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, aminoethyl-dextran (AED), causes a cortical $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]_i$ transient (while injected La^{3+} causes a sustained fluorescence signal). (iii) Simply increasing $[\text{Ca}^{2+}]_o$ causes a similar rapid, short-lived $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]$ signal increase to a level required for full exocytosis activation. In addition, we show formation of $[\text{Ca}^{2+}]$ microdomains ($\leq 0.5 \mu\text{m}$, ≤ 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

(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, aminoethyl-dextran, 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. $[\text{Ca}^{2+}]_o < [\text{Ca}^{2+}]_i^{\text{rest}}$ which is ~65 nM [40]. Cortical $[\text{Ca}^{2+}]$ transients achieved at $[\text{Ca}^{2+}]_o = 30$ 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 $[\text{Ca}^{2+}]_o$ 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 $[\text{Ca}^{2+}]_i$ 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 $\lambda_{\text{excitation}} = 360$ and 380 nm, while $\lambda_{\text{emission}}$ evaluated was ≥ 515 nm.

Results

VOLTAGE-DEPENDENT Ca^{2+} CHANNELS ARE NOT INVOLVED IN AED PRODUCED $[\text{Ca}^{2+}]_i$ TRANSIENTS

We analyzed $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_o$ 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 $2\ \mu\text{M}$ AED at $[\text{Ca}^{2+}]_o = 50\ \mu\text{M}$. Evaluated was a $\sim 5\ \mu\text{m}$ broad cortical zone.

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

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

Characteristics of the cortical $[\text{Ca}^{2+}]$ 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 $\pm$ SEM, n = number of cells analyzed.

PARAMECIUM CELLS REACT TO HIGH $[\text{Ca}^{2+}]_o$ OR TO EXTRACELLULAR La^{3+} BY A $[\text{Ca}^{2+}]_i$ TRANSIENT—EVIDENCE OF A CaSR?

May there exist a CaSR in *Paramecium* cells? If so, could it potentially explain generation of AED-mediated $[\text{Ca}^{2+}]_i$ transients? The prediction would be that an instantaneous increase of $[\text{Ca}^{2+}]_o$ 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 $[\text{Ca}^{2+}]_o$ in the medium to 10 mM, in order to look for any rapid change in $[\text{Ca}^{2+}]_i$. While we know that simply increasing $[\text{Ca}^{2+}]_o$ to this level does not induce exocytosis [67], for reasons analyzed in the Discussion, we do find a rapid cortical $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ increase not only in response to AED or to high $[\text{Ca}^{2+}]_o$, but also in response to extracellular La^{3+} application [87]. Yet its

Fig. 3. A cortical $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_o = 10$ mM. Top left: conventional 2λ Fura Red analysis; anterior Ca^{2+}_o application. Top right: fast 1λ CLSM Fluo-3 analysis; anterior Ca^{2+}_o application. Bottom: Fluo-3 CLSM analysis of anterior (left) or posterior (right) $[\text{Ca}^{2+}]_i$ transients after Ca^{2+}_o application at the respective cell pole. Values $\pm$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ increase by rapid application of high $[\text{Ca}^{2+}]_o$, though causing a swift $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_o = 50 \mu\text{M}$, 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 $[\text{Ca}^{2+}]_i$ registration by 1λ CLSM, (b') quantitative evaluation. Values $\pm$ SEM, $n = 4$. Bars = $10 \mu\text{m}$.

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 $[\text{Ca}^{2+}]_i$ transient progressing laterally and centropetally. Data retrieved, at sites indicated in the scheme, by 1 λ 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 $[\text{Ca}^{2+}]_o$ 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 $[\text{Ca}^{2+}]_i$ 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 enforced by Ca^{2+} influx. Clearly $[\text{Ca}^{2+}]_i$ signals are generated also at $[\text{Ca}^{2+}]_o = 30 \text{ nM}$, yet they are larger and they last longer at $[\text{Ca}^{2+}]_o = 50 \mu\text{M}$.

To substantiate even further or to disprove our previous view of partial activation of exocytotic membrane fusion in our cells even at low $[\text{Ca}^{2+}]_o$ [41, 67], we now additionally applied the novel Ca^{2+} releasing agent,

4CmC (Figs. 7–12, Table 3). This drug, applied at $[\text{Ca}^{2+}]_o = 500 \mu\text{M}$, yields a Ca^{2+} signal at the site of application, where it also causes trichocyst exocytosis (Fig. 7). Under appropriate conditions, microdomains of $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_o$. Clearly the effect achieved depends on the concentration of each of the components present. With $[\text{Ca}^{2+}]_o = 30 \text{ nM}$, 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 $[\text{Ca}^{2+}]_o$, with subsequent addition of high $[\text{Ca}^{2+}]_o$ (Fig. 11). If stores were emptied in the first phase at low $[\text{Ca}^{2+}]_o$, any signal intensification upon readdition of high $[\text{Ca}^{2+}]_o$ could demonstrate occurrence of SOC. This is what we see in Fig. 11, showing an example of a f/f_0 recording. In this figure, data points indicated at 0 sec for f/f_0 recording correspond to t_0 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 $[\text{Ca}^{2+}]_i$ transient which sweeps centropetally after the first application at 0 sec. When repeated after 60 sec, no $[\text{Ca}^{2+}]_i$ increase can be registered, for reasons indicated in the text. 1 λ CLSM Fluo-3 analysis.

Table 2. $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ measurement	Maximum f/f_0 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 $\pm$ SD, $n = 7$.

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

4CmC concentration (μM), (n)	$[\text{Ca}^{2+}]_o$ (M)	Cortical $[\text{Ca}^{2+}]_i$ rise (f/f_0)				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 $[\text{Ca}^{2+}]_o = 10^{-3}$ M after store depletion by 2 min exposure to 4CmC. Remarkably the response is much smaller with $[\text{Ca}^{2+}]_o = 10^{-3}$ M in the absence of any previous 4CmC application (nonhatched column, bottom middle). (The outermost right part of Fig. 11 shows $[\text{Ca}^{2+}]_i$ signal decay when $[\text{Ca}^{2+}]_o$ is diluted to $\sim 10^{-6}$ M within 11 sec.) In sum, 4CmC induces a SOC-type reaction immediately following addition of $[\text{Ca}^{2+}]_o = 10^{-3}$ 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 thapsigargin 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 $[\text{Ca}^{2+}]_o = 30$ nM, but a considerable signal intensification by Ca^{2+} influx at higher $[\text{Ca}^{2+}]_o$ (Table 4). When compared with the effects of caffeine [41] and particu-

Fig. 7. Local 4CmC application (arrowhead) at t_0 causes local transient $[\text{Ca}^{2+}]_i$ increase and trichocyst (t) exocytosis. Fura-2 loaded 7S cell in $[\text{Ca}^{2+}]_o = 50 \mu\text{M}$, $500 \mu\text{M}$ 4CmC. Note delay of reaction during permeation of the drug. Bar = $10 \mu\text{m}$.

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

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

EVIDENCE OF $[\text{Ca}^{2+}]_i$ MICRODOMAINS IN *PARAMECIUM*

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

Fig. 9. Typical example of a $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_o = 30 \text{ nM}$, 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 $[\text{Ca}^{2+}]_i$ 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 $\sim 15 \text{ nm}$ [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 $[\text{Ca}^{2+}]_i$ transients induced by increased $[\text{Ca}^{2+}]_o$ 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 $[\text{Ca}^{2+}]_o$, while unexpectedly producing the well known “Ca paradox” in *Paramecium*, i.e., membrane depolarization with increased forward 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_o$ application. Though yielding a $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_o$ [7]. We assume exocytosis inhibition by too long exposure (~ 1 min) to high $[\text{Ca}^{2+}]_o$, 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 $[\text{Ca}^{2+}]_o$, in despite of formation of a regular $[\text{Ca}^{2+}]_i$ transient. As previously shown [67], high $[\text{Ca}^{2+}]_o$ applied for ≤ 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 Ca²⁺ from stores in Fluo-3 injected 7S cells by 4CmC preincubation (2 min) at [Ca²⁺]_o = 10⁻⁷ M induces massive Ca²⁺ influx upon addition of 10⁻³ M Ca²⁺_o. Scheme at top right: Mobilization of Ca²⁺ from stores by 2 min 4CmC preincubation in presence of BAPTA was followed by readdition of [Ca²⁺]_o = 10⁻³ M, before [Ca²⁺]_o was allowed to drop to 10⁻⁶ M during a subsequent time of 11 sec. Bottom left: example of a [Ca²⁺]_i transient generated at t₀ (as indicated in scheme at top right). Bottom right: Cortical [Ca²⁺]_i signal amplitudes (hatched columns: with 4CmC, clear columns: without 4CmC preincubation) were obtained at [Ca²⁺]_o = 10⁻⁷ M (left column pair), after adding [Ca²⁺]_o = 10⁻³ M (middle column pair), or after decay of [Ca²⁺]_o to 10⁻⁶ M (right column pair). Values ± SEM, n = 4 (hatched columns) or 2 (clear columns).

Table 4. [Ca²⁺]_i transients generated in Fluo-3 loaded 7S cells by application of inhibitors of the SERCA-type Ca²⁺-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)
Thapsigargin, 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 IP₃-mediated Ca²⁺ 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 IP₃. 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $\text{Ca}^{2+}_o = 50 \mu\text{M}$, $\pm\text{MnCl}_2 = 5 \text{ mM}$, AED = $2 \mu\text{M}$ (left), 4CmC = $500 \mu\text{M}$ (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 ($\text{EC}_{100} = 1 \mu\text{M}$, [72]) or lysozyme (EC_{100}

$\geq 100 \mu\text{M}$, [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].

$[\text{Ca}^{2+}]_i$ MICRODOMAINS AND POSSIBLE SITE-DIRECTED Ca^{2+} FLUX

In muscle cells, Ca^{2+} imaging by fluorochromes allowed to observe $[\text{Ca}^{2+}]_i$ 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 $0.5\ \mu\text{m}/5\text{--}20\ \text{msec}$ [14], $1.4\ \mu\text{m}/30\ \text{msec}$ [17] and $1.5\ \mu\text{m}/13\ \text{msec}$ [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 $\leq 33\ \text{msec}$, i.e., below the time required for image recording. Our present data are also compatible with the range of the lifetime ($t_{1/2} = 21\ \text{msec}$) 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 $[\text{Ca}^{2+}]_i$ 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 accommodate a trichocyst. Characterization of molecular components involved in AED stimulation remain open so far.

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References

1. Adebajo, O.A., Igietsme, J., Huang, C.L.H., Zaidi, M. 1998. The effect of extracellularly applied divalent cations on cytosolic Ca^{2+} in murine Leydig cells: evidence for a Ca^{2+} -sensing receptor. *J. Physiol.* **513**:399–410
2. Barritt, G.J. 1999. Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signaling requirements. *Biochem. J.* **337**:153–169
3. Beisson, J., Lefort-Tran, M., Pouphe, M., Rossignol, M., Satir, B. 1976. Genetic analysis of membrane differentiation in *Paramecium*. Freeze-fracture study of the trichocyst cycle in wild-type and mutant strains. *J. Cell Biol.* **69**:126–143
4. Bennett, E., Urcan, M.S., Tinkle, S.S., Koszowski, A.G., Levinson, S.R. 1997. Contribution of sialic acid to the voltage dependence of sodium channel gating. A possible electrostatic mechanism. *J. Gen. Physiol.* **109**:327–343
5. Berridge, M.J. 1997. Elementary and global aspects of calcium signaling. *J. Physiol.* **499**:291–306
6. Berridge, M.J. 1998. Neuronal calcium signaling. *Neuron* **21**:13–26
7. Blanchard, M.-P., Klauke, N., Plattner, H. 1999. Veratridine-mediated Ca^{2+} dynamics and exocytosis in *Paramecium* cells. *J. Membrane Biol.* **169**:155–165
8. Bootman, M.D., Berridge, M.J. 1995. The elemental principles of calcium signaling. *Cell* **83**:675–678
9. Bruce, J.I.E. 1998. Identification of an extracellular calcium-sensing receptor in rat pancreatic acinar cells. *J. Physiol.* **513**:50P
10. Bruce, J.I.E., Yang, X., Ferguson, C.J., Elliott, A.C., Steward, M.C., Case, R.M., Riccardi, D. 1999. Molecular and functional identification of a Ca^{2+} (polyvalent cation)-sensing receptor in rat pancreas. *J. Biol. Chem.* **274**:20561–20568
11. Chattopadhyay, N., Yamaguchi, T., Brown, E.M. 1998. Ca^{2+} receptor from brain to gut: common stimulus, diverse actions. *Trends Endocrinol. Metab.* **9**:354–359
12. Cheek, T.R., Barry, V.A. 1993. Stimulus-secretion coupling in excitable cells: a central role for calcium. *J. Exp. Biol.* **184**:183–196
13. Cheek, T.R., Morgan, A., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J., Burgoyne, R.D. 1993. Spatial localization of agonist-induced Ca^{2+} entry in bovine adrenal chromaffin cells. Different patterns induced by histamine and angiotensin II, and relationship to catecholamine release. *J. Cell Sci.* **105**:913–921
14. Cheng, H., Song, L.S., Shirokova, N., González, A., Lakatta, E.G., Ríos, E., Stern, M.D. 1999. Amplitude distribution of calcium sparks in confocal images: theory and studies with an automated detection method. *Biophys. J.* **76**:606–617
15. Clapham, D.E. 1995. Calcium signaling. *Cell* **80**:259–268
16. Cohen, J., Kerboeuf, D. 1993. Calcium and trichocyst exocytosis in *Paramecium*: Genetic and physiological studies. In: Membrane Traffic in Protozoa. H. Plattner, editor. pp. 61–81. JAI Press, Greenwich, CT
17. Collier, M.L., Thomas, A.P., Berlin, J.R. 1999. Relationship between L-type Ca^{2+} current and unitary sarcoplasmic reticulum Ca^{2+} release events in rat ventricular myocytes. *J. Physiol.* **516**:117–128
18. Du, G.G., Ashley, C.C., Lea, T.J. 1996. Ca^{2+} effluxes from the sarcoplasmic reticulum vesicles of frog muscle: effects of cyclopiazonic acid and thapsigargin. *Cell Calcium* **20**:355–359
19. Erxleben, C., Klauke, N., Flötenmeyer, M., Blanchard, M.-P., Braun, C., Plattner, H. 1997. Microdomain Ca^{2+} activation during exocytosis in *Paramecium* cells. Superposition of local subplasmalemmal calcium store activation by local Ca^{2+} influx. *J. Cell Biol.* **136**:597–607
20. Erxleben, C., Plattner, H. 1994. Ca^{2+} release from subplasmalemmal stores as a primary event during exocytosis in *Paramecium* cells. *J. Cell Biol.* **127**:935–945

21. Fasolato, C., Hoth, M., Matthews, G., Penner, R. 1993. Ca^{2+} and Mn^{2+} influx through receptor-mediated activation of nonspecific cation channels in mast cells. *Proc. Natl. Acad. Sci. USA* **90**:3068–3072
22. Frankenhaeuser, B., Hodgkin, A.G. 1957. The action of calcium on the electric properties of squid giant axons. *J. Physiol.* **137**:218–244
23. Franzini-Armstrong, C., Protasi, F. 1997. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol. Rev.* **77**:699–729
24. Fried, R.M., Tashjian, A.H. 1986. Unusual sensitivity of cytosolic free Ca^{2+} to changes in extracellular Ca^{2+} in rat C-cells. *J. Biol. Chem.* **261**:7669–7674
25. Gamberucci, A., Fulceri, R., Marcolongo, P., Pralong, W.F., Benedetti, A. 1998. Histones and basic polypeptides activate Ca^{2+} /cation influx in various cell types. *Biochem. J.* **331**:623–630
26. Gustin, M., Hennessey, T.M. 1988. Neomycin inhibits the calcium current of *Paramecium*. *Biochim. Biophys. Acta* **940**:99–104
27. Haga, N., Forte, M., Saimi, Y., Kung, C. 1982. Microinjection of cytoplasm as a test of complementation in *Paramecium*. *J. Cell Biol.* **92**:559–564
28. Hauser, K., Pavlovic, N., Kissmehl, R., Plattner, H. 1998. Molecular characterization of a sarco(endo)plasmic reticulum Ca^{2+} -ATPase gene from *Paramecium tetraurelia* and localization of its gene product to subplasmalemmal calcium stores. *Biochem. J.* **334**:31–38
29. Hennessey, T.M., Kim, M.Y., Satir, B.H. 1995. Lysozyme acts as a chemorepellent and secretagogue in *Paramecium* by activating a novel receptor-operated Ca^{++} conductance. *J. Membrane Biol.* **148**:13–25
30. Herrmann-Frank, A., Richter, M., Sarkozi, S., Mohr, U., Lehmann-Horn, F. 1996. 4-chloro-m-cresol, a potent and specific activator of the skeletal muscle ryanodine receptor. *Biochim. Biophys. Acta* **1289**:31–40
31. Hinrichsen, R.D., Fraga, D., Russell, C. 1995. The regulation of calcium in *Paramecium*. *Adv. Sec. Mess. Phosphoprot. Res.* **30**:311–338
32. Hughes, B.P., Auld, A.M., Barritt, G.J. 1988. Evidence that neomycin inhibits plasma membrane Ca^{2+} inflow in isolated hepatocytes. *Biochem. Pharmacol.* **37**:1357–1361
33. Inesi, G., Sagara, Y. 1994. Specific inhibitors of intracellular Ca^{2+} transport ATPases. *J. Membrane Biol.* **141**:1–6
34. Kabbara, A.A., Allen, D.G. 1999. Measurement of sarcoplasmic reticulum Ca^{2+} content in intact amphibian skeletal muscle fibres with 4-chloro-m-cresol. *Cell Calcium* **25**:227–235
35. Kao, J.P.Y., Tsien, R.Y. 1988. Ca^{2+} binding kinetics of fura-2 and azo-1 from temperature-jump relaxation measurements. *Biophys. J.* **53**:635–639
36. Keith, R.A., Horn, M.B., Piser, T.M., Mangano, T.J. 1993. Effects of stimulus intensity on the inhibition by ω -conotoxin GVIA and neomycin of K^{+} -evoked [^3H]norepinephrine release from hippocampal brain slices and synaptosomal calcium influx. *Biochem. Pharmacol.* **45**:165–171
37. Kerboeuf, D., Cohen, J. 1990. A Ca^{2+} influx associated with exocytosis is specifically abolished in a *Paramecium* exocytotic mutant. *J. Cell Biol.* **111**:2527–2535
38. Kim, M.Y., Kuruvilla, H.G., Hennessey, T.M. 1997. Chemosensory adaptation in *Paramecium* involves changes in both repellent binding and the consequent receptor 39. potentials. *Comp. Biochem. Physiol.* **118A**:589–597
39. Kissmehl, R., Huber, S., Kottwitz, B., Hauser, K., Plattner, H. 1998. Subplasmalemmal Ca-stores in *Paramecium tetraurelia*. Identification and characterisation of a sarco(endo)plasmic reticulumlike Ca^{2+} -ATPase by phosphoenzyme intermediate formation and its inhibition by caffeine. *Cell Calcium* **24**:193–203
40. Klauke, N., Plattner, H. 1997. Imaging of Ca^{2+} transients induced in *Paramecium* cells by a polyamine secretagogue. *J. Cell Sci.* **110**:975–983
41. Klauke, N., Plattner, H. 1998. Caffeine-induced Ca^{2+} transients and exocytosis in *Paramecium* cells. A correlated Ca^{2+} imaging and quenched-flow/freeze-fracture analysis. *J. Membrane Biol.* **161**:65–81
42. Knoll, G., Braun, C., Plattner, H. 1991. Quenched flow analysis of exocytosis in *Paramecium* cells: time course, changes in membrane structure, and calcium requirements revealed after rapid mixing and rapid freezing of intact cells. *J. Cell Biol.* **113**:1295–1304
43. Knoll, G., Grässle, A., Braun, C., Probst, W., Höhne-Zell, B., Plattner, H. 1993. A calcium influx is neither strictly associated with nor necessary for exocytotic membrane fusion in *Paramecium* cells. *Cell Calcium* **14**:173–183
44. Kung, C., Saimi, Y. 1985. Ca^{2+} channels of *Paramecium*: a multidisciplinary study. In: Genes and Membranes: Transport Proteins and Receptors. F. Bronner and A. Kleinzeller, editors. pp. 45–66. Academic Press, Orlando
45. Kuruvilla, H.G., Hennessey, T.M. 1998. Purification and characterization of a novel chemorepellent receptor from *Tetrahymena thermophila*. *J. Membrane Biol.* **162**:51–57
46. Kwan, C.Y., Putney, J.W. 1990. Uptake and intracellular sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells. *J. Biol. Chem.* **265**:678–684
47. Lacampagne, A., Ward, C.W., Klein, M.G., Schneider, M.F. 1999. Time course of individual Ca^{2+} sparks in frog skeletal muscle recorded at high time resolution. *J. Gen. Physiol.* **113**:187–198
48. Länge, S., Klauke, N., Plattner, H. 1995. Subplasmalemmal Ca^{2+} stores of probable relevance for exocytosis in *Paramecium*. Alveolar sacs share some but not all characteristics with sarcoplasmic reticulum. *Cell Calcium* **17**:335–344
49. Lewis, R.S. 1999. Store-operated calcium channels. *Adv. Sec. Mess. Phosphoprot. Res.* **33**:279–307
50. Lu, W.Y., Xiong, Z.G., Oser, B.A., MacDonald, J.F. 1998. Multiple sites of action of neomycin, Mg^{2+} and spermine on the NMDA receptors of rat hippocampal CA1 pyramidal neurones. *J. Physiol.* **512**:29–46
51. Machemer, H. 1988. Electrophysiology. In: *Paramecium*. H.-D. Görtz, editor. pp. 185–215. Springer-Verlag, Berlin, Heidelberg, New York
52. Machemer, H., Ogura, A. 1979. Ionic conductances of membranes in ciliated and deciliated *Paramecium*. *J. Physiol.* **296**:49–60
53. Machemer-Röhnisch, S., Machemer, H. 1989. A Ca paradox: electric and behavioral responses of *Paramecium* following changes in external ion concentration. *Eur. J. Protistol.* **25**:45–59
54. Mackrill, J.J. 1999. Protein-protein interactions in intracellular Ca^{2+} -release channel function. *Biochem. J.* **337**:345–361
55. Malgaroli, A., Fesce, R., Meldolesi, J. 1990. Spontaneous $[\text{Ca}^{2+}]_i$ fluctuations in rat chromaffin cells do not require inositol 1,4,5-trisphosphate elevations but are generated by a caffeine- and ryanodine-sensitive intracellular Ca^{2+} store. *J. Biol. Chem.* **265**:3005–3008
56. Meissner, G. 1994. Ryanodine receptor/ Ca^{2+} release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.* **56**:485–508
57. Meldolesi, J., Villa, A. 1993. Endoplasmic reticulum and the control of Ca^{2+} homeostasis. *Subcell. Biochem.* **21**:189–207
58. Metuzals, J., Chang, D., Hammar, K., Reese, T.S. 1997. Organi-

- zation of the cortical endoplasmic reticulum in the squid giant axon. *J. Neurocytol.* **26**:529–539
59. Ogura, A., Machemer, H. 1980. Distribution of mechanoreceptor channels in the *Paramecium* surface membrane. *J. Comp. Physiol. A* **135**:233–242
 60. Ogura, A., Takahashi, K. 1976. Artificial deciliation causes loss of calcium-dependent responses in *Paramecium*. *Nature* **264**:170–172
 61. Papahadjopoulos, D. 1978. Calcium-induced phase changes and fusion in natural and model membranes. In: Membrane Fusion, G. Poste and G.L. Nicolson, editors. pp. 765–790. Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford
 62. Parekh, A.B., Penner, R. 1997. Store depletion and calcium influx. *Physiol. Rev.* **77**:901–930
 63. Peeters, G.A., Kohmoto, O., Barry, W.H. 1989. Detection of La^{3+} influx in ventricular cells by indo-1 fluorescence. *Am. J. Physiol.* **256**:C351–C357
 64. Peters, C., Mayer, A. 1998. Ca^{2+} /calmodulin signals the completion of docking and triggers a late step of vacuole fusion. *Nature* **396**:575–580
 65. Phillippe, M. 1994. Neomycin inhibition of hormone-stimulated smooth muscle contractions in myometrial tissue. *Biochem. Biophys. Res. Commun.* **205**:245–250
 66. Pillai, S., Bickle, D.D. 1992. Lanthanum influx into cultured human keratinocytes: effect on calcium flux and terminal differentiation. *J. Cell. Physiol.* **151**:623–629
 67. Plattner, H., Braun, C., Hentschel, J. 1997a. Facilitation of membrane fusion during exocytosis and exocytosis-coupled endocytosis and acceleration of “ghost” detachment in *Paramecium* by extracellular calcium. A quenched-flow/freeze-fracture analysis. *J. Membrane Biol.* **158**:197–208
 68. Plattner, H., Habermann, A., Kissmehl, R., Klauke, N., Majoul, I., Söling, H.-D. 1997b. Differential distribution of calcium stores in *Paramecium* cells. Occurrence of a subplasmalemmal store with a calsequestrin-like protein. *Eur. J. Cell Biol.* **72**:297–306
 69. Plattner, H., Knoll, G., Pape, R. 1993. Synchronization of different steps of the secretory cycle in *Paramecium tetraurelia*: trichocyst exocytosis, exocytosis-coupled endocytosis, and intracellular transport. In: Membrane Traffic in Protozoa. H. Plattner, editor. pp. 123–148. JAI Press, Greenwich, CT
 70. Plattner, H., Lumpert, C.J., Knoll, G., Kissmehl, R., Höhne, B., Momayezi, M., Glas-Albrecht, R. 1991. Stimulus-secretion coupling in *Paramecium* cells. *Eur. J. Cell Biol.* **55**:3–16
 71. Plattner, H., Matt, H., Kersken, H., Haacke, B., Stürzl, R. 1984. Synchronous exocytosis in *Paramecium* cells. I. A novel approach. *Exp. Cell Res.* **151**:6–13
 72. Plattner, H., Stürzl, R., Matt, H. 1985. Synchronous exocytosis in *Paramecium* cells. IV. Polyamino compounds as potent trigger agents for repeatable trigger-redocking cycles. *Eur. J. Cell Biol.* **36**:32–37
 73. Powis, D.A., Clark, C.L., O'Brien, K.J. 1994. Lanthanum can be transported by the sodium-calcium exchange pathway and directly triggers catecholamine release from bovine chromaffin cells. *Cell Calcium* **16**:377–390
 74. Pozzan, T., Rizzuto, R., Volpe, P., Meldolesi, J. 1994. Molecular and cellular physiology of intracellular calcium stores. *Pharmacol. Rev.* **74**:595–636
 75. Preston, R.R. 1990. Genetic dissection of Ca^{2+} -dependent ion channel function in *Paramecium*. *BioEssays* **12**:273–281
 76. Preston, R.R., Kink, J.A., Hinrichsen, R.D., Saimi, Y., Kung, C. 1991. Calmodulin mutants and Ca^{2+} -dependent channels in *Paramecium*. *Annu. Rev. Physiol.* **53**:309–319
 77. Preston, R.R., Saimi, Y., Kung, C. 1992a. Calcium current activated upon hyperpolarization of *Paramecium tetraurelia*. *J. Gen. Physiol.* **100**:233–251
 78. Preston, R.R., Saimi, Y., Kung, C. 1992b. Calcium-dependent inactivation of the calcium current activated upon hyperpolarization of *Paramecium tetraurelia*. *J. Gen. Physiol.* **100**:253–268
 79. Quinn, S.J., Ye, C.P., Diaz, R., Kifor, O., Bai, M., Vassilev, P., Brown, E. 1997. The Ca^{2+} -sensing receptor: a target for polyamines. *Am. J. Physiol.* **273**:C1315–C1323
 80. Reiss, M., Lipsey, L.R., Zhou, Z.L. 1991. Extracellular dependent-calcium regulation of transmembrane calcium fluxes in murine keratinocytes. *J. Cell. Physiol.* **147**:281–291
 81. Riccardi, D., Hebert, S.C. 1997. Neomycin and gentamycin act as agonists at the extracellular Ca^{2+} -sensing receptor isolated from rat kidney. *J. Physiol.* **504**:139P–140P
 82. Saimi, Y. 1986. Calcium-dependent sodium currents in *Paramecium*: mutational manipulations and effects of hyper- and depolarization. *J. Membrane Biol.* **92**:227–236
 83. Saimi, Y., Kung, C. 1980. A Ca-induced Na-current in *Paramecium*. *J. Exp. Biol.* **88**:305–325
 84. Saitow, F., Nakaoka, Y., Oosawa, Y. 1997. A calcium-activated, large conductance and nonselective cation channel in *Paramecium* cell. *Biochim. Biophys. Acta* **1327**:52–60
 85. Schneider, M.F. 1994. Control of calcium release in functioning skeletal muscle fibers. *Annu. Rev. Physiol.* **56**:463–484
 86. Schultz, J.E., Guo, Y.L., Kleefeld, G., Völkel, H. 1997. Hyperpolarization- and depolarization-activated Ca^{2+} currents in *Paramecium* trigger behavioral changes and cGMP formation independently. *J. Membrane Biol.* **156**:251–259
 87. Shorte, S.L., Schofield, J.G. 1996. The effect of extracellular polyvalent cations on bovine anterior pituitary cells. Evidence for a Ca^{2+} -sensing receptor coupled to release of intracellular calcium stores. *Cell Calcium* **19**:43–57
 88. Stelly, N., Halpern, S., Nicolas, G., Fragu, P., Adoutte, A. 1995. Direct visualization of a vast cortical calcium compartment in *Paramecium* by secondary ion mass spectrometry (SIMS) microscopy: possible involvement in exocytosis. *J. Cell Sci.* **108**:1895–1909
 89. Stelly, N., Mauger, J.P., Keryer, G., Claret, M., Adoutte, A. 1991. Cortical alveoli of *Paramecium*: a vast submembranous calcium storage compartment. *J. Cell Biol.* **113**:103–112
 90. Takemura, H., Hughes, A.R., Thastrup, O., Putney, J.W. 1989. Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.* **264**:12266–12272
 91. Taylor, C.W. 1990. Receptor-regulated Ca^{2+} entry: secret pathway or secret messenger? *Trends Neurosci.* **11**:269–271
 92. Tse, F.W., Tse, A., Hille, B., Horstmann, H., Almers, W. 1997. Local Ca^{2+} release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron* **18**:121–132
 93. Vassilev, P.M., Ho-Pao, C.L., Kanazirska, M.P.V., Ye, C., Hong, K., Seidman, C.E., Seidman, J.G., Brown, E.M. 1997. Ca_v -sensing receptor (CaR)-mediated activation of K^+ channels is blunted in CaR gene-deficient mouse neurons. *NeuroRep.* **8**:1411–1416
 94. Warnat, J., Philipp, S., Zimmer, S., Flockerzi, V., Cavalié, A. 1999. Phenotype of a recombinant store-operated channel: highly selective permeation of Ca^{2+} . *J. Physiol.* **518**:631–638
 95. Weiger, T.M., Langer, T., Hermann, A. 1998. External action of di- and polyamines on maxi calcium-activated potassium channels: an electrophysiological and molecular modeling study. *Biophys. J.* **74**:722–730
 96. Westerblad, H., Andrade, F.H., Islam, M.S. 1998. Effects of ryanodine receptor agonist 4 chloro-m-cresol on myoplasmic free Ca^{2+} concentration and force of contraction in mouse skeletal muscle. *Cell Calcium* **24**:105–115

97. Williams, K. 1997. Interactions of polyamines with ion channels. *Biochem. J.* **325**:289–297
98. Yamaguchi, T., Olozak, I., Chattopadhyay, N., Butters, R.R., Kifor, O., Scadden, D.T., Brown, E.M. 1998. Expression of extracellular calcium (Ca^{2+}_o)-sensing receptor in human peripheral blood monocytes. *Biochem. Biophys. Res. Commun.* **246**:501–506
99. Yano, M., El-Hayek, R., Antoniu, B., Ikemoto, N. 1994. Neomycin: a novel potent blocker of communication between T-tubule and sarcoplasmic reticulum. *FEBS Lett.* **351**:349–352
100. Ye, C., Ho-Pao, C.L., Kanazirska, M. 1997. Deficient cation channel regulation in neurons from mice with targeted disruption of the extracellular Ca^{2+} sensor receptor gene. *Brain Res. Bull.* **44**:75–84
101. Zaidi, M., Shankar, V.S., Tunwell, R., Adebajo, O.A., Mackrill, J., Pazianas, M., O'Connell, D., Simon, B.J., Rifkin, B.R., Venkitaraman, A.R., Huang, C.L.H., Lai, F.A. 1995. A ryanodine receptorlike molecule expressed in the osteoclast plasma membrane functions in extracellular Ca^{2+} sensing. *J. Clin. Invest.* **96**:1582–1590
102. Zhou, X.L., Chan, C.W.M., Saimi, Y., Kung, C. 1995. Functional reconstitution of ion channels from *Paramecium* cortex into artificial liposomes. *J. Membrane Biol.* **144**:199–208
103. Zhu, D.L., Peng, H.B. 1988. Increase in intracellular calcium induced by the polycation-coated latex bead, a stimulus that causes postsynaptic-type differentiation in cultured *Xenopus* muscle cells. *Dev. Biol.* **126**:63–70