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Refilling of cortical calcium stores in *Paramecium* cells: in situ analysis in correlation with store-operated calcium influx

I. Mohamed[1], M. Husser, I. Sehring, J. Hentschel, C. Hentschel, H. Plattner*

Universität Konstanz, Fachbereich Biologie, P.O. Box 5560, 78457 Konstanz, Germany

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Abstract

This is the first thorough study of refilling of a cortical calcium store in a secretory cell after stimulation in which we combined widely different methodologies. Stimulation of dense-core vesicle ("trichocysts") exocytosis in *Paramecium* involves a Ca^{2+}-influx" superimposed to Ca^{2+}-release from cortical stores ("alveolar sacs" (ASs)). In quenched-flow experiments, membrane fusion frequency rose with increasing $[Ca^{2+}]_o$ in the medium, from ~20–25% at $[Ca^{2+}]_o \leq 0.25\ \mu M$ to 100% at $[Ca^{2+}]_o$ between 2 and 10 μM, i.e. close to the range of estimated local intracellular $[Ca^{2+}]$ during membrane fusion. Next, we analyzed Ca^{2+}-specific fluorochrome signals during stimulation under different conditions. Treatment with actin-reactive drugs had no effect on Ca^{2+}-signaling. In double trigger experiments, with BAPTA in the second secretagogue application (BAPTA only for stimulation and analysis), the cortical Ca^{2+}-signal (due solely to Ca^{2+} released from cortical stores) recovered with $t_{1/2}$ ~65 min. When ASs were analyzed in situ by X-ray microanalysis after different trigger times ($+Ca^{2+}{}_o$), $t_{1/2}$ for store refilling was similar, ~60 min. These values are similar to previously measured $^{45}Ca^{2+}$-uptake by isolated ASs. In sum we find, (i) exogenous Ca^{2+} increases exocytosis/membrane fusion performance with $EC_{50} = 0.7\ \mu M$, (ii) Ca^{2+}-signaling in this system is not sensitive to actin-reactive drugs, and (iii) refilling of these cortical calcium stores goes on over hours and thus is much slower than expected.

Keywords: Calcium; Ciliates; Exocytosis; *Paramecium*; Secretion

1. Introduction

Ca^{2+} is a second messenger for many processes in eukaryotic cells [1,2], including exocytosis [3–5], from protozoa to mammals. Thereby, Ca^{2+} may originate from influx from the outside medium and/or from release from internal stores. One possibility is that, in a first step, a secretagogue causes mobilization of Ca^{2+} from stores located in the cell cortex, superimposed by a Ca^{2+}-influx as a second step [6]—a process called "store-operated Ca^{2+}-influx" (SOC). Signal coupling between the two steps is poorly understood [5–8]. Depletion of stores is followed by refilling via the activity of a SERCA-type Ca^{2+}-pump [9,10], whereby SERCA stands for sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase.

Paramecium, which belongs to the ciliated protozoa and thus is closely related to parasitic species, like *Plasmodium* and *Toxoplasma* [11], is an established model system to study exocytosis of dense-core (DC) vesicles called "trichocysts" [12–14]. Meanwhile, we have elucidated in *Paramecium* some essential features of Ca^{2+}-signaling during exocytosis [15]. Cells can be stimulated for trichocyst exocytosis by the polyamine secretagogue aminoethyldextran (AED) at 1 μM concentrations, usually at an extracellular Ca^{2+}-concentration ($[Ca^{2+}]_o$) up to 1 mM [16]. This is paralleled by Ca^{2+}-specific fluorochrome signals [17,18]. Due to the occurrence of up to 1000 preformed primed exocytosis sites, these cells react by much faster and much more synchronous exocytosis [12,19] than any other DC vesicle system [4]. We have developed a quenched-flow device for quantitative analysis of membrane fusion events by subsequent quantitative freeze-fracture/electron microscope (EM) analysis [20]. Under the standard conditions specified earlier, exocytotic events occur with an apparent $t_{1/2} = 57$ ms, followed by exocytosis-coupled endocytotic membrane resealing ($t_{1/2} = 126$ ms), all being accomplished within 350 ms [19,20]. When $[Ca^{2+}]_o$ is reduced to a value slightly below intracellular levels at rest ($[Ca^{2+}]_i$) = 65 nM [17] up to ~25 or slightly more of the preformed exocytotic sites

* Corresponding author. Tel.: +7531-88-2228; fax: +7531-88-2245.
E-mail addresses: ihabkmohamed@yahoo.com (I. Mohamed), helmut.plattner@uni-konstanz.de (H. Plattner).
[1] Present address: Department of Zoology, Faculty of Science, Ain-Shams University, Cairo, Egypt.

can still undergo membrane fusion (this paper, in accordance with previous analyses [21,22]). However, increasing $[Ca^{2+}]_o$ clearly accelerates all steps of the exo-endocytotic cycle [23]. In fact, a significant Ca^{2+}-influx normally accompanies AED-triggered trichocyst exocytosis [24,25]. More scrutinized analyses have established the occurrence of an SOC-type mechanism in *Paramecium* [18,26], as discussed in more detail in the following.

The regular design of the cortex of a *Paramecium* cell contains not only regularly arranged trichocyst docking sites for immediate release, but also intermittent "alveolar sacs" (ASs) which encircle each trichocyst (see [14,15]). ASs are flat sacs, ~100 nm wide, which represent cortical Ca^{2+}-stores [27,28] attached at the cell membrane at a distance of ~15 nm. We found by energy-dispersive X-ray (EDX) microanalysis that up to 80% of the stored Ca^{2+} is released upon AED stimulation [29]. Channels involved are only partially identified—they respond to the ryanodine-receptor activators, caffeine and 4-chloro-*meta*-cresol (4Cl-m-C) [18], but not to inositol 1,4,5-trisphosphate ($InsP_3$) [28]. ASs possess a SERCA-type pump [30,31] located in that part of the ASs which faces the cell center, as we found by immuno- [32] and green fluorescent protein (GFP)-transfection labeling [33].

In order to re-establish the capability to respond to external stimuli, stores have to be refilled. While there are ample data on refilling of isolated fragments derived from intracellular Ca^{2+}-stores, like ER and SR, measurements in situ have not been reported for any (cortical) store in any secretory system. Here, we have combined widely different methods, including (i) stimulation of synchronous exocytosis, combined with quenched-flow/freeze-fracture analysis at varying $[Ca^{2+}]_o$, (ii) EDX analysis of total calcium concentrations ([Ca]) contained in ASs at different times after stimulation, (iii) fluorochrome analyses, combined with double trigger experiments. When performed at low $[Ca^{2+}]_o$, this allows to analyze the contribution selectively of store activation after different refilling times, while the SOC-type component is seen only during stimulation at high $[Ca^{2+}]_o$. Finally, (iv) we tested the applicability to our system of the hypothesis (derived from higher eukaryotic cells) that F-actin may bind Ca^{2+}-stores to the plasma membrane and thus mediate an SOC-mechanism [5,7,34–36]. For this purpose, we used drugs shifting the G/F-actin equilibrium.

2. Materials and methods

2.1. Cell materials

Wildtype cells strain 7S were grown as described [17]. Normally, $[Ca^{2+}]_o$ was 1 and 0.5 mM after mixing with the secretagogue, AED, except when indicated that EGTA or BAPTA were added during stimulation, to produce $[Ca^{2+}]_o$ ~30 nM (slightly below $[Ca^{2+}]_i$ at rest [17]). Note that cells were exposed to Ca^{2+}-chelators only briefly, e.g. for 0.5 s during quenched-flow or during simultaneous application with the trigger agent. Under these conditions of low $[Ca^{2+}]_o$, chelators were removed immediately after stimulation.

2.2. Dependency of exocytotic membrane fusion on extracellular Ca^{2+}

For analyzing membrane fusion on freeze-fracture replicas, cells were stimulated with AED in a quenched-flow apparatus [20]. Since this served the detailed analysis in the EM of the dependency of membrane fusion on the availability of extracellular Ca^{2+}, $[Ca^{2+}]_o$ was quickly adjusted to different levels in the quenched-flow device, by mixing cells for 0.5 s with EGTA (like in previous experiments [21,23]) in a first mixing chamber, before AED stimulation was performed for 80 ms in a second mixing chamber. Further, processing and quantitative analysis were as described previously [23].

2.3. Double trigger fluorochrome analyses of intracellular Ca^{2+}-transients

After injection of Fura Red (both from Molecular Probes, Eugene, OR) for double wavelength recording as described [17], exocytosis was stimulated with AED [16], eventually with the addition of BAPTA. In double trigger experiments, cells were stimulated by AED twice. The second time was at different time intervals after the first trigger and after a shortly preceding Fura Red injection. Fluorochrome experiments with the ultrafast Ca^{2+}-chelator, BAPTA, added to the trigger medium were designed to adjust $[Ca^{2+}]_o$ to ~30 nM, i.e. slightly below $[Ca^{2+}]_i$ at rest [17]. Changes of cortical $[Ca^{2+}]_i$ in the area closest to AED application were evaluated, also as described [17,18]. As mentioned, Ca^{2+}-chelators were eventually added only during stimulation, in quenched-flow experiments 0.5 s before. The Ca^{2+}-chelator was present in fluorochrome recordings under conditions of low $[Ca^{2+}]_o$. In double trigger experiments at low $[Ca^{2+}]_o$, with a time interval of up to 1 h, the chelator was added only during —but not before—the second secretagogue application. No deleterious effects could be recognized during the brief time of Ca^{2+}-chelator application used.

2.4. EDX analysis of total calcium content in ASs

For EDX, cells contained in their medium with $[Ca^{2+}]_o$ = 1 mM were stimulated for different times with an equal part of 2 μM AED (removed in long-time stimulation experiments) in the quenched-flow device [20,23] for cryofixation in melting propane and subsequent freeze-substitution under conditions appropriate to retain Ca^{2+} in place according to the method of Poenie and Epel [37], modified as previously described [21,26,29,38]. Then, cells were embedded in Spurr's resin and semithin sections of 0.5 μm were ana-

lyzed in an analytical EM, type Zeiss/Leo EM912 Omega operated in the STEM mode. Analysis of strictly cross-cut ASs (width ~98 nm) was done in the spot mode (diameter at entry: 63 nm, exit: 72 nm) and net Ca Kα peaks were quantified as previously described [29,38].

2.5. Actin-reactive drugs

We used the following drugs: cytochalasin B (CytB) from Sigma (Deisenhofen, Germany) and latrunculin A (LatA) as well as jasplakinolide (Jas) from Molecular Probes (Eugene, OR, USA). They were dissolved as a stock solution in DMSO which was diluted to the concentrations indicated, resulting in a DMSO concentration of <2% (without side-effects in *Paramecium*).

Considering the limited knowledge of the effect of such drugs in *Paramecium* and in order to find out appropriate drug concentrations, we first tested effects on phagocytosis—the best known process requiring F-actin [39–41]. First, *Saccharomyces cerevisiae* cells were stained by boiling in Congo Red solution. When ingested by *Paramecium*, a color change to blue indicates phagocytotic internalization. We counted the number of yeast cells phagocytosed within 3 min by 30 cells after exposure to the respective drugs for the time indicated. Reversibility of phagocytosis inhibition was also tested in wash-out experiments. Second, using appropriate CytB concentrations, we analyzed cortical Ca^{2+}-transients generated by a single AED stimulus at $[Ca^{2+}]_o$ = 1 mM. For digital $[Ca^{2+}]_i$ analyses, see the earlier description.

3. Results

3.1. Dependency of exocytotic membrane fusion on $[Ca^{2+}]_o$

In the first mixing chamber of the quenched-flow apparatus, we briefly (0.5 s) adjusted $[Ca^{2+}]_o$ to values between 30 nM and 0.5 mM, followed by AED stimulation for 80 ms in the second mixing chamber. This is the time normally required to accomplish membrane fusion at all potential trichocyst exocytosis sites [20]. Subsequent quantitative EM evaluation of freeze-fracture replicas is presented in Fig. 1. Up to $[Ca^{2+}]_o$ = 0.25 μM, a basal level of 22–26%, average ~25%, of sites competent for exocytosis (as recognizable by their ultrastructural organization [23]) undergo fusion. From >0.25 μM on the frequency of fusion events increases to near plateau values at $[Ca^{2+}]_o$ = 2 μM, with only 14% additional increase up to $[Ca^{2+}]_o$ = 0.5 mM. Half-value of $[Ca^{2+}]_o$ for the increase is 0.6 and 0.7 μM, respectively, for the range of $[Ca^{2+}]_o$ between 0.25 and 2 μM, and for the range between 0.25 and 0.5 mM, respectively (Fig. 1).

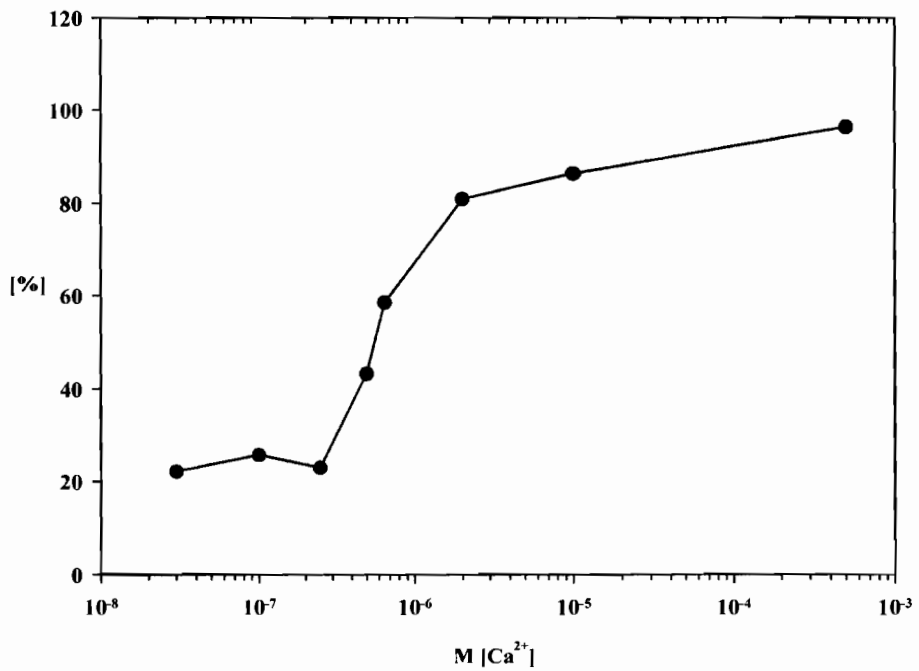

Fig. 1. Increase of exocytotic membrane fusion with increasing $[Ca^{2+}]_o$ during AED stimulation. In the quenched-flow apparatus, $[Ca^{2+}]_o$ has been adjusted to different values 0.5 s before and during 80 ms AED stimulation, by adding EGTA at different concentrations (yielding the calculated $[Ca^{2+}]_o$ values indicated; see "Section 2" and [23]). EM analysis of typical freeze-fracture/ultrastructural transformations is presented as medians of trichocyst docking sites described in Refs. [20,23]. Note that a fairly constant proportion (~25%) of membrane fusion occurs already at very low $[Ca^{2+}]_o$, increasing from ~0.25 μM on to a plateau at >2 μM. Number of cells (N) and of fusion sites (n) analyzed per data point are, in the order of rising $[Ca^{2+}]_o$ indicated on the basis, as follows: N = 28, 24, 29, 25, 30, 44, 29, and 32; n = 460, 443, 394, 548, 806, 410, and 636 (from $[Ca^{2+}]_o = 3 \times 10^{-8}$–$5 \times 10^{-4}$ M).

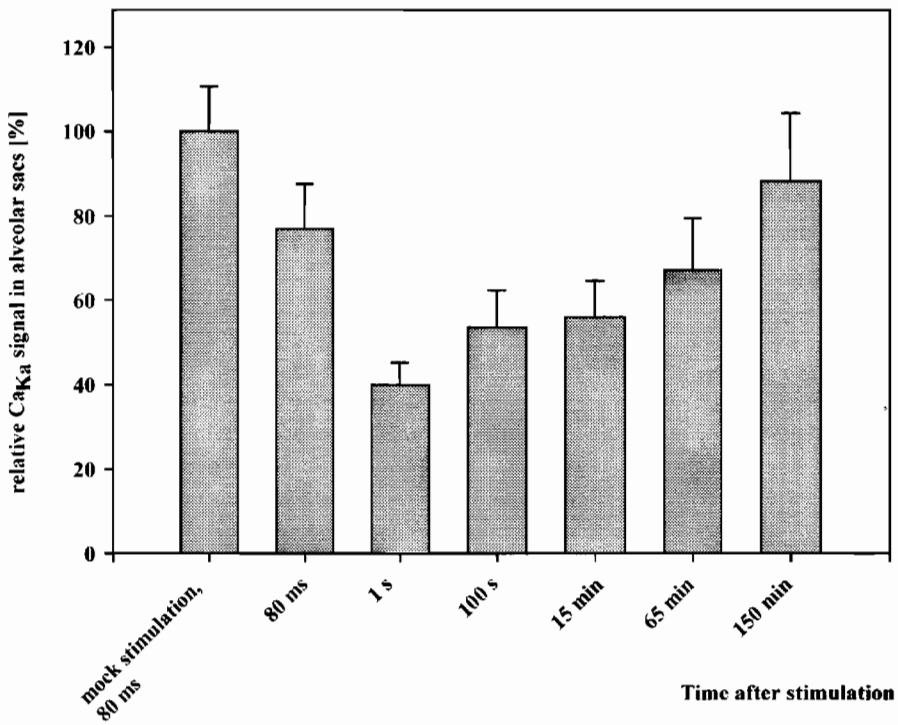

Fig. 2. Relative intensity of Ca Kα signals (normalized to control = 80 ms mock stimulation with buffer), corresponding [Ca], recorded in ASs by EDX at different times after AED-stimulated exocytosis. Values for controls and 150 min refilling are significantly different from value after 80 ms stimulation ($P < 0.05$); bars = standard error of the mean (S.E.M.). Note that Ca^{2+}-release, which is only partial, is followed by rather slow refilling. From left to right: N (cells analyzed) = 4, 4, 6, 6, 5, 4, and 4; n (ASs analyzed) = 16, 26, 24, 30, 40, 30, and 28.

3.2. Analysis of AS refilling by EDX

The state of filling and refilling after stimulation could be directly monitored by quantitative evaluation of the Ca Kα net peaks contained in the EDX spectra recorded in the spot mode. These values represent concentrations of total calcium, [Ca], i.e. free and bound. Data were collected from ASs in many cells, as indicated in Fig. 2. In agreement with previous analyses, AED caused rapid depletion of ASs of their stored calcium, from a calibrated original value of total [Ca] = 43 mM in ASs before stimulation to ~25% residual [Ca] after 1 s AED stimulation [29]. After full stimulation, AED was removed and cells were kept under "normal" culture conditions ($+Ca^{2+}{}_o$, see "Section 2"). Aliquots were subjected to fast-freezing, freeze-substitution, and EDX analysis at different times after AED stimulation. Under these conditions, [Ca] in AS was seen to increase with a half-time of ~60 min, as one can derive from Fig. 2.

3.3. Double trigger experiments and fluorochrome analysis

We have compared local cortical fluorochrome signals achieved in Fura Red injected cells at the site of AED application with an extracellular micropipette (see [17]), as documented in Fig. 3. Before this step, cells in suspension had been fully stimulated by AED at $[Ca^{2+}]_o$ = 1 mM, AED was removed and Fura Red had been injected immediately before cells were immobilized for local application of a second AED stimulus at different time intervals after the first stimulus. The second stimulus was applied under two different conditions, (i) at $[Ca^{2+}]_o$ = 1 mM, in experiments further on designated "$+Ca^{2+}{}_o$" (Fig. 3a–e), and with 1 mM BAPTA added to yield $[Ca^{2+}]_o$ ~30 nM, designated "$-Ca^{2+}{}_o$" (Fig. 3f–h). Note that $[Ca^{2+}]_i$ in unstimulated cells is ~65 nM [17]. Therefore, any SOC-type mechanism can occur only in experiments $+Ca^{2+}{}_o$, but not in those $-Ca^{2+}{}_o$. We also analyzed the time required to achieve peak values of cortical $[Ca^{2+}]_i$ (see the following description).

It should be noted that only AED stimulation $-Ca^{2+}{}_o$ can give information, though indirect, on the extent of store refilling. Experiments $+Ca^{2+}{}_o$ have also been included in order to control any potential side-effects of double triggering, such as any possible refractoriness of Ca^{2+}-influx channels, and thus to obtain some additional information on the largely unknown mechanisms governing SOC-type responses. We emphasize again the brief time of Ca^{2+}-chelator application, without any recognizable side-effects (see "Section 2").

Let us first consider experiments of the type "$+Ca^{2+}$". Control experiments (one AED stimulus only, $+Ca^{2+}{}_o$), show a swift increase of cortical Ca^{2+}-signals, with a peak at 1.5 s ± 0.0 (standard error of the mean (S.E.M.)) after AED stimulation (Fig. 3a). Considering the time required for filter change (0.5 s) in double wavelength recordings, the real rise time may be smaller. In double trigger experiments, with an increasing time interval between the first and the

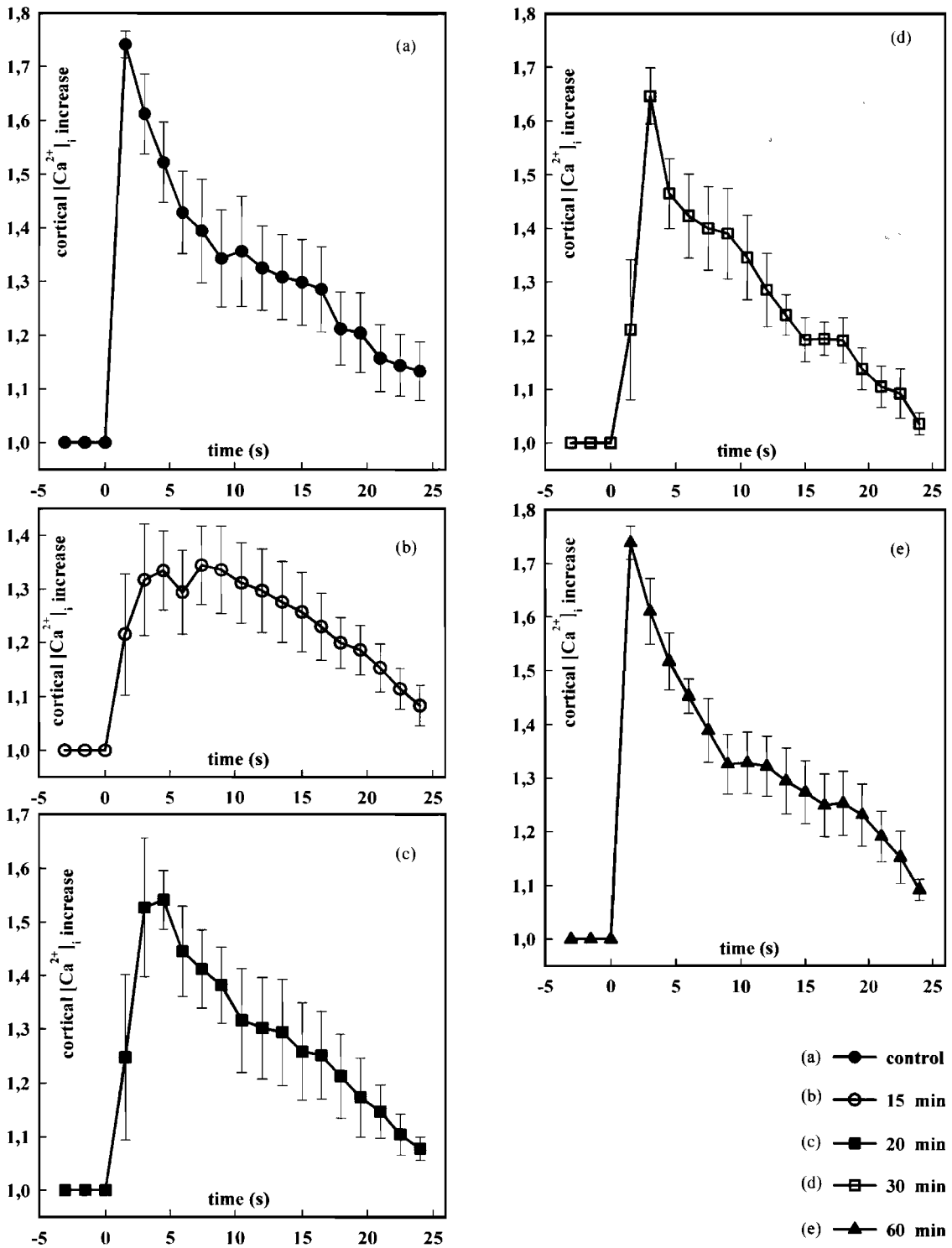

Fig. 3. Time course of fluorochrome signals recorded in the cortex of Fura Red-loaded cells at the site of AED stimulation (at time 0). Abscissa: time scale (s); ordinate: relative $[Ca^{2+}]_i$-signal recorded in cell cortex at stimulation site. Panels a–e were obtained under conditions "$+Ca^{2+}_o$", after one AED stimulus only (0 min), and after double triggering in 15, 20, 30, and 60 min intervals, respectively. Panels f–h were obtained under conditions "$-Ca^{2+}_o$" (with BAPTA in the AED trigger medium), also after one AED stimulus only (0 min), and after double stimulation in 15 min and 60 min intervals, respectively. Bars = S.E.M.; missing S.E.M. bars means no statistical fluctuations recorded. Number of cells analyzed per data point $N = 5$.

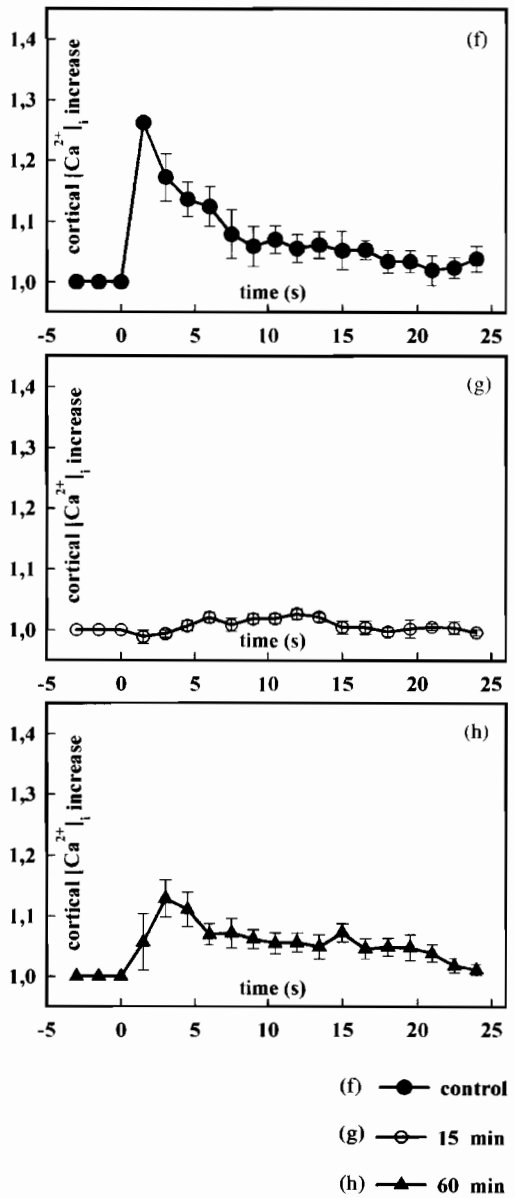

Fig. 3. (*Continued*).

second trigger, the cortical Ca^{2+}-signal increase becomes more and more pronounced, but the rise time becomes longer than recorded in controls. In detail, the relative fluorescence signal increase was 1.74 ± 0.03 for the control (one stimulus), 1.43 ± 0.04 (15 min interval between first and second stimulus), 1.65 ± 0.04 (20 min), 1.68 ± 0.03 (30 min), and 1.74 ± 0.03 (60 min). Rise times were ≤1.5 s ± 0.0 for the control (one stimulus), 3.9 s ± 1.0 (15 min interval), 3.0 s ± 0.5 (20 min), 2.7 s ± 0.3 (30 min), and ≤1.5 s ± 0.0 (60 min). We want to comment these data as follows. The shortest double trigger experiment technically possible was with a 15 min interval. This is the minimum time required for handling, i.e. wash-out of AED, fluorochrome injection, and immobilization for the second AED stimulation and subsequent signal recording. When the second AED stimulus followed in a 15 min interval after the first stimulus, the signal generated was much weaker and it rose less rapidly than in controls (Fig. 3b). Then, with increasing intervals between the two stimuli, the $[Ca^{2+}]_i$ peak achieved increases (Fig. 3a–e) and rise time to peak value decreases.

Fig. 3f–h contain results from experiments of the type "$-Ca^{2+}{}_o$". (Recall that $Ca^{2+}{}_o$ was chelated only during the second AED application and that 1.5 s is again the minimum recording time due to filter change.) The relative fluorescence signal increase achieved was 1.27 ± 0.005 (control, only one AED + BAPTA application), 1.03 ± 0.000 (15 min interval between AED and AED + BAPTA application), and 1.13 ± 0.03 (60 min interval). The rise time was ≤1.5 s ± 0.0, 7.5 s ± 1.6 (15 min), and 2.4 s ± 0.4 (60 min interval between AED and AED + BAPTA application). In summary, control cells, stimulated by AED + BAPTA, yield only 35% of the cortical Ca^{2+}-signal when compared with stimulation experiments under conditions $+Ca^{2+}{}_o$. Under conditions $-Ca^{2+}{}_o$, the signal generated by a second AED stimulus after 15 min is extraordinarily small (7% of that $+Ca^{2+}{}_o$). Half of the signal of controls at $-Ca^{2+}{}_o$ was found when the time interval between the two stimuli was 65 min.

Comparison of the data "$+Ca^{2+}{}_o$" and "$-Ca^{2+}{}_o$" contained in Fig. 3 reveals some additional aspects. In "$+Ca^{2+}{}_o$" experiments, there is a strong Ca^{2+}-influx component in controls which, in double trigger experiments, slowly recovers with increasing time between the two stimuli. Under conditions "$-Ca^{2+}{}_o$", the Ca^{2+}-signal is small in single trigger experiments (controls, 0 s). In 15 min double trigger experiments, there is considerable response under conditions "$+Ca^{2+}{}_o$", but a negligibly small one under conditions "$-Ca^{2+}{}_o$". Evidently, 15 min is much too short for any substantial refilling of ASs. Since at $-Ca^{2+}{}_o$, the signal clearly reaches 50% after 65 min, this indicates the half-time of store refilling. This signal increase parallels the store refilling determined directly by EDX analysis (Fig. 2)—an alternative approach based on a widely different principle.

3.4. *Effects of actin-reactive drugs on SOC*

In order to elucidate any effect of cortical F-actin, as reported for higher eukaryotes (see "Section 1"), we first tested in pilot experiments the effects of different F-actin de-stabilizing (CytB, LatA) and stabilizing (Jas) drugs on phagocytosis (data not shown). We considered this important taking into account the aberrant or even missing effect of many drugs in lower eukaryotes. Phagocytosis was taken as a positive control since in *Paramecium* this is the best known process requiring F-actin [39–43]. We also tested reversibility to exclude toxic side-effects. Concentrations of CytB required are relatively high, just as in previous work with paramecia, when different cell functions had been analyzed [39,44,45]. We achieved 50% inhibition with CytB at 100 μg/ml, 30 min; 90 and 95% inhibition was achieved with 200 μg/ml applied for 30 and 60 min, respectively. To ascertain specificity even more, we also used some newly

Table 1
Effect of CytB (200 μg/ml) on $[Ca^{2+}]_i$ fluorescence signal generated in the cell cortex upon AED stimulation

Duration of CytB treatment (min)	Relative $[Ca^{2+}]_i$ increase (%)
0 (control)	100 ± 3
30	96 ± 4
60	106 ± 2
60 → 90 (wash-out)	97 ± 1

Controls = 100% ± standard deviation. Number of cells analyzed, $N = 5$ per data point.

available drugs, Jas and LatA, respectively. They all inhibited phagocytosis at relatively high concentrations. All these effects were fully reversible in wash-out experiments; for instance, after application of CytB at 200 μg/ml for 60 min, the time required for 50 and 100% recovery was 40 and 90 min, respectively. This made us confident to see any potential effect on Ca^{2+}-signaling, if it would exist, without any side-effects.

Using routine ultrathin section electron microscopy, we first analyzed the effect of CytB on the positioning not only of ASs but also of mitochondria—another organelle of relevance for Ca^{2+}-signaling [15]. Yet, no effect on their positioning was found in quantitative evaluations (data not shown). Secondly, we analyzed fluorochrome signals generated by AED after CytB treatment under conditions "$+Ca^{2+}_o$". As documented in Table 1, we found no change in Ca^{2+}-signaling when CytB-treated cells were stimulated with AED, even under conditions when phagocytosis was considerably inhibited.

4. Discussion

4.1. Relevance of Ca^{2+}_o for maximal exocytotic response

Previously, we had found with wildtype cells that mobilization of Ca^{2+} from internal (cortical) stores can activate only ~22–37% of trichocyst docking sites to undergo membrane fusion [21,22]. While this is in agreement with the data presented here, we now have analyzed in more detail the dependency of membrane fusion on $[Ca^{2+}]_o$ (Fig. 1, Table 3). We find that maximal response occurs with $[Ca^{2+}]_o$ close to, or slightly above the ~5 μM estimated at the activation sites [15,17]. Above a threshold level of $[Ca^{2+}]_o$, i.e. >0.25 μM, the exocytotic membrane fusion response steadily increases, with half-maximal activation at $[Ca^{2+}]_o = 0.7$ μM (Table 2).

Is Ca^{2+} the relevant ion for AED stimulation or can the phenomena observed be explained merely by the charge of the secretagogue? Our answer is clearly in favor of the relevance of Ca^{2+} because of different reasons. (i) The polycationic compound, AED, can act as a secretatogue only in conjunction with Ca^{2+}, as reviewed in Ref. [15], or with the quite similar Sr^{2+} ion [29], but not with Mg^{2+} which is inhibitory [46]. (ii) Injection of highly selective Ca^{2+}-buffers inhibits trichocyst exocytosis [17]. (iii) Neither depolarization nor hyperpolarization can cause trichocyst exocytosis [47].

Table 2
Characteristics of alveolar sacs (ASs), $[Ca^{2+}]_i$ required for exocytosis, Ca^{2+}-release from ASs, and refilling of ASs in situ (following maximal AED stimulation) in comparison to in vitro $^{45}Ca^{2+}$ uptake by isolated AS

Total calcium concentration [Ca] in AS	43 mM [38]
Ca^{2+} released from AS upon stimulation (1 s)	80% [29]
$[Ca^{2+}]_o$ required for SOC-type effect	
Minimum effect	0.25 μM (this paper)
Half-maximal effect	0.7 μM (this paper)
Maximal effect	~2–10 μM (this paper)
Local $[Ca^{2+}]_i$ at maximal stimulation	~5 μM [17]
Refilling half-time[a]	
EDX	~60 min (this paper)
Double trigger experiments + BAPTA	~65 min (this paper)
$^{45}Ca^{2+}$-uptake in vitro	~60 min [56][b]

[a] Assuming logarithmic increase.
[b] Derived from extensive series presented as Fig. 3 in Länge et al. [57].

Are our current data compatible with the SOC-mechanism mentioned? An SOC-mechanism occurring during stimulated trichocyst exocytosis in *Paramecium* has been implied indirectly from quenched-flow/freeze-fracture analysis [21] and from whole cell-patch recording of Ca^{2+}-activated currents [47]. More stringent evidence came from work with a double mutant devoid of any $^{45}Ca^{2+}$-influx [24], showing generation of cytosolic fluorochrome signals and decreasing CaKα net peaks in EDX analyses after AED stimulation [26].

Storage of Ca^{2+} in ASs depends on the presence of and binding to a calsequestrin-like high capacity/low affinity Ca^{2+}-binding protein [48]. Ca^{2+} can rapidly dissociate, and according to EDX data, ~80% are released during 1 s AED stimulation [29]. While local $[Ca^{2+}]_i$ at exocytosis sites can only indirectly and roughly be estimated, a balance calculation of total Ca^{2+}-fluxes reveals that mobilization from ASs and SOC-type influx may contribute about equally to Ca^{2+}-signaling [15]. Clearly both components have to act in concert to achieve optimal response. Our present fluorochrome analyses (Fig. 3) clearly support this concept for the following reasons. Double trigger experiments, conditions "$+Ca^{2+}_o$", yield much higher signals upon a second AED stimulation at different time intervals (15, 20, 30, and 60 min) than under conditions "$-Ca^{2+}_o$". When the two conditions, $+Ca^{2+}_o$ and $-Ca^{2+}_o$, are compared, values obtained with double trigger intervals of 0 (control), 15, and 60 s under "$-Ca^{2+}_o$" conditions represent 35, 7, and 18%, respectively, of those obtained under "$+Ca^{2+}_o$" conditions (see "Section 3"). The very low value at 15 min, $-Ca^{2+}_o$, is clearly expected if an SOC-type mechanism occurs. The same holds for the proportion of the influx component which is lowest at 15 min in the series "$+Ca^{2+}_o$", but in relative terms it is highest when put in relation to the signal under "$-Ca^{2+}_o$" conditions, also at 15 min. This agrees well with the SOC-mechanism described.

How do we explain the relative low signal in 15 min double trigger experiments at "$+Ca^{2+}_o$" and the relatively long time required to reach peak values at "$-Ca^{2+}_o$"? A tentative explanation could be the refractoriness of the Ca^{2+}-influx channels involved, although this is not known, e.g. for the unspecific cation-conducting channels of the type described by Saitow et al. [49] which we had envisaged as candidates [18].

Another aspect of our work is the absence of any effects of "actin-reactive" drugs on SOC-type signaling in *Paramecium*, in contrast to several reports on higher eukaryotic cells [5,7,34–36]. We believe that in *Paramecium*, linkage of ASs to the plasma membrane is unlikely due to F-actin. Although we know that some F-actin does occur in the cell periphery [40,41], its precise localization remains to be established. Any potential contribution to positioning of SERCA molecules and possibly of cation exchangers relative to each other [50], as well as to the total Ca^{2+}_i-balance remain unknown so far.

4.2. *Refilling of ASs*

During Ca^{2+}-signaling, as in other cells, a very large excess of total calcium is sweeping over the cell cortex into the *Paramecium* cell [15]. Before re-uptake into ASs, Ca^{2+} will be bound to cytosolic Ca^{2+}-binding proteins with high Ca^{2+}-binding capacity [51]. Gradual dissociation allows for slowly ongoing transfer from the cytosolic compartment into stores.

How fast are stores refilled with Ca^{2+}? Most publications consider only changes of free Ca^{2+}, $[Ca^{2+}]$, in stores. Fluorochrome analyses show recovery of $[Ca^{2+}]$ within the order of magnitude of 30 s in the SR smooth muscle [52] to 1–3 min in the SR of skeletal muscle [53], when measured in vitro. To assess refilling of stores, measurements of total calcium concentrations [Ca] is required. Such data are essentially restricted to the SR of muscle cells. EDX analysis of [Ca] in SR of skeletal muscle, which also operates by an SOC-type mechanism [1,2,10,54], revealed that ~40% are released during normal contraction activity and ~60% during tetanic stimulation [55]. The percentage of Ca^{2+}-released is comparable to what we found during one cycle of trichocyst exocytosis [29]. Also [Ca] values in stores before stimulation are quite similar in the two systems, i.e. 43 mM in ASs [38] and 33 mM in skeletal muscle SR [54], both estimated on the basis of wet weight. However, refilling times evidently are widely different between the SR of skeletal muscle and our system. How can this be explained?

In a more scrutinized analysis, we would have to consider two very different types of Ca^{2+}-uptake into ASs, a fast and a slow component. (i) In quenched-flow/EDX experiments, we found that Ca^{2+} or Sr^{2+} as a substitute (with clearly resolved Kα energy) enters ASs within 1 s, i.e. already during ongoing exocytosis and release of endogenous Ca^{2+} [29]. (ii) As a candidate for the long-term re-uptake mechanism analyzed in the present paper, we have to consider the SERCA-pump of ASs [30,31]. This is localized specifically in that part of ASs which faces the interior of the cell, i.e. the "inner AS membrane" [32]. For aspect (i), some authors envisage a Na^+/Ca^{2+} exchanger system, e.g. for the SR in smooth muscle cells (as discussed by Hellstrand [56]), but no information along these lines exists for *Paramecium*. In striated muscle cells, rapid re-uptake of Ca^{2+} into the SR is mediated by mechanism (ii), i.e. by its SERCA activity. In the SR, this is due to higher activity and to the much larger membrane area containing SERCA molecules (in the extensive longitudinal system [9]) as compared to the area containing Ca^{2+}-release channels (restricted to the relatively small terminal cisternae [53]). This microanatomical principle, small release versus large uptake area, is not applicable to *Paramecium* [32] and concomitantly refilling by SERCA activity is slow (see Table 2). Therefore, over longer periods after AED stimulation, Ca^{2+} in ASs is replenished only slowly by the activity of their SERCA-type Ca^{2+}-pump/ATPase [30,31]. The half-time of 60–65 min for refilling, as reported in the present paper, is the same as that determined for $^{45}Ca^{2+}$-uptake by isolated ASs in vitro ($t_{1/2}$ ~60 min), [56]. In total, Ca^{2+}-pumping activity of ASs from *Paramecium* [56] is much slower than that of SR from skeletal muscle [9].

4.3. *Implications for the Paramecium system and outlook to related protozoan species*

In contrast to the extensive genome cloning, little is known about Ca^{2+}-signaling in related pathogenic species which also contain cortical alveoli (see "Section 1"). Their invasion mechanism involves sequential exocytotic processes which in part depend on Ca^{2+} [58]. Our analysis may facilitate further insight into this system. In *Paramecium*, rapid trichocyst exocytosis serves defense against predators, as first shown by Harumoto and Miyake [59] and then by Knoll et al. [60]. After total depletion of trichocysts, re-installation of a complete new set requires up to 10 h [12,61]. Even though Ca^{2+}-re-uptake is slow, sufficient Ca^{2+} will be available in ASs as new trichocysts are gradually inserted.

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