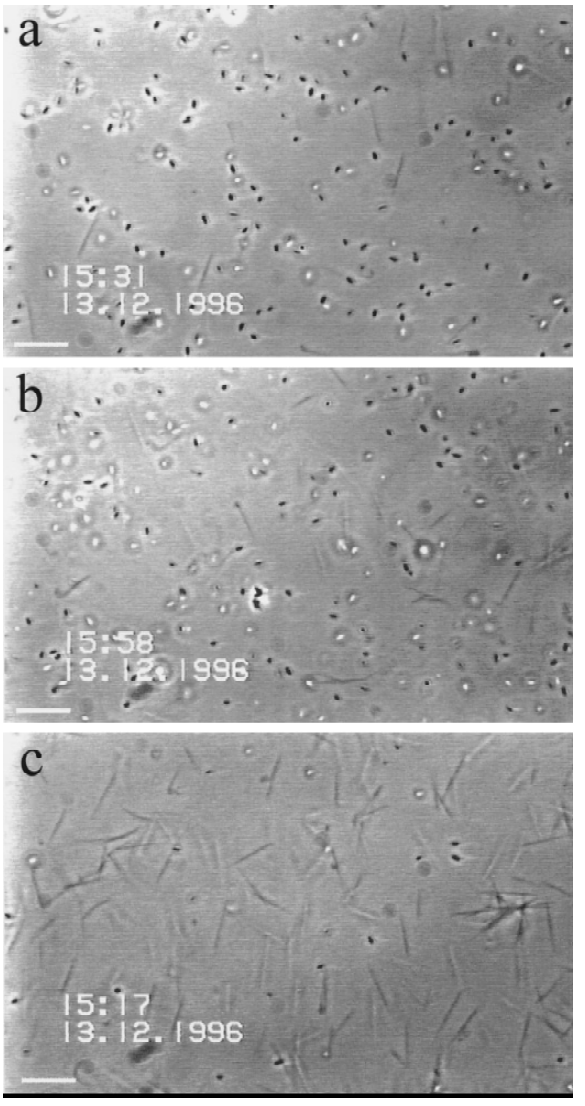

Fig. 17. Immediate response of trichocysts, isolated with intact membranes, to caffeine in presence of (a) 10 nM, (b) 80 nM, or (c) 330 nM free Ca^{2+} in phase contrast imaging. Note that in (*a*, *b*) condensed trichocysts (dense points) by far outnumber decondensed ones (rods of moderate density), while the opposite is true for (c). Bar = 20 μm.

The reduced signal we see in the absence of Ca_o^{2+} also excludes unspecific fluorochrome signals by caffeine (Tanaka & Tashjian, 1993). Similarly, our findings are compatible with the recording of Ca^{2+}-activated plasmalemmal currents during caffeine triggered exocytosis (Erxleben & Plattner, 1994).

How to Explain Internal Trichocyst Decondensation?

Explosive matrix stretching normally causes ejection of trichocyst contents through an exocytotic opening. As

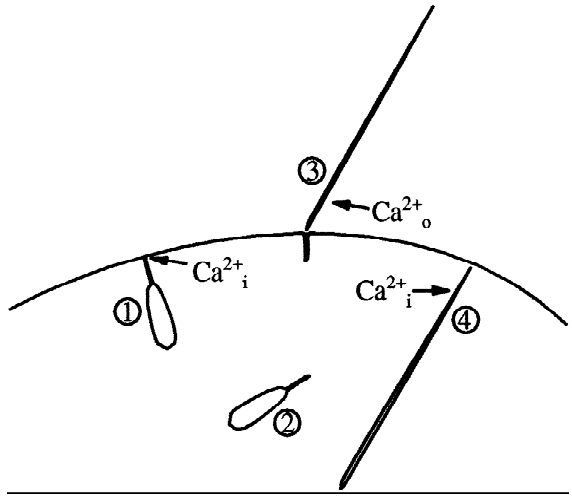

Fig. 18. Stages of trichocyst morphology — explanation of terminology used and effects of Ca^{2+}. (1) Docked condensed trichocyst, Ca^{2+}_i causing membrane fusion. (2) Free condensed trichocyst, without Ca^{2+} effects. (3) External decondensation of a trichocyst (during exocytosis), Ca_o^{2+} causing decondensation. (4) Internal decondensation of a docked trichocyst (Ca_i^{2+} entering the organelle).

we now observed with caffeine, trichocyst contents can undergo decondensation without membrane fusion under two conditions. This occurs (i) with most of the trichocysts in nondischarge strains, whether $[Ca^{2+}]$ increase is small or large, and (ii) with some trichocysts in wildtype cells at regular $[Ca^{2+}]_o$ and with many more when $[Ca^{2+}]_i$ increase is too small to cause membrane fusion (*see above*). We also show that decondensation *in situ* depends on the local $[Ca^{2+}]_i$ increase in presence of caffeine, thus indicating caffeine-mediated Ca^{2+} permeation into trichocysts. These are normally devoid of any detectable Ca^{2+}, opposite to most other secretory organelles (*see* Introduction). Ca^{2+} channels seem to occur more widely in secretory organelle membranes than generally assumed (Kasai, Li & Miyashita, 1993). For instance, zymogen granules can be depleted of their internal Ca^{2+} by activation of putative organellar Ca^{2+}-channels by $InsP_3$ or cADPR (Gerasimenko et al., 1996). Possibly the trichocyst membrane also contains Ca^{2+} channels opening in response to the agonist caffeine. Remarkably, this effect is inhibited by Mg^{2+} (Table 1), as in caffeine-activation of Ca^{2+} channels in the SR (Sitsapesan & Williams, 1990).

Conclusions

What data can we add to the field? Essentially, we show the following: (i) In wildtype cells, caffeine stimulates a rapid $[Ca^{2+}]_i$ increase and, in parallel, trichocyst secretion. (ii) Also just as in other cell types, caffeine mobilizes Ca^{2+} from internal pools and this is normally su-

perimposed by a Ca^{2+}-influx from the medium. (iii) A novel finding is the action of caffeine on secretory vesicles and induction of secretory contents decondensation in nondischarge strains (incapable of membrane fusion) by Ca^{2+} entry into organelles. (iv) In a similar way, subthreshold activation in wildtype cells can cause aberrant internal decondensation and aberrant membrane restructuring, as shown by quenched-flow/freeze-fracturing and quantitative evaluation, which also show normal exocytosis under standard conditions.

The detailed mechanism of Ca^{2+} mobilization by caffeine from cortical pools still has to be elucidated. In smooth muscle cells caffeine can activate Ca^{2+}-permeable nonselective cation channels (Guerro, Fay & Singer, 1994). Such putative Ca^{2+} release channels were also found by reconstitution studies using cortices from *Paramecium* (Zhou et al., 1995). The most likely interpretation of the $[Ca^{2+}]_i$ transients we see, with their precise localization and timing, would be mobilization of Ca^{2+} from cortical stores superimposed by Ca^{2+} influx. This can produce normal exocytosis. Internal trichocyst decondensation may be caused by caffeine-mediated Ca^{2+} permeation into the secretory organelles, particularly in strains which cannot produce exocytotic openings. Our data suggest a different sensitivity of the two Ca^{2+}-dependent steps, membrane fusion and trichocyst decondensation. Subthreshold increase of $[Ca^{2+}]_i$ can cause internal trichocyst decondensation and dispersal of rosette IMPs without membrane fusion.

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