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Polyamine Triggering of Exocytosis in *Paramecium* Involves an Extracellular Ca^{2+}/(Polyvalent Cation)-Sensing Receptor, Subplasmalemmal Ca-Store Mobilization and Store-Operated Ca^{2+}-Influx via Unspecific Cation Channels

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Abstract. The polyamine secretagogue, aminoethyldextran (AED), causes a cortical [Ca^{2+}] transient in *Paramecium* cells, as analyzed by fluorochrome imaging. Our most essential findings are: (i) Cortical Ca^{2+} signals also occur when AED is applied in presence of the fast Ca^{2+} chelator, BAPTA. (ii) Extracellular La^{3+} application causes within seconds a rapid, reversible fluorescence signal whose reversibility can be attributed to a physiological $[Ca^{2+}]_i$ transient (while injected La^{3+} causes a sustained fluorescence signal). (iii) Simply increasing $[Ca^{2+}]_o$ causes a similar rapid, short-lived $[Ca^{2+}]_i$ transient. All these phenomena, (i–iii), are compatible with activation of an extracellular "Ca^{2+}/(polyvalent cation)-sensing receptor" known from some higher eukaryotic systems, where this sensor (responding to Ca^{2+}, La^{3+} and some multiply charged cations) is linked to cortical calcium stores which, thus, are activated. In *Paramecium*, such subplasmalemmal stores ("alveolar sacs") are physically linked to the cell membrane and they can also be activated by the Ca^{2+} releasing agent, 4-chloro-m-cresol, just like in Sarcoplasmic Reticulum. Since this drug causes a cortical Ca^{2+} signal also in absence of $Ca^{2+}{}_o$ we largely exclude a "Ca^{2+}-induced Ca^{2+} release" (CICR) mechanism. Our finding of increased cortical Ca^{2+} signals after store depletion and re-addition of extracellular Ca^{2+} can be explained by a "store-operated Ca^{2+} influx" (SOC), i.e., a Ca^{2+} influx superimposing store activation. AED stimulation in presence of $Mn^{2+}{}_o$ causes fluorescence quenching in Fura-2 loaded cells, indicating involvement of unspecific cation channels. Such channels, known to occur in *Paramecium*, share some general characteristics of SOC-type Ca^{2+} influx channels. In conclusion, we assume the following sequence of events during AED stimulated exocytosis: (i) activation of an extracellular Ca^{2+}/polyamine-sensing receptor, (ii) release of Ca^{2+} from subplasmalemmal stores, (iii) and Ca^{2+} influx via unspecific cation channels. All three steps are required to produce a steep cortical [Ca^{2+}] signal increase to a level required for full exocytosis activation. In addition, we show formation of [Ca^{2+}] microdomains ($\leq$0.5 μm, $\leq$33 msec) upon stimulation.

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

Introduction

In different systems, Ca^{2+}, a universal regulator of stimulated exocytosis, may originate from influx via Ca^{2+} carrying channels and/or from stores, whereby Ca^{2+} from both sources may exert mutual control in different ways. Variations to this complicated interplay have extensively been reviewed [2, 5, 6, 8, 15, 54]. Some systems operate via receptor-activated Ca^{2+} channels, while some others use voltage sensitive Ca^{2+} channels. Ca^{2+} influx may cause "Ca^{2+}-induced Ca^{2+} release" (CICR), or vice versa Ca^{2+} mobilization from stores may cause "store-operated Ca^{2+} influx" (SOC). Another possible feedback mechanism is a current generated by "Ca^{2+} release-activated Ca^{2+} influx" (I_{CRAC}), [62], involving inositol 1,4,5-trisphosphate (IP_3) formation, while structural or functional coupling of stores to plasmalemmal I_{CRAC}-type Ca^{2+} conductances is not well known. In general, Ca stores may be of the IP_3- or ryanodine-sensitive type, although the latter more generally respond to caffeine [12], and, even at ~100 times lower concentrations, to the recently described activator, 4-chloro-meta-cresol

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(4CmC, [30, 34, 96]) which in Sarcoplasmic Reticulum (SR) also activates mutated Ca^{2+} release channels of low ryanodine-sensitivity. Another new concept emerging in Ca^{2+} signaling is the existence of extracellular "Ca^{2+}/(polyvalent cation)-sensing receptors" (CaSR) which sometimes are linked to IP_3 formation. However, recently some forms of CaSR have been described which do not involve IP_3 formation for mobilization of Ca^{2+} from cell membrane-coupled stores [1, 11, 79, 93].

In our work with the ciliated protozoan, *Paramecium tetraurelia*, we can now, in conjunction with a series of new experiments, try to put together the complex puzzle from many details pertinent to regulation of exocytosis of dense-core vesicles ("trichocysts"). In brief, the background is as follows. Trichocysts can be released synchronously (80 msec) in great numbers in response to the polyamine secretagogue, aminoethyldextran, AED, $M_r = 40$ kDa [42, 69, 71, 72]. Though all trichocysts docked at the cell membrane (~95% of all) are competent for exocytosis, only ~40% of all exocytosis sites are activated by AED [19, 67] when extracellular Ca^{2+} concentration is reduced to levels slightly below intracellular concentrations at rest, i.e. $[Ca^{2+}]_o < [Ca^{2+}]_i^{rest}$ which is ~65 nM [40]. Cortical $[Ca^{2+}]$ transients achieved at $[Ca^{2+}]_o = 30$ nM by 1–2 μM AED [40] or 50 mM caffeine [41] could be primarily due to mobilization from alveolar sacs, and secondarily to overlapping Ca^{2+} influx — an aspect to be analyzed here in more detail. Increasing $[Ca^{2+}]_o$ allows all trichocysts to undergo exocytosis and it greatly accelerates all steps of the exo-endocytosis cycle triggered by AED [67]. Alveolar sacs are well established vast subplasmalemmal Ca storage compartments [43, 48, 88, 89] which are physically linked to the cell membrane at a distance of ~15 nm [70]. Among some features in common with muscle SR (*c.f.* [23, 56, 85]) or ER (*c.f.* [57, 74]) are the occurrence of calsequestrin-like protein in alveolar sacs [68] and of a SERCA-type Ca^{2+}-pump [28, 39].

We now address the following questions. While lack of any role of IP_3 [48] excludes a I_{CRAC}-type mechanism, could a CICR- or a SOC-type mechanism be involved in our system? In fact, there has been some controversy about this aspect in *Paramecium*, some papers arguing in favor of CICR [16, 37], some against it [19, 43, 48, 67, 70]. Since Ca^{2+} channels in alveolar sacs can be activated by caffeine [41], but not by ryanodine [48], we now tried 4CmC, the alternative, meanwhile well established agent used to mobilize Ca^{2+} from SR [30, 34, 96]. So far we could not establish for *Paramecium* cells the precise interplay between Ca^{2+} from both potential sources, Ca_o^{2+} and Ca_i^{2+}. We try to find out whether a CaSR may be involved in AED-stimulated trichocyst exocytosis. The situation is complicated because *Paramecium* possesses a variety of plasmalemmal Ca^{2+} carrying channels and because for most channels specific activators or inhibitors are not established in our system. Therefore, we had to keep in mind many candidates, like depolarization- [52, 60], hyperpolarization- [77, 78] and mechanosensitive Ca^{2+} channels [59], as well as Ca^{2+} carrying Na^+ channels [82], and some unspecific cation channels [84]. Detailed reviews are available [31, 44, 51, 75, 76]. Also feasibility of a CaSR-type signal transduction mechanism, as described above, had to be tested in the context of Ca^{2+} channel candidates in stores and in the plasma membrane.

Materials and Methods

Strains used were 7S (wildtype), nondischarge strain nd9-28°C [3], and "pawn" strain d4-500r [27], all cultivated monoxenically, with *Enterobacter aerogenes* added, at 25°C (7S and pawn) or 28°C (nd9). 7S and pawn cells perform exocytosis of almost all of their trichocysts, of which ~95% are attached at the cell surface, in response to AED [71, 72], in contrast to nd9 cells cultivated at the nonpermissive temperature of 28°C. Cells were used as indicated in [40, 41], where we also described materials used, methods of microinjection, fluorochromes, equipment used, manipulation of the extracellular medium, calibration and evaluation. As additional materials we used BAPTA, $LaCl_3$ and $MnCl_2$, each p.A. grade, 4CmC from Fluka (Deisenhofen, Germany, 1 M stock solution in DMSO, diluted 1:200 in final use), and Fura-2 as an additional fluorochrome from Molecular Probes (Eugene, OR). The injection vehicle was 5 mM Pipes/NaOH pH 7.0. All concentrations indicated mean final concentrations seen by the cell.

Since trichocyst exocytosis occurs on a subsecond time scale [42, 69] we included fast CLSM f/f_o ratio analysis of Fluo-3 loaded cells, to determine $[Ca^{2+}]_i$ transients generated by the different compounds tested. Parameters, as defined in Fig. 1, were used. Single wavelength Fluo-3 measurements were evaluated as f/f_o ratio, using a confocal laser scanning microscope (CLSM, type Odyssey, from Noran, Bruchsal, Germany) with a fast opto-acoustic beam deflection system, all as described [40, 41]. To demonstrate involvement of unspecific cation channels in the plasmalemma, shown by Mn^{2+} quenching of Fura-2 [13, 21, 87], also used at 100 μM, we applied $\lambda_{excitation} = 360$ and 380 nm, while $\lambda_{emission}$ evaluated was ≥515 nm.

Results

Voltage-Dependent Ca^{2+} Channels are Not Involved in AED Produced $[Ca^{2+}]_i$ Transients

We analyzed $[Ca^{2+}]_i$ transients in wildtype (7S) and pawn (d4-500r) cells in response to AED (Fig. 2). These strains were compared since the latter has no ciliary voltage-dependent Ca^{2+} channels [27]. As summarized in Table 1, (i) f/f_o ratios rise faster and to a higher and longer lasting plateau level in 7S cells and (ii) the $[Ca^{2+}]_i$ transient decays more slowly than in pawn cells. We did not analyze in more detail any possible significance of this difference between the two strains. Yet the mere fact that both strains, each one with established exocytosis competence [71], develop a clearly defined $[Ca^{2+}]_i$ transient in parallel to AED-mediated exocytosis, strictly argues against any essential role of voltage-sensitive Ca^{2+} channels in regulation of trichocyst secretion.

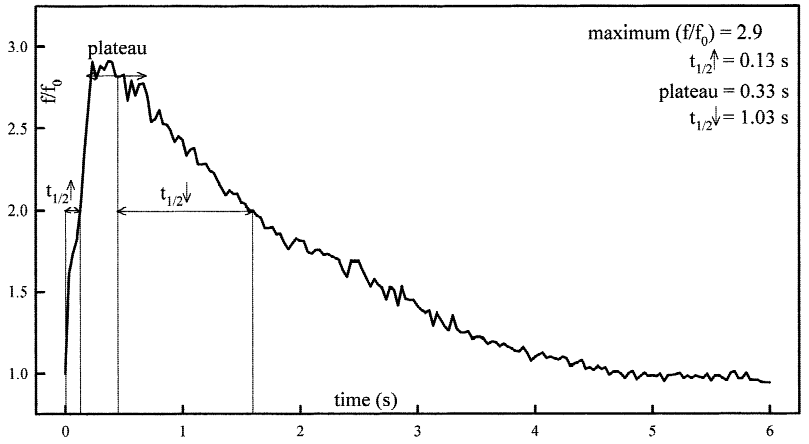

Fig. 1. Typical form of a cortical $[Ca^{2+}]_o$ transient during activation, with definitions used in this paper. This example represents a CLSM analysis of a Fluo-3 loaded 7S cell anteriorly stimulated by 2 μM AED at $[Ca^{2+}]_o$ = 50 μM. Evaluated was a ~5 μm broad cortical zone.

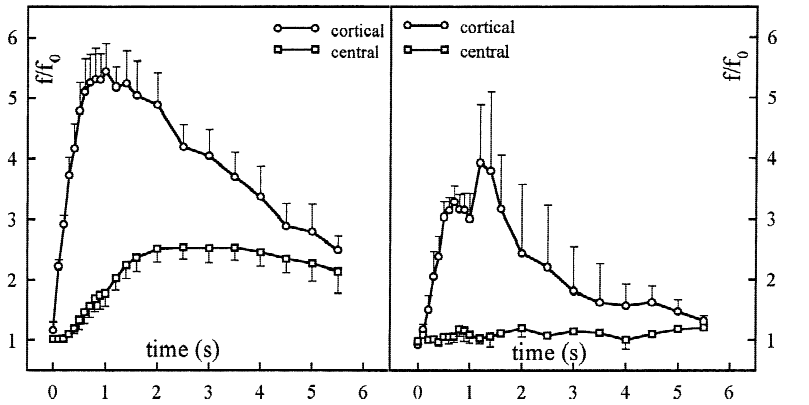

Fig. 2. Occurrence of a cortical $[Ca^{2+}]_i$ transient in both, 7S (left) and pawn cells (right). Fluo-3 loaded cells, anteriorly AED (2 μM) stimulated at $[Ca^{2+}]_o$ = 50 μM. Note more pronounced $[Ca^{2+}]_i$ transient in 7S cells as compared to pawn cells, and spillover into central regions in 7S. Values ± SEM, n = 6.

Table 1. Cortical $[Ca^{2+}]$ transients (f/f_o ratio) in Fluo-3 loaded 7S and pawn cells stimulated with AED at the posterior cell pole at $[Ca^{2+}]_o$ = 50 μM

Characteristics of the cortical $[Ca^{2+}]$ transient	Strain 7S n = 6	Strain d4-500r n = 6
Maximal f/f_o	5.4 ± 0.5	3.9 ± 0.9
$t_{1/2}$ rise time (sec)	0.26 ± 0.08	0.75 ± 0.46
Plateau duration (sec)	1.00 ± 0.48	0.50 ± 0.23
$t_{1/2}$ decay time (sec)	1.94 ± 0.94	0.51 ± 0.02
Exocytosis	Normal response	Normal response

Values ± SEM, n = number of cells analyzed.

Paramecium Cells React to High $[Ca^{2+}]_o$ or to Extracellular La^{3+} by a $[Ca^{2+}]_i$ Transient—Evidence of a CaSR?

May there exist a CaSR in *Paramecium* cells? If so, could it potentially explain generation of AED-mediated $[Ca^{2+}]_i$ transients? The prediction would be that an instantaneous increase of $[Ca^{2+}]_o$ would cause a cortical Ca^{2+} signal, just as one would expect also for extracellular application of La^{3+}, according to previous work with some mammalian cells [87].

In experiments with 7S cells (Fig. 3), we rapidly increased $[Ca^{2+}]_o$ in the medium to 10 mM, in order to look for any rapid change in $[Ca^{2+}]_i$. While we know that simply increasing $[Ca^{2+}]_o$ to this level does not induce exocytosis [67], for reasons analyzed in the Discussion, we do find a rapid cortical $[Ca^{2+}]$ increase (Fig. 3). By analyzing separately anterior and posterior cell regions we also took into account the uneven distribution of some channels in the cell surface membrane, with mechanosensitive Ca^{2+} channels [59] and Ca^{2+} carrying Na^+ channels [75] enriched towards the anterior cell pole. (We emphasize that cells are not mechanically triggered by the procedure used, while we wanted to explicitly exclude even an auxiliary role for mechanosensitive Ca^{2+} channels). Yet $[Ca^{2+}]_i$ responses were practically the same in anterior and posterior regions. Therefore, we exclude that activation of any of the two nonuniformly distributed types of channels mentioned would be involved in a primary reaction to AED. Clearly exocytotic reaction to AED is not or only slightly different in the different cell regions.

If a CaSR-linked mechanism would occur, one would expect activation of cortical $[Ca^{2+}]_i$ increase not only in response to AED or to high $[Ca^{2+}]_o$, but also in response to extracellular La^{3+} application [87]. Yet its

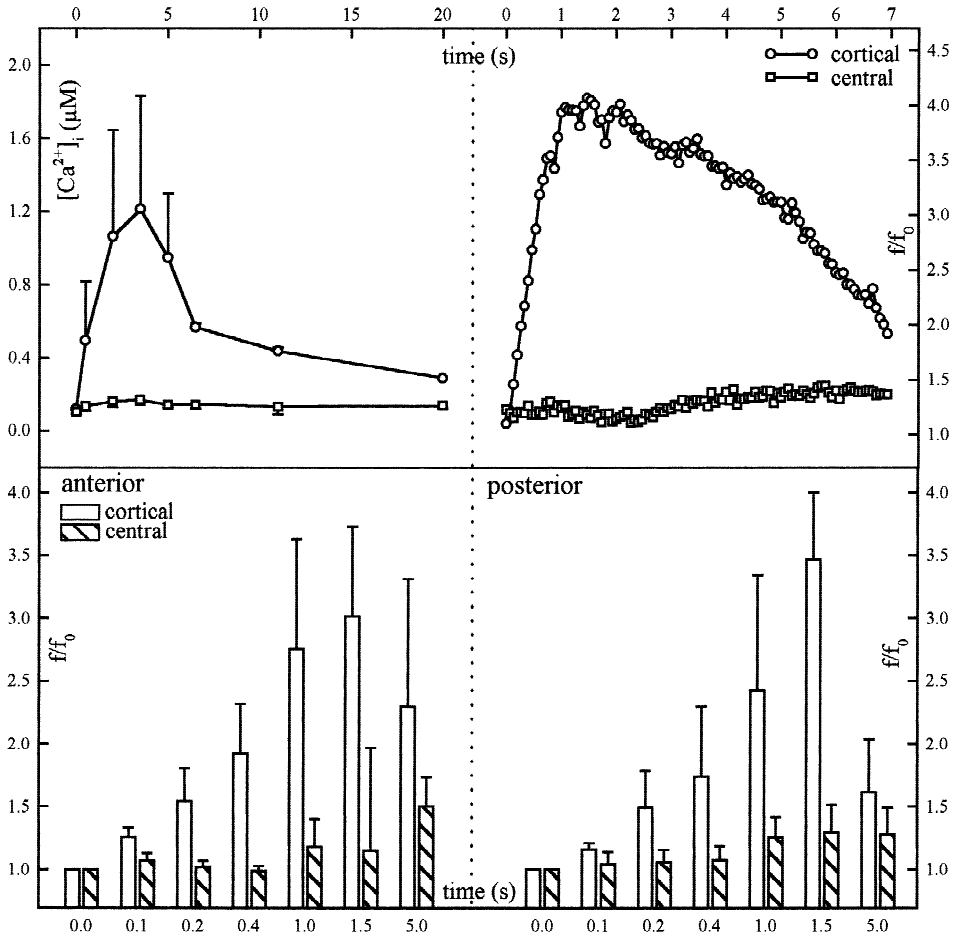

Fig. 3. A cortical $[Ca^{2+}]_i$ transient forms in 7S cells (top left: Fura Red loaded; top right, bottom left and right: Fluo-3 loaded) in response to quick application of $[Ca^{2+}]_o$ = 10 mM. Top left: conventional 2λ Fura Red analysis; anterior $Ca^{2+}{}_o$ application. Top right: fast 1 λ CLSM Fluo-3 analysis; anterior $Ca^{2+}{}_o$ application. Bottom: Fluo-3 CLSM analysis of anterior (left) or posterior (right) $[Ca^{2+}]_i$ transients after $Ca^{2+}{}_o$ application at the respective cell pole. Values ± SEM, n = 3 (top left), 4 (bottom left) or 5 (bottom right).

use is problematic since La^{3+} can enter a cell and then per se yield a fluorescence signal [46, 63]. Furthermore, La^{3+} can affect also some other functions after entering a cell [66, 73]. Therefore, we have applied La^{3+} (i) either quickly to the medium (Fig. 4), or, (ii) in controls, by microinjection into the cytosol (*not shown*) in order to compare the respective reactions obtained. In case (i) we received an intense, transient Fluo-3 signal, while in (ii) we recorded a sustained signal since intracellular La^{3+} will permanently activate, and thus block, Fluo-3. In controls with longer times of extracellular La^{3+} application, we registered a gradually developing sustained Fluo-3 signal (*data not shown*), as to be expected. Only the reversible fluorescent signal occurring immediately after brief extracellular La^{3+} application will represent a true $[Ca^{2+}]_i$ signal which also is accompanied by trichocyst exocytosis (Fig. 4).

As an interim statement, arguments collected so far would largely be compatible with occurrence of a CaSR, though with some restrictions. (i) Internal Ca^{2+} mobilization may precede influx. (ii) Full exocytosis stimulation occurs only by an extracellular La^{3+} effect and, to a much larger extent, by the polycationic secretagogue, AED, and either compound may activate a CaSR-type mechanism. (iii) Mere $[Ca^{2+}]_i$ increase by rapid application of high $[Ca^{2+}]_o$, though causing a swift $[Ca^{2+}]_i$ increase, does not entail any exocytosis. Could this be considered an argument against a CICR-type mechanism? Would it be compatible with a CaSR involvement? Would one have to assume that a positive exocytotic response may be mediated only by Ca^{2+} mobilization from cortical stores as a primary step (instead of a CICR) and enforcement by coupling of a SOC-type mechanisms as a secondary step? As discussed below, the answer largely depends on whether Ca^{2+} release from cortical stores can be proved to occur. We therefore, tried to substantiate this aspect in the following two ways.

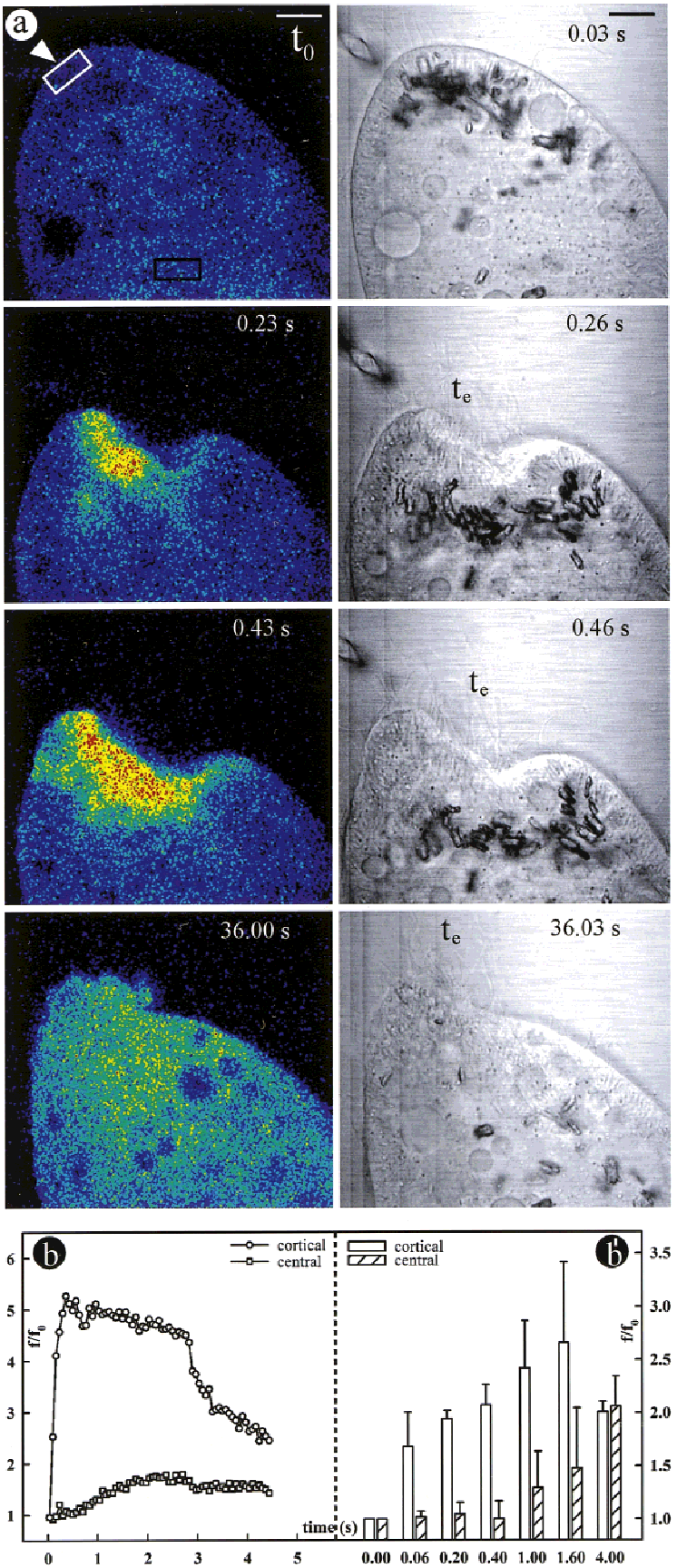

Fig. 4. Quick extracellular La^{3+} (1 mM) application causes a local cortical $[Ca^{2+}]_i$ transient (0.23 and 0.43 sec) and local trichocyst exocytosis seen in transmitted light (0.26 and 0.46 sec) in (*a*). Fluo-3 injected 7S cells in $[Ca^{2+}]_o = 50$ μM, anterior La^{3+} application (arrowhead, close to application pipette seen at top left in figure series at the right side). Note typical cell deformation upon La^{3+}-induced explosive trichocyst release. (*b*) Example of a fast $[Ca^{2+}]_i$ registration by 1λ CLSM, (*b'*) quantitative evaluation. Values ± SEM, $n = 4$. Bars = 10 μm.

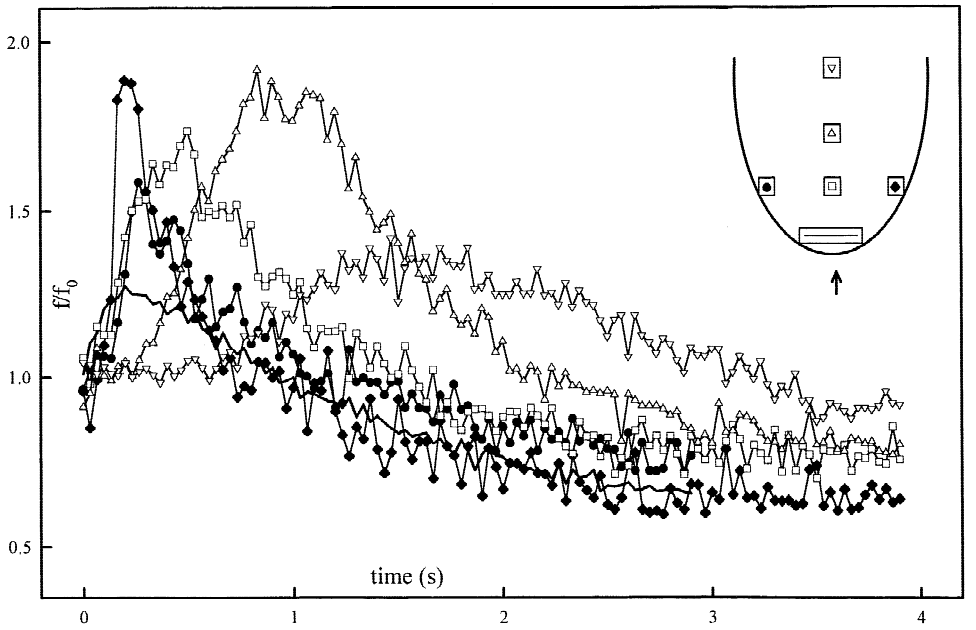

Fig. 5. Simultaneous application of AED (2 μM) and BAPTA (1 mM) by superfusion of a 7S cell at its anterior pole (arrow) causes a $[Ca^{2+}]_i$ transient progressing laterally and centropetally. Data retrieved, at sites indicated in the scheme, by 1λ CLSM Fluo-3 analysis.

Ca^{2+} Mobilization from Cortical Stores

First we analyzed the Ca^{2+} releasing capacity of AED in presence of a very fast Ca^{2+} chelator, BAPTA (Fig. 5). The rationale was to exclude more stringently a CICR mechanism since we previously had used only the slower Ca^{2+} chelator, EGTA [40, 41]. When cells were superfused with a mixture of AED + BAPTA (1 mM), we saw a clear Ca^{2+} signal developing with some delay along the cell surface and, with even more delay, towards the interior of the cell. Since BAPTA is a much smaller molecule than AED and since it binds Ca^{2+} within 0.5 μs time [35], we can reasonably assume complete complexation of $Ca^{2+}{}_o$ during stimulation. Therefore, the Ca^{2+} signal originating in Fig. 5 from the cell cortex must come from subplasmalemmal stores. Application of BAPTA only does not induce any cortical or central $[Ca^{2+}]_i$ changes. When application of a mixture of AED + BAPTA was repeated after 1 min (Fig. 6), Ca^{2+} could no more be mobilized from stores. Evaluation in Table 2 (without $Ca^{2+}{}_o$), in comparison to Table 1 (with $Ca^{2+}{}_o$), reveals that store mobilization may be just one component of the AED triggered Ca^{2+} signal and that this normally would be inforced by Ca^{2+} influx. Clearly $[Ca^{2+}]_i$ signals are generated also at $[Ca^{2+}]_o$ = 30 nM, yet they are larger and they last longer at $[Ca^{2+}]_o$ = 50 μM.

To substantiate even further or to disprove our previous view of partial activation of exocytotic membrane fusion in our cells even at low $[Ca^{2+}]_o$ [41, 67], we now additionally applied the novel Ca^{2+} releasing agent, 4CmC (Figs. 7–12, Table 3). This drug, applied at $[Ca^{2+}]_o$ = 500 μM, yields a Ca^{2+} signal at the site of application, where it also causes trichocyst exocytosis (Fig. 7). Under appropriate conditions, microdomains of $[Ca^{2+}]_i$ increase can be observed (Fig. 8), as described in more detail below. In a typical superfusion experiment with 4CmC + 1 mM BAPTA (Fig. 9) the cortical signal may be dual. An interim decrease after formation of the first peak may perhaps be due to internal chelation of Ca^{2+} by Ca-binding proteins before 4CmC permeates further and generates a second signal, perhaps by activation of ER, in more central regions. Time- and space-dependent analysis of signal spread into deeper layers (Fig. 10) shows this intermediate attenuation at about 15 to 17 μm from the surface. Table 3 summarizes results achieved with different 4CmC concentrations at different $[Ca^{2+}]_o$. Clearly the effect achieved depends on the concentration of each of the components present. With $[Ca^{2+}]_o$ = 30 nM, more 4CmC is required to induce exocytosis which evidently depends on a rapidly rising Ca^{2+} signal.

In an attempt to find optimal conditions for demonstrating involvement of a SOC-type mechanism we exposed cells to 4CmC at low $[Ca^{2+}]_o$, with subsequent addition of high $[Ca^{2+}]_o$ (Fig. 11). If stores were emptied in the first phase at low $[Ca^{2+}]_o$, any signal intensification upon readdition of high $[Ca^{2+}]_o$ could demonstrate occurrence of SOC. This is what we see in Fig. 11, showing an example of a f/f_o recording. In this figure, data points indicated at 0 sec for f/f_o recording correspond to t_o in the scheme at top right, and to the large

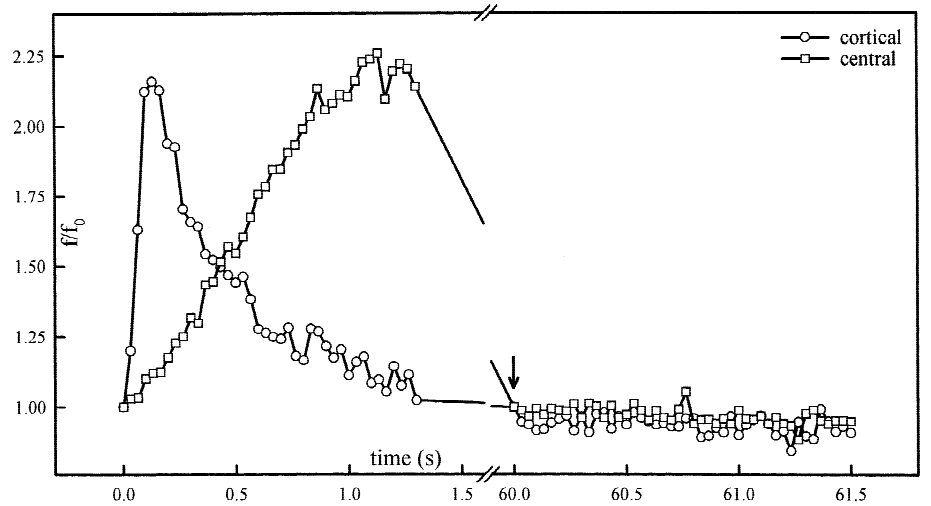

Fig. 6. Simultaneous application of AED (2 μM) and BAPTA (1 mM) by superfusion of a 7S cell at its anterior pole causes a cortical $[Ca^{2+}]_i$ transient which sweeps centropetally after the first application at 0 sec. When repeated after 60 sec, no $[Ca^{2+}]_i$ increase can be registered, for reasons indicated in the text. 1λ CLSM Fluo-3 analysis.

Table 2. $[Ca^{2+}]$ transients generated in Fluo-3 loaded 7S cells in response to superfusion of the anterior cell pole by a mixture of AED (2 μM) + BAPTA (1 mM)

Site of $[Ca^{2+}]_i$ measurement	Maximum f/f_o ratio	$t_{1/2}$ rise time (sec)	Duration of plateau (sec)	$t_{1/2}$ decay time (sec)
Cortical	2.7 ± 0.7	0.16 ± 0.07	0.15 ± 0.11	0.87 ± 0.59
Central	2.3 ± 1.1	0.39 ± 0.15	0.33 ± 0.15	1.52 ± 0.78

Values ± SD, $n = 7$.

Table 3. Cortical $[Ca^{2+}]$ transients and exocytotic response generated in Fluo-3 loaded 7S cells in response to 4CmC at different $[Ca^{2+}]_o$

4CmC concentration (μM), (*n*)	$[Ca^{2+}]_o$ (M)	Cortical $[Ca^{2+}]_i$ rise (f/f_o)				Exocytosis	
		Maximum (1–10 sec)	$t_{1/2}$ rise time (sec)	Decay (30–60 sec)	Recovery (>120 sec)	Contents release	Delay (sec)
100, (3)	5×10^{-5}	2.4 ± 0.5	1.1 ± 0.8	1.9 ± 0.1	1.1 ± 0.1	−	−
500, (5)	5×10^{-5}	4.3 ± 0.8	0.8 ± 0.2	3.6 ± 0.9	1.2 ± 0.1	+	0.5 ± 0.2
500, (3)	3×10^{-8}	3.1 ± 0.4	4.4 ± 3.0	2.5 ± 0.4	1.3 ± 0.2	−	−
1000, (5)	3×10^{-8}	3.2 ± 0.7	0.7 ± 0.2	2.7 ± 0.8	1.8 ± 0.3	+	0.7 ± 0.2

Values + SEM, n = number of cells analyzed.

hatched column (below at bottom right) generated by adding $[Ca^{2+}]_o = 10^{-3}$ M after store depletion by 2 min exposure to 4CmC. Remarkably the response is much smaller with $[Ca^{2+}]_o = 10^{-3}$ M in the absence of any previous 4CmC application (nonhatched column, bottom middle). (The outermost right part of Fig. 11 shows $[Ca^{2+}]_i$ signal decay when $[Ca^{2+}]_o$ is diluted to ~10^{-6} M within 11 sec.) In sum, 4CmC induces a SOC-type reaction immediately following addition of $[Ca^{2+}]_o = 10^{-3}$ M after previous store depletion under conditions of $Ca^{2+}{}_o$ chelation.

We then also explored a SOC-type reaction in a more conventional way, i.e., by application of SERCA inhibitors (Table 4). Yet concentrations to be used particularly with thapsigargicin were very high, similar to those required with yeast [64], and much higher than required for higher eukaryotic systems. CPA at a concentration of 500 μM, as usually applied also with other cells [18, 33, 90], causes a moderate cortical Ca^{2+} signal at $[Ca^{2+}]_o = 30$ nM, but a considerable signal intensification by Ca^{2+} influx at higher $[Ca^{2+}]_o$ (Table 4). When compared with the effects of caffeine [41] and particu-

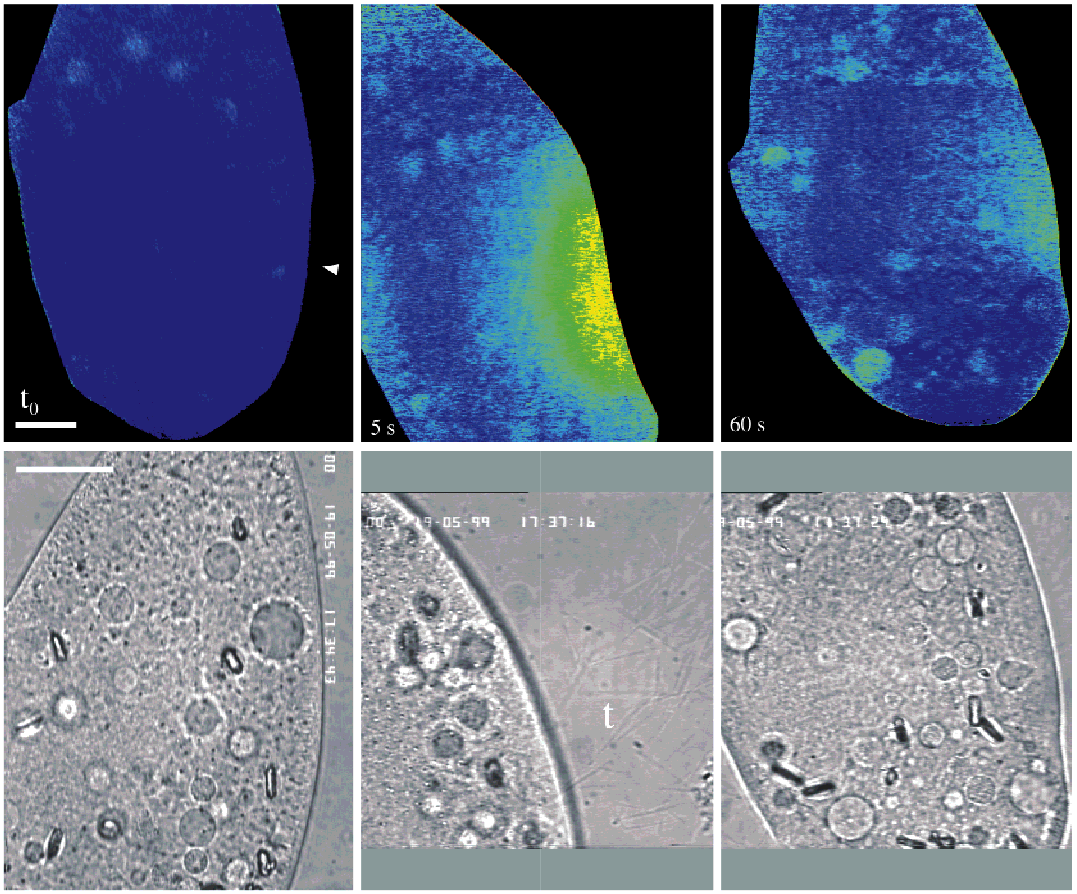

Fig. 7. Local 4CmC application (arrowhead) at t_o causes local transient $[Ca^{2+}]_i$ increase and trichocyst (*t*) exocytosis. Fura-2 loaded 7S cell in $[Ca^{2+}]_o$ = 50 μM, 500 μM 4CmC. Note delay of reaction during permeation of the drug. Bar = 10 μm.

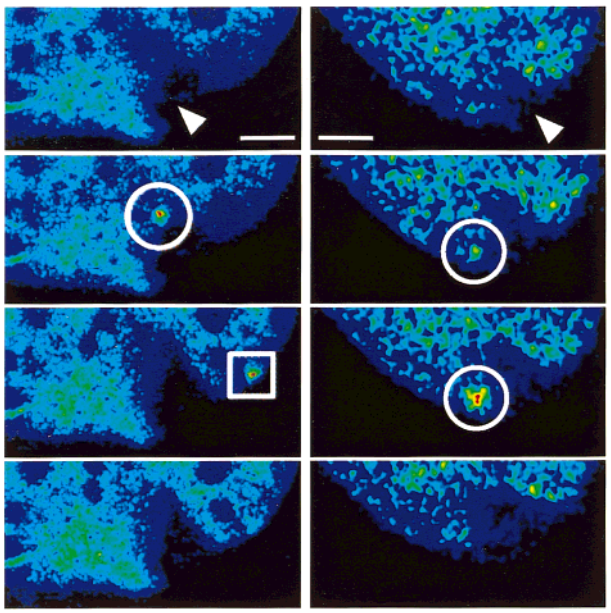

Fig. 8. AED (left) as well as 4CmC (right), applied at arrowheads at $[Ca^{2+}]_o \leq 30$ nM, form microdomain Ca^{2+} signals (circles, squares) lasting ≤33 msec, i.e., the time required to collect an image. Note eventual local cell deformation in the course of vigorous trichocyst expulsion. Bars = 5 μm.

larly of AED [40], $[Ca^{2+}]_i$ values achieved are similar, but rise time is much slower and $[Ca^{2+}]_i$ recovery values are still higher after removing SERCA inhibitors. Thus, Table 4 indicates slow and only partially reversible effects of the inhibitors tested on $[Ca^{2+}]_i$ homeostasis.

Evidence of $[Ca^{2+}]_i$ Microdomains in *Paramecium*

We looked whether AED or the new stimulant, 4CmC, would generate $[Ca^{2+}]_i$ microdomains. Fig. 8 shows two such domains formed by AED and one by 4CmC at $[Ca^{2+}]_o \leq 30$ nM, i.e., intensely fluorescent spots of ≤0.5

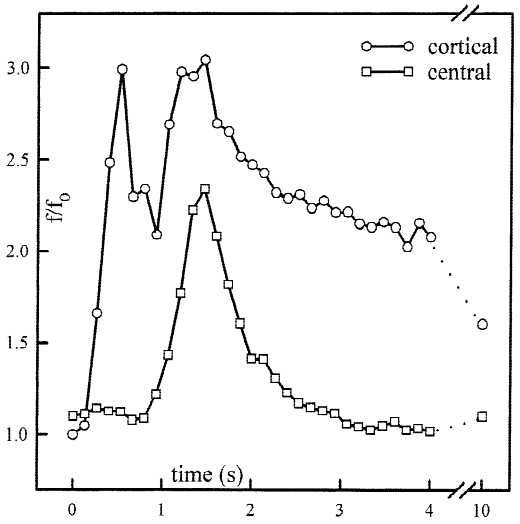

Fig. 9. Typical example of a $[Ca^{2+}]_i$ signal caused by anterior application of a mixture, 4CmC (500 μM) + 1 mM BAPTA, in a Fluo-3 loaded 7S cell. Note formation of a dual signal peak in cell cortex and signal propagation into central regions. Remarkably the signal forms at $[Ca^{2+}]_o$ = 30 nM, due to cortical store mobilization.

μm diameter. During stimulation microdomains are visible for only 33 msec, i.e., they have a shorter life-time than required for collecting an image. Eventually a cell is deformed at such sites — an infallible indication of vigorous trichocyst ejection. We also ascertained for 4CmC occurrence of exocytotic membrane fusion by the fluorescent dye, FM1-43 (*data not shown*).

Evidence of Involvement of Unspecific Cation Channels

Finally, we determined, for AED and 4CmC, involvement of unspecific cation channels during Ca^{2+} influx, shown above to accompany store mobilization, by Mn^{2+}-quenching of Fura-2 fluorescence. The rationale of Fig. 12 is that Fura-2 has a Ca^{2+}-sensitive emission at 380 nm which decreases as $[Ca^{2+}]_i$ increases. In contrast, the Ca^{2+}-insensitive emission at 360 nm is quenched by Mn^{2+} when entering cells from the outside medium [13, 21]. Since we see a cortical Ca^{2+} signal which is quenched in presence of Mn^{2+}, this standard assay clearly indicates involvement of unspecific cation channels during AED or 4CmC stimulation, respectively.

Discussion

No Evidence of a CICR-type Mechanism

Alveolar sacs are tightly coupled to the cell membrane at a distance of ~15 nm [70] and, thus, remind SR-sarcolemma connection in striated muscle cells [23]. This arrangement mediates CICR in vivo in cardiac cells, though only in vitro in skeletal muscle cells [56, 85, 91]. Also some neurons show such tight structural [58] or functional coupling [92] of cortical Ca-stores to the cell membrane. Up to now caffeine was the only "conventional" drug capable of releasing Ca^{2+} from alveolar sacs [41]. Now we show the same effect, at the usual submillimolar concentrations, with 4CmC – a much more specific activator of ryanodine-sensitive Ca^{2+} release channels in SR (*see* Introduction). So far we have most clear evidence against CICR involvement in our system only from in vitro experiments [48, 102]. Alternatively, mobilization of Ca^{2+} from cortical stores by AED in absence of $Ca^{2+}{}_o$ can activate a large number of exocytosis sites in vivo [19, 67]. Therefore, mobilization of Ca^{2+} from alveolar sacs is considered the primary step during AED stimulation. Rapid and intense cortical $[Ca^{2+}]_i$ transients induced by increased $[Ca^{2+}]_o$ application does not entail exocytosis, as we find. A priori this would represent a stringent argument against involvement of CICR in trichocyst exocytosis, but corollaries of this observation have to be dissected in more detail, below.

Which Established Channels May Deliver Ca^{2+} from the Outside?

Based on results with d4-500r, we exclude ciliary voltage-dependent Ca^{2+} channels. Involvement of any such channels in the somatic cell membrane [86] is also unlikely, mainly for the following reason. Increasing $[Ca^{2+}]_o$, while unexpectedly producing the well known "Ca paradox" in *Paramecium*, i.e., membrane depolarization with increased foreward swimming [53], causes a strong cortical Ca^{2+} signal, but electrical depolarization does not cause trichocyst release [20]. Similarly electrical hyperpolarization does not induce exocytosis [20]. Therefore, hyperpolarization-sensitive Ca^{2+} channels, as specified by Preston et al. [77, 78] are unlikely to be involved, particularly since their typical inhibition by Ba^{2+} or amiloride does not apply, e.g., to AED stimulated exocytosis and to electrical current responses to AED [20]. Since we always observe, with AED or with 4CmC, equal $[Ca^{2+}]_i$ transient formation and exocytotic reaction over the entire cell surface, we also exclude anteriorly enriched mechano-sensitive Ca^{2+} channels [59] or Ca^{2+}-conducting Na^+ channels [82]. Still to be considered are unspecific cation channels of the type described in *Paramecium* by Saitow et al. [84]. The conductance induced by AED or 4CmC does carry Mn^{2+}, as we show in Fig. 12. Channels of this type are unspecific cation channels considered to participate in SOC-type mechanisms [54], as discussed below.

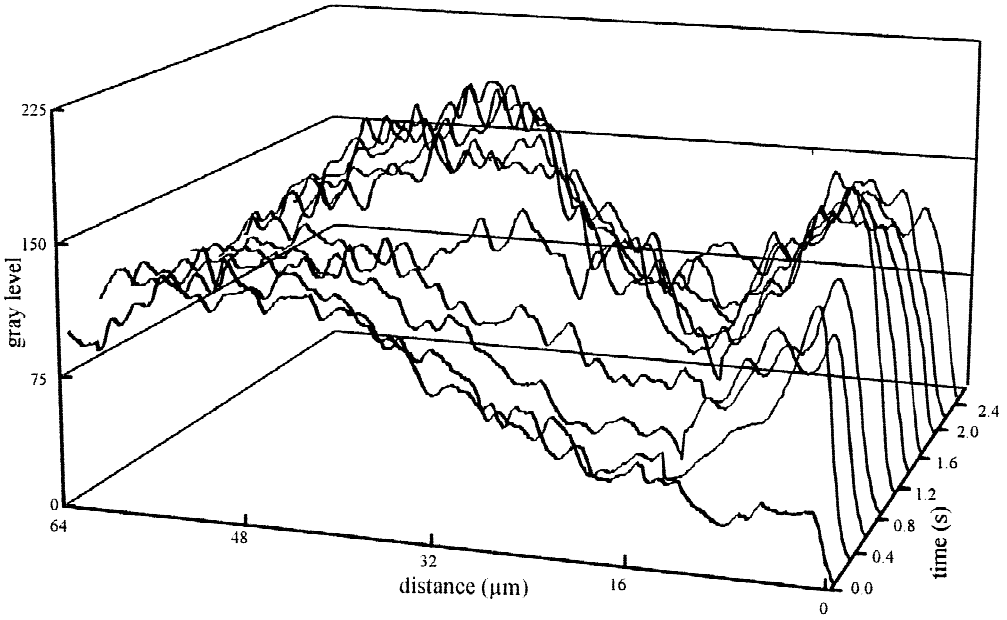

Fig. 10. Time-space resolved signal development at different distances from the cell surface under conditions specified in Fig. 9.

CaSR AND/OR SOC IN *PARAMECIUM*?

In the context discussed above with regard to unspecific cation channels in the cell membrane, CaSR-coupled conductances are activated by lanthanides, like Gd^{3+} or La^{3+}. In some higher eukaryotes, lanthanides not only cause Ca^{2+} release from IP_3-insensitive, ryanodine-sensitive stores, but they also activate, from the outside, unspecific Ca^{2+} conducting channels [1, 9, 10, 24, 80, 94, 100]. However, due to different extra- and intracellular effects of lanthanides, global effects finally observed may greatly vary depending on cell type and protocol used. In *Paramecium*, brief extracellular La^{3+} application produces a cortical $[Ca^{2+}]_i$ transient (Fig. 4) and such an effect has been shown in anterior pituitary cells to be due to activation of a CaSR [87].

Theoretically this should also hold for high $[Ca^{2+}]_o$ application. Though yielding a $[Ca^{2+}]_i$ transient with the usual characteristics, just as obtained with La^{3+} or AED, this entails no exocytosis. The response is the same as with veratridine stimulation at high $[Ca^{2+}]_o$ [7]. We assume exocytosis inhibition by too long exposure (~1 min) to high $[Ca^{2+}]_o$, i.e., 10 mM, and we attribute this effect to the well established membrane stabilizing effect by Ca^{2+} [22] caused by rigidification of membrane lipids in the course of equally well established Ca^{2+}-mediated lipid phase transition [61]. In this context one can easily explain absence of exocytosis (membrane fusion) after 1 min application of high $[Ca^{2+}]_o$, in despite of formation of a regular $[Ca^{2+}]_i$ transient. As previously shown [67], high $[Ca^{2+}]_o$ applied for $\leq$0.5 sec or simultaneously with the trigger agent has no such effect, thus explaining our current results with quick application of $La^{3+}{}_o$ (*see below*). For the intriguing effects of La^{3+} in SOC analysis, see review by Lewis [49].

Similar CaSR-related activities are achieved by exogenous polyamine stimulation [25, 79] which also induces exocytosis in a variety of cell types, like in neuronal and in pituitary cells [87] which also contain cortical Ca stores [92]. Different types of CaSR are recognized to be widely distributed, e.g., in leukocytes, pancreatic cells or CNS neurons [11, 93, 100], let alone "professional" Ca^{2+}-regulating cell types, like osteocytes [98, 102]. In some systems, polyamines can directly activate different plasmalemmal channels, including Ca^{2+} channels [4, 25, 95, 97, 103]. If this would apply to *Paramecium*, unspecific cation channels of a similar type as described by Saitow et al. [84] could theoretically be directly activated by AED, even if a CaSR would not be involved. However, the capability of AED to produce a cortical Ca^{2+} signal again points toward a mechanism involving a CaSR to which an unspecific cation channel activity could be connected.

By implication, a CaSR-type function has been postulated previously already for ciliated protozoa, *Paramecium* and *Tetrahymena*, though under different designation. Hennessey et al. [29], using lysozyme (pI ~10), confirmed that highly positively charged compounds induce exocytosis, just like some other polycations with sufficiently densely packed cationic groups [72]. However, lysozyme may also affect ciliary voltage-dependent Ca^{2+} channels since it acts as a repellent at 10^3 times

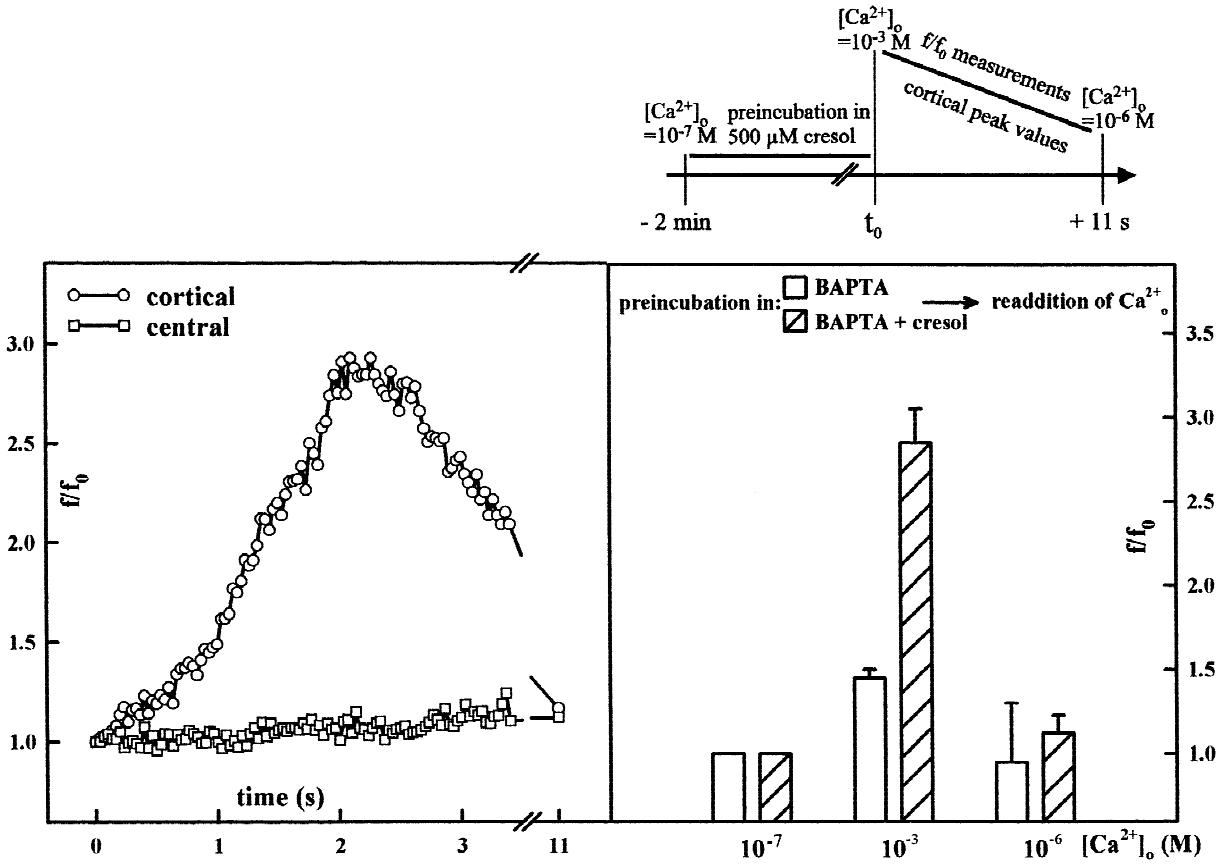

Fig. 11. Deprivation of Ca^{2+} from stores in Fluo-3 injected 7S cells by 4CmC preincubation (2 min) at $[Ca^{2+}]_o = 10^{-7}$ M induces massive Ca^{2+} influx upon addition of 10^{-3} M $Ca^{2+}{}_o$. Scheme at top right: Mobilization of Ca^{2+} from stores by 2 min 4CmC preincubation in presence of BAPTA was followed by readdition of $[Ca^{2+}]_o = 10^{-3}$ M, before $[Ca^{2+}]_o$ was allowed to drop to 10^{-6} M during a subsequent time of 11 sec. Bottom left: example of a $[Ca^{2+}]_i$ transient generated at t_o (as indicated in scheme at top right). Bottom right: Cortical $[Ca^{2+}]_i$ signal amplitudes (hatched columns: with 4CmC, clear columns: without 4CmC preincubation) were obtained at $[Ca^{2+}]_o = 10^{-7}$ M (left column pair), after adding $[Ca^{2+}]_o = 10^{-3}$ M (middle column pair), or after decay of $[Ca^{2+}]_o$ to 10^{-6} M (right column pair). Values ± SEM, $n = 4$ (hatched columns) or 2 (clear columns).

Table 4. $[Ca^{2+}]_i$ transients generated in Fluo-3 loaded 7S cells by application of inhibitors of the SERCA-type Ca^{2+}-pump

Inhibitor concentration	$[Ca^{2+}]_o$ (M)	*n*	Cortical $[Ca^{2+}]_i$ transient				
			Rest (nM)	Maximum (nM)	Rise time (sec)	Recovery (nM)	Decay time (sec)
Thapsigargicin, 100 µM	5×10^{-5}	6	101 ± 19	225 ± 11	42 ± 14	147 ± 21	20–60
Cyclopiazonic acid, 500 µM	5×10^{-5}	4	117 ± 17	680 ± 81	20 ± 7	375 ± 68	20–60
Cyclopiazonic acid, 500 µM	3×10^{-8}	3	44 ± 4	133 ± 7	16 ± 4	89 ± 11	20–60

Values ± SEM, *n* = number of cells analyzed. For comparison with data achieved with AED and caffeine, respectively, see refs. [40, 41] and text.

lower concentration than required for exocytosis [29, 38], i.e., millimolar *vs.* micromolar, as required with AED [72].

These effects of lysozyme can be abolished by the aminoglycoside, neomycin, 1 mM [29]. However, any effects of this drug are difficult to judge since neomycin can permeate into cells within 1–2 min [55, 65] and then exert its "classical" function by inhibiting IP_3-mediated Ca^{2+} release, although we have no evidence of the latter mechanism in *Paramecium* [48]. In pilot experiments, 2 min extracellular application of neomycin before AED stimulation inhibits trichocyst exocytosis. This may occur from the inside, though unrelated to IP_3. One possibility is inhibition by neomycin of communication be-

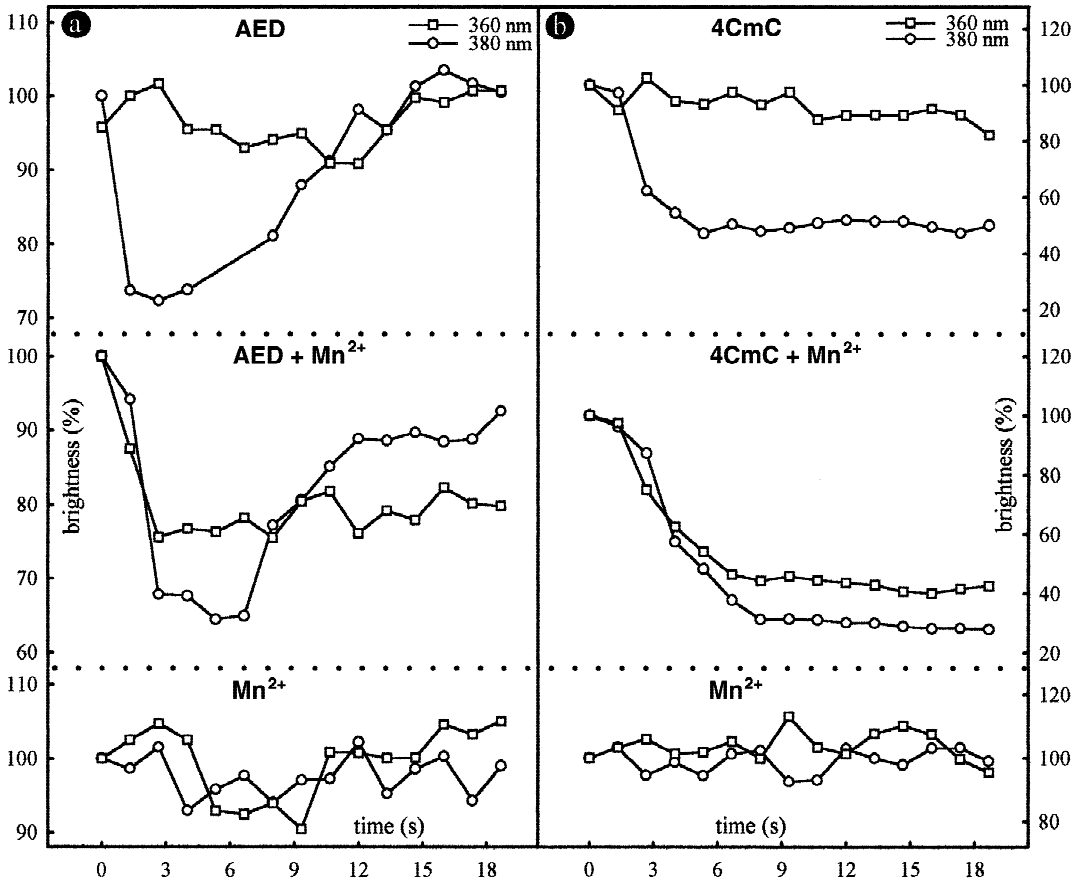

Fig. 12. Evaluation of cortical regions of Fura-2 loaded cells shows Ca^{2+}-mediated fluorescence decrease (indicating $[Ca^{2+}]_i$ increase), but Mn^{2+}-mediated fluorescence quenching, indicating involvement of unspecific cation channels in the cell membrane, in response to AED (*a*) or 4CmC (*b*) at the wavelengths indicated. For AED (*a*) we used nd9-28°C cells, known to yield similar $[Ca^{2+}]_i$ signals as 7S cells, but without cell dislocation (due to vigorous trichocyst expulsion in 7S). In (*b*), 7S cells were exposed to 4CmC. Concentrations used were Ca^{2+}_o = 50 μM, ±$MnCl_2$ = 5 mM, AED = 2 μM (left), 4CmC = 500 μM (right).

tween cell membrane and cortical stores, as found in muscle cells [99]. Would our conclusions be compatible with previous extensive experiments by other groups applying neomycin to ciliates? In *Paramecium*, neomycin also inhibits ciliary voltage-dependent Ca^{2+} currents [26], an effect clearly unrelated to exocytosis (Fig. 2). The "receptor potential" ascribed by Hennessey et al. [29] to activation of a "lysozyme receptor" may be interpreted as an effect on ciliary Ca^{2+} channels since we, too, have recognized occasional brief depolarization of some cells in response to AED, but only in connection with occasional occurrence of ciliary reversal [20]. Our interpretation agrees with activation of widely different plasmalemmal cation channels by exogenous polyamines [4, 36, 95, 97, 103] and its inhibition by neomycin, or with activation of a CaSR of a broad-range activity by polyamines. It is also unlikely to have a different receptor for AED (EC_{100} = 1 μM, [72]) or lysozyme (EC_{100} ≥ 100 μM, [29]). Neomycin can also activate CaSR in kidney cells [81] and Ca^{2+} influx in liver cells (devoid of voltage-dependent Ca^{2+} channels), as shown by Hughes et al. [32]. However, as in our system, neomycin does not activate CaSR in pancreatic cells [10]. Furthermore, one has to differentiate more stringently between extra- and intracellular effects occurring during neomycin application. As stated, neomycin can permeate into cells when applied for min [55, 65]. It then can block some intracellular functions, like communication between cell membrane and SR in striated muscle [99] or in smooth muscle cells [65], as it could in our unpublished results with *Paramecium*. Particularly the multiple effects of neomycin, e.g., in pyramidal neurons [50], restrain us from any further interpretation of any effects seen in ciliates. Along these lines it would be desirable to further characterize the "lysozyme receptor" isolated by Kuruvilla and Hennessey [45]. Interestingly in osteo-

clasts an extracellular domain of the CaSR has been demonstrated to resemble in part a ryanodine receptor [101].

$[Ca^{2+}]_i$ Microdomains and Possible Site-Directed Ca^{2+} Flux

In muscle cells, Ca^{2+} imaging by fluorochromes allowed to observe $[Ca^{2+}]_i$ microdomains originating from SR and to follow their temporal development by rapid line scans. Examples of values thus determined for size and duration are about 0.5 μm/5–20 msec [14], 1.4 μm/30 msec [17] and 1.5 μm/13 msec [47]. Although all values depend on the cell and assay conditions used, of course, they are well comparable to the microdomains we see in response to AED or 4CmC (Fig. 8). While their duration in *Paramecium* cannot be easily determined by line scans, their lifetime must be ≤33 msec, i.e., below the time required for image recording. Our present data are also compatible with the range of the lifetime ($t_{1/2}$ = 21 msec) of Ca^{2+}-activated "minimal" currents occurring in parallel to exocytosis of single trichocysts [19]. Though we could also not yet determine any more precise relationship between microdomain formation and trichocyst release sites, eventual simultaneous local cell deformation strongly suggests such a correlation. Similarly, we did not analyze induction of membrane fusion in response to 4CmC and AED + BAPTA application, respectively. This may be inferred, however, from similar experiments with caffeine [41] and AED + EGTA [67].

Conclusions

Our present data and those achieved by others with polycationic compounds appear compatible with a CaSR, connected to a SOC-type mechanism, as we postulate throughout this paper on the basis of widely different evidence. Mobilization of Ca^{2+} from alveolar sacs has been ascertained by application of 4CmC. From the $[Ca^{2+}]_i$ microdomains seen during AED or 4CmC stimulation we derive the possibility of a site-directed Ca^{2+}-flux at sites where the alveolar sacs system is interrupted to accomodate a trichocyst. Characterization of molecular components involved in AED stimulation remain open so far.

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