Sub-second calcium coupling between outside medium and subplasmalemmal stores during overstimulation/depolarisation-induced ciliary beat reversal in *Paramecium* cells

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Abstract

As amply documented by electrophysiology, depolarisation in *Paramecium* induces a Ca^{2+} influx selectively via ciliary voltage-dependent Ca^{2+} -channels, thus inducing ciliary beat reversal. Subsequent downregulation of ciliary Ca^{2+} has remained enigmatic. We now analysed this aspect, eventually under overstimulation conditions, by quenched-flow/cryofixation, combined with electron microscope X-ray microanalysis which registers total calcium concentrations, $[\text{Ca}]$. This allows to follow Ca-signals within a time period (≥ 30 ms) smaller than one ciliary beat (~ 50 ms) and beyond. Particularly under overstimulation conditions ($\sim 10^{-5}$ M Ca^{2+} before, 0.5 mM Ca^{2+} during stimulation) we find in cilia a $[\text{Ca}]$ peak at ~ 80 ms and its decay to near-basal levels within 110 ms (90%) to 170 ms (100% decay). This $[\text{Ca}]$ wave is followed, with little delay, by a $[\text{Ca}]$ wave into subplasmalemmal Ca-stores (alveolar sacs), culminating at ~ 100 ms, with a decay to original levels within 170 ms. Also with little delay $[\text{Ca}]$ slightly increases in the cytoplasm below. This implies rapid dissipation of Ca^{2+} through the ciliary basis, paralleled by a rapid, transient uptake by, and release from cortical stores, suggesting fast exchange mechanisms to be analysed as yet. This novel type of coupling may be relevant for some phenomena described for other cells.

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1. Introduction

Ca^{2+} -transients govern a variety of cellular phenomena, some occurring on a sub-second time scale [1–3], by different mechanisms for Ca^{2+} mobilisation and subsequent re-establishment of Ca^{2+} homeostasis [4–6]. In ciliated protozoans, specifically in *Paramecium*, Ca^{2+} regulates dense-core secretory vesicle (trichocyst) exocytosis [7,8] and ciliary beat [9–11], notably depolarisation-induced ciliary beat reversal [12,13], among other phenomena [14]. Ciliary reversal parallels electrically or chemically induced depolarisation, causing activation of voltage-dependent Ca^{2+} -channels which subsequently are rapidly inactivated by the very same Ca^{2+} [15]. In *Paramecium*, depolarisation-induced Ca^{2+} -currents

may last only several milliseconds, e.g., during short depolarisation pulses [16]. Remarkably, this phenomenon of Ca^{2+} -channel inactivation by an increase of intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, has been recognised, though considerably later, in some neurons [17–19]. In both systems it may help to reduce the Ca^{2+} signal to the minimum required for cell activation. Moreover, in *Paramecium*, voltage-dependent Ca^{2+} -channels are localised exclusively in the ciliary membranes [20]. All this may help to restrict the Ca^{2+} -signal in cilia during depolarisation and, thus, contribute to optimise energetic requirements for re-establishment of $[\text{Ca}^{2+}]_i$ homeostasis.

How Ca^{2+} in cilia of *Paramecium* – or any comparable system – is downregulated after activation has not been analysed so far. Plausible assumptions would be, e.g., inactivation by binding to Ca^{2+} -binding proteins [14], including calmodulin [21] and some other functional target molecules, and/or

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diffusion to the ciliary basis and then into the nearby cortical cytosol for subsequent elimination. This may be particularly important when Ca^{2+} influx goes on for longer periods of time, for instance when *Paramecium* cells are exposed to depolarising solutions [22].

Two types of Ca^{2+} -pumps (Ca^{2+} -ATPases) have been found in *Paramecium*, a plasmalemmal type activated by Ca^{2+} /calmodulin [23] and a SERCA-type occurring in alveolar sacs [24,25], the established cortical Ca -stores of *Paramecium* [26–30]. The presence of a Ca^{2+} -ATPase (pump) in ciliary membranes has been denied [23]. So far, there was also no evidence of any role for alveolar sacs in ciliary activity regulation. These sacs line the entire surface of *Paramecium* (and of any other pathogenic or harmless member of the phylum Alveolata), with the exception of trichocyst docking sites and the emergence site of cilia.

Any analysis of Ca^{2+} -dynamics in cilia by fluorochromes, particularly over short times, remains problematic. As an alternative, we have previously established a quenched-flow/cryofixation procedure appropriate to achieve time-dependent cell stimulation [31] and retention of calcium in place [27] for subsequent analysis by electron microscopic (EM) energy-dispersive X-ray microanalysis, EDX [29,30]. EDX allows to measure locally total calcium concentrations, $[\text{Ca}]$, by recording the main element-specific peak, $\text{Ca K}\alpha$, with high spatial and temporal resolution. In our case this is possible from 30 ms on, the dead-time of the quenched-flow machine where depolarisation can be induced by rapid mixing cells with high $[\text{K}^+]$ medium. With an electron probe of ~ 63 nm, in the EM operated in the scanning transmission mode, STEM, $[\text{Ca}]$ can be conveniently registered in cilia of ~ 300 nm diameter as well as in alveolar sacs (average width ~ 100 nm), but not in the 15 nm wide subplasmalemmal space between these sacs and the plasma membrane.

In brief, using this methodology we now find, under overstimulation conditions (see Section 2), a rapid increase of $[\text{Ca}]$ in cilia, culminating within the second stroke and decaying with the third stroke after stimulation. Quite unexpectedly we then see a rapid $[\text{Ca}]$ increase in alveolar sacs within the same time range, paralleled by Ca^{2+} dissipation into the nearby cytosol. These novel findings will also be interesting in the context of current problems concerning the crosstalk between cortical stores and the outside medium via the cell membrane in a variety of cell types.

2. Materials and methods

2.1. Cells

Paramecium tetraurelia wildtype strain 7S and, for controls, strain *d4-500r* were cultivated as indicated previously [30]. Strain *d4-500r* contains no functional Ca^{2+} -influx channels in its cilia [32]. Before each experiment, performance of ciliary beat reversal upon adding KCl (to yield a final con-

centration of 20 mM) was tested, as well as its absence in *d4-500r* cells.

2.2. Stimulation conditions and quenched-flow/freeze-substitution

Under “standard conditions” 7S cells were contained in the medium specified previously [29,30], including Pipes buffer (pH 7.2), with CaCl_2 and KCl added, 1 mM each. To achieve a higher extra- to intracellular gradient, $[\text{Ca}^{2+}]_o/[\text{Ca}^{2+}]_i$, $[\text{Ca}^{2+}]_o$ was eventually reduced to $\sim 10^{-5}$ M for ~ 75 min before stimulation, and $[\text{Ca}^{2+}]_o$ was increased together with KCl selectively during stimulation by rapid mixing to yield 0.5 mM CaCl_2 and 20 mM KCl (final concentrations seen by the cells), respectively. This is designated further on as “overstimulation”. The mutant, *d4-500r*, was processed under “standard conditions”, yet with $[\text{Ca}^{2+}]_o = 10$ mM to intensify any potential Ca^{2+} signal (if any). The corresponding details are indicated in the respective figure captions. For controls (0 ms data points), cells were mixed for 80 ms with the same solution in which they were before the respective stimulation experiments. Rapid mixing was performed in the quenched-flow apparatus previously described [31].

Retention of calcium in samples, with the exclusion of any detectable redistribution during freeze-substitution, was achieved by the method previously described in detail [27,29,30].

2.3. EDX analysis and statistics

Analyses were conducted in a Zeiss/Leo EM912 Omega electron microscope equipped with a scanning transmission (STEM) unit, type DSA unit STEM digital attachment, and an energy-dispersive X-ray microanalysis unit, type INCA from Oxford Instruments. Sections (500 nm thick) were analysed in the EM, operated in the scanning transmission mode (STEM) at 80 kV acceleration voltage, ~ 12 μA beam current and a spot size adjusted to 63 nm. Net $\text{Ca K}\alpha$ counts were registered by spot measurements, e.g., in 0.5 μm intervals along longitudinally cut cilia, in strictly cross-cut alveolar sacs, over longitudinally cut ciliary basal bodies (containing also closely surrounding alveolar sacs), in the cytosol at a distance of 0.5 and 1.0 μm , respectively, from ciliary bases, and in cortical mitochondria. Measurements in alveolar sacs were randomised irrespective of the location relative to cilia which is feasible considering the rapid spread of Ca^{2+} inside stores [33]. $[\text{Ca}]$ in alveolar sacs of unstimulated cells, previously estimated as ~ 43 mM [29], can serve as an internal reference value. An example of positions analysed is shown in Fig. 1. Statistical analysis was performed by paired Student's *t*-test for the data set obtained under “standard” stimulation conditions, but no significant differences within the time course in a given compartment were found. For “overstimulated” samples, to achieve higher accuracy, we applied the SigmaStat 2.03 program (Jandel Scientific, San Rafael, CA).

Fig. 1. STEM image of a semi-thin section used for EDX analysis, with positions of point measurements visible as contamination spots over a cilium (ci), alveolar sac (as) and a ciliary basal body (bb), as well as in cytoplasmic domains at 0.5 and 1.0 μm distance (cy0.5, cy1.0), respectively, from a ciliary basal body. Scale bar = 0.5 μm .

3. Results

Fig. 1 gives an example of how 500 nm-thick sections were analysed by STEM/EDX, with data acquisition points labelled by contamination spots formed during analysis. The probe diameter of 63 nm, with a calculated top/bottom spread to 72 nm [29] is smaller than any of the structural details analysed, including cilia (~ 300 nm diameter) and alveolar sacs with an average width of 98 ± 11 nm [30]. Data collected over ciliary basal bodies include counts from closely surrounding alveolar sacs whose volume contribution is estimated as $\sim 30\%$ within the 500 nm-sections at data acquisition points. Fig. 1 also shows how values were collected in the cytosolic compartment at 0.5 and 1.0 μm distance, respectively, from basal bodies.

Fig. 2 presents the calcium-specific EDX signals, i.e., the Ca K α readings obtained with wildtype cells at different times after depolarisation under “standard conditions” (see Section 2). This implies that cells were contained in a medium including 1 mM CaCl₂ before and during stimulation by rapid mixing with 40 mM KCl to yield a final concentration of $[\text{K}^+] = 20$ mM and $[\text{Ca}^{2+}] = 0.5$ mM, respectively. Data points indicated include bars for S.E.M. Further statistical evaluation included determination of P values (see Section 2).

Remarkably, under “standard conditions”, one can hardly recognise any $[\text{Ca}]$ increase in cilia (with none of the P values below 0.05) with only a very weak increase 80 ms after depolarisation ($P = 0.21$ for 80 ms versus 0 ms). Much more pronounced are $[\text{Ca}]$ changes in alveolar sacs where Ca K α readings first decline (30 ms, $P = 0.05$) and then cul-

Fig. 2. Time course of Ca K α signals upon stimulation of ciliary reversal under “standard conditions”. Wildtype (7S) cells (contained in 5 mM Na-Pipes + 1 mM KCl + 1 mM CaCl₂, pH 7.2) were stimulated by mixing with the same buffer and increasing KCl to yield 20 mM KCl (in the presence of 0.5 mM CaCl₂) in the quenched-flow machine for the times indicated. All curves are normalised to 100% for Ca K α signals in alveolar sacs before stimulation. Bars = S.E.M. N , number of cells; n , individual data points. For 0 s (unstimulated control), $N = 10$ for all samples, $n = 26, 11, 36, 18, 18$ (data sequence for alveolar sacs, basal bodies, cilia, cytoplasm 0.5 and 1.0 μm , respectively, below basal bodies). For 30 ms activation, $N = 9$ for all, $n = 27, 8, 35, 14, 13$ (same sequence). For 80 ms activation, $N = 10$ for all, $n = 33, 13, 43, 18, 14$ (same sequence). For 110 ms activation, $N = 8$ for all samples, $n = 19, 13, 43, 23, 17$ (same sequence as for 0 s [control]). No significant differences could be established by Student's t -test for experimental values compared to controls.

minate 80 ms after depolarisation ($P = 0.67$ for 80 ms versus 0 ms). Although much weaker, a similar tendency is recognised in “basal bodies” with their surrounding alveolar sacs. There was also some indication of $[\text{Ca}]$ increase in the nearby cytosol (Fig. 2), but this also could not be statistically ascertained.

Any of the tendencies shown in Fig. 2 are much more pronounced when cells are brought into a low calcium medium, $[\text{Ca}^{2+}]_o \sim 10^{-5}$ M, before stimulation, followed by stimulation in the presence of $[\text{Ca}^{2+}]_o = 0.5$ mM which was added only during depolarisation with $[\text{K}^+]_o = 20$ mM for different times in the quenched-flow apparatus. Data retrieved under such “overstimulation” conditions are presented in Fig. 3. For a critical appraisal of this set-up, see Section 4.

In detail, Fig. 3 reveals a remarkable increase of Ca K α signals in cilia (values averaged from all measurements over the entire length), with a maximum 80 ms after depolarisation ($P < 0.006$ up to 0.008 for 80 ms versus 30 ms, $P < 0.001$ up to 0.041 for 80 ms versus 0 ms; ranges for P values are given to compare data for the individual positions along the ciliary shaft as indicated in Fig. 1). For a trigger time ≥ 110 ms, e.g.,

Fig. 3. Time course of Ca K α signals upon “overstimulation” of ciliary reversal. Wildtype (7S) cells (contained in 5 mM Na-Pipes + 1 mM KCl [pH 7.2], $[Ca^{2+}]_o \sim 10^{-5}$ M) were stimulated by mixing with the same solution but with 1 mM $CaCl_2$ added only during stimulation, when $[K^+]$ was increased to yield 20 mM in the quenched-flow machine for times indicated. Controls (0 s) were processed without increasing $[Ca^{2+}]_o$ and $[K^+]_o$, respectively. All curves are normalised to 100% for Ca K α signals in alveolar sacs before stimulation. Bars = S.E.M. N , number of cells; n , individual data points. For 0 s (unstimulated control), $N=27$, 12, 155, 30, 30, 6 for samples in the following sequence: alveolar sacs, basal bodies; cilia, cytosol at 0.5 μ m from ciliary basal body, cytosol at 1.0 μ m from basal body, cortical mitochondria; in the same sequence, $n=38$, 16, 257, 55, 55, 18. For 30 ms activation, in the same sequence, $N=22$, 12, 170, 29, 29, 0 (mitochondria not analysed), and $n=30$, 13, 256, 41, 40, 0 (mitochondria not analysed). For 80 ms stimulation, in the same sequence, $N=21$, 13, 176, 24, 22, 8, and $n=30$, 13, 306, 43, 42, 20. For 110 ms activation, in the same sequence, $N=16$, 9, 148, 20, 19, 6, and $n=22$, 9, 183, 25, 22, 27. For 170 ms stimulation, in the same sequence, $N=14$, 6, 143, 19, 19, 6, and $n=18$, 7, 197, 28, 26, 18. For significance values, see text.

at 170 ms stimulation, values recorded in cilia decrease again to original levels and the values show no more any statistically significant difference (P value = 0.922 when the 170 ms value is referred to the 0 ms value). Considering the duration of one ciliary beat, i.e. ~ 50 ms, $[Ca]$ values recorded rise within one ciliary stroke, culminate within the second stroke and are reset to resting levels within the third stroke after depolarisation.

In Fig. 3, $[Ca]$ values steadily increase in “basal bodies”, culminating at ~ 110 ms, i.e., slightly after the maximum recorded in cilia (80 ms), before values decline. This delay is difficult to ascertain statistically, but resembles the indication of a small delay occurring in alveolar sacs (which also contribute to “basal body” data points). In alveolar sacs, values first decline slightly ($P=0.014$ for 30 ms versus 0 ms), and then increase to a maximum at ~ 95 ms (between 80 and 110 ms, $P<0.001$ and <0.001 , respectively, for 80 ms versus 0 ms and 110 ms versus 0 ms, respectively).

Fig. 4. Diagram showing “overstimulation” of the mutant, *d4-500r* (devoid of ciliary voltage-dependent Ca^{2+} -channels; grey blocks) under control conditions (unstimulated) and after “overstimulation” (for 7S cells, see also Fig. 3) for 80 ms. For comparison key-values obtained from wildtype cells (7S; white blocks), as reported previously [45], are included. The *d4-500r* cells were exposed to conditions similar to those indicated in Fig. 2, but with $[Ca^{2+}]_o$ increased to 10 mM (see Section 2). Bars = S.E.M. N , number of cells; n , individual data points. For *d4-500r* cells, for 0 s (unstimulated control), $N=3$ and $n=10$ for alveolar sacs samples; $N=5$, $n=64$ for cilia. For 80 ms activation, $N=4$ and $n=8$ for alveolar sacs; $N=6$, $n=50$ for cilia.

Already 80 ms after depolarisation Ca K α counts increase in the cytosol, 0.5 and 1.0 μ m off basal bodies ($P=0.007$ for 0.5 μ m and 0.125 for 1.0 μ m, referred to 0 ms values, respectively). This increase looks higher at 0.5 μ m distance from basal bodies than at 1.0 μ m distance, though this cannot be statistically ascertained ($P=0.529$ for 0.5 μ m versus 1.0 μ m distance). No increase is recognised in nearby mitochondria (Fig. 3).

In sum, the data contained in Fig. 3 would be compatible with the following sequence of events after depolarisation: influx of Ca^{2+} into cilia, followed by dissipation and transient uptake into nearby alveolar sacs. From there Ca^{2+} is dissipated into the cortical cytosol with a considerable dilution effect to levels approaching detection limit already in the outermost cytosolic layer of only a few micrometre thickness. However, this logical sequence would be difficult to ascertain from the statistics underlying the curves presented. Therefore, we resorted to the mutant lacking any functional Ca^{2+} -channels in ciliary membranes, as it was used in the following experiments.

Using *d4-500r* cells and applying “overstimulation”, we have analysed $[Ca]$ values in alveolar sacs and cilia (Fig. 4). The aim was to see how $[Ca]$ would change in a strain which is unable to react to a depolarisation by Ca^{2+} -influx (see Section 2). When compared to parallel experiments conducted with 7S cells, *d4-500r* cells contain much lower $[Ca]$ in alveolar sacs and in cilia before stimulation. This is not unexpected since in different *P. tetraurelia* strains, under resting conditions, $[Ca^{2+}]_i$ may also vary to a similar extent [7], the reasons being unknown. Interestingly, during 80 ms stimulation, $[Ca]$

values in alveolar sacs increase only in 7S cells, while they decrease in *d4-500r* cells, though statistically not significant ($P=0.024$). It looks as if *d4-500r* cells would maintain the depression in alveolar [Ca] seen in 7S cells shortly after depolarisation (Fig. 3), the cause of this depression remaining to be settled also with the *d4-500r* strain. In cilia of *d4-500r* cells, [Ca] under depolarisation conditions does not change significantly, i.e., $P>0.5$ (80 ms stimulated versus unstimulated), as shown in Fig. 4. This is in clear contrast to [Ca] signals recorded in cilia of 7S cells under comparable conditions of “overstimulation” (see also Fig. 3). All these findings with the *d4-500r* strain are compatible with the lack of any significant Ca^{2+} -influx into cilia and beyond, e.g., into alveolar sacs.

Mock stimulation by subjecting wildtype cells to any of the procedures outlined above, but without increasing [KCl] in the medium during passage of the quenched-flow apparatus, did not cause any of the [Ca] changes described above for the different organelles.

4. Discussion

The fate of Ca^{2+} entering a *Paramecium* cell during depolarisation-induced ciliary beat reversal has not been analysed up to now. As it was routinely drawn in corresponding schemes, mainly metabolic removal by pump activities has been more or less tacitly assumed. We now show that an excess of Ca^{2+} after stimulation may be removed from cilia by a much more intricate mechanism, with high efficiency and speed. The elucidation of this pathway is greatly facilitated by applying conditions of “overstimulation”. This does not preclude that under mild stimulation conditions Ca^{2+} -binding to ciliary proteins may be a dominant aspect.

4.1. Is “overstimulation” a feasible procedure?

As outlined above, we increase the gradient between intra- and extracellular Ca^{2+} in some of the experiments designated as “overstimulation”. Although the $[\text{Ca}^{2+}]_o$ level applied in such experiments before stimulation is low, $\sim 10^{-5}$ M, this is well above $[\text{Ca}^{2+}]_i$ -values in unstimulated cells [7]. Since *Paramecium* is notoriously known as being sensitive to $[\text{Ca}^{2+}]_o=0$ [34] it should be noted that $[\text{Ca}^{2+}]_o=10^{-6}$ M is well tolerated and that exocytosis – a Ca^{2+}_o -dependent process – can take place at still lower $[\text{Ca}^{2+}]_o$ -values [27,35]. *Paramecium* cells rapidly react to any changes in $[\text{Ca}^{2+}]_o$ by a change of $[\text{Ca}^{2+}]_i$, e.g., by an increase of $[\text{Ca}^{2+}]_i$ in parallel to an increase of $[\text{Ca}^{2+}]_o$ [36], but with no exocytotic or behavioural response in the absence of a specific stimulus [37]. This may also hold for the stores whose filling state is gradually influenced by $[\text{Ca}^{2+}]_o$, e.g., in muscle [38]. An overstimulation effect is, therefore, expected from an increase of $[\text{Ca}^{2+}]_o$ precisely during stimulation. Thus, an increased concentration gradient can be exploited to increase

the Ca^{2+} -influx during stimulation and, hence, to increase the [Ca] signal to be recorded by EDX in cilia and alveolar sacs. Remarkably, the time course is similar in experiments under “overstimulation” (Fig. 3) and under “standard” conditions (Fig. 2). In sum, we are confident that “overstimulation” is a feasible way to exaggerate a physiological response which otherwise may remain much more difficult to detect.

4.2. Comparison with electrophysiology and in vitro experiments

In vitro, using extracted cell models, ciliary beat reversal requires only 1 μM [39]. Extrapolation showed similar requirements in vivo [34]. Considering that, inside the cells, the usual ratio of total calcium, [Ca], to free calcium $[\text{Ca}^{2+}]$, generally differs by about three to four orders of magnitude [1,3], it is not surprising that we see [Ca] in cilia to rise to a level well above detectability by our EDX system, particularly during overstimulation.

Ciliary reversal starts already within one ciliary stroke, with all cilia over the entire cell surface [40]. Concomitantly, we see [Ca] to rise from 30 ms on and to culminate at 80 ms, i.e., within the second stroke. Calmodulin, considered as a link between $[\text{Ca}^{2+}]_i$ increase and ciliary reversal [41], can bind Ca^{2+} within milliseconds [42]. In fact, calmodulin is tightly associated with the ciliary membrane and to the axoneme [43].

From the currents measured during minimal depolarisation steps (e.g., 20 mV/4 ms) at $[\text{Ca}^{2+}]_o=1$ mM, the number of equivalents transferred into the estimated volume of all cilia (1.7×10^{-12} l) has been estimated as 8×10^{-17} during the time required to inactivate presumably all ciliary Ca^{2+} channels [16]. This would yield a $[\text{Ca}^{2+}]$ of 2 μM in cilia, if one disregards any further dissipation. Estimations under varied conditions were slightly above this value [15]. Assuming 80 ms channel activity (the time we observe [Ca] in cilia to culminate), extrapolation would result in a ciliary $[\text{Ca}^{2+}]=0.08\text{--}0.16$ mM during depolarisation under conditions indicated by others [11,44]. Clearly such values could yield [Ca] values at the limits of detectability by our EDX recordings. Therefore, we resorted to “overstimulation” experiments. In this case, one has to consider that [Ca] increases may be higher than in electrophysiological recordings. In fact, chemical depolarisation over longer times combined with $^{45}\text{Ca}^{2+}$ flux measurements shows much higher Ca^{2+} uptake since Ca^{2+} flux into cells (unstimulated background subtracted) lasts much longer than recorded during electrical stimulation [22]. This was explained by masked Ca^{2+} flux during ongoing depolarisation [13]. Concomitantly, pilot calculations from $^{45}\text{Ca}^{2+}$ flux data published with chemical depolarisation [22] would result in [Ca]-values approaching those we see by EDX.

Electrophysiological and EDX data are compatible if one assumes rapid Ca^{2+} dissipation into deeper layers or extrusion. In fact, removal of Ca^{2+} from cilia during

depolarisation-induced ciliary reversal has been a postulate in electrophysiological papers [11,12,40], although it was not possible experimentally to determine how this would work. Our “overstimulation” experiments show how calcium can be removed from cilia after depolarisation (at least when ciliary Ca^{2+} -binding proteins would be saturated). The way we find is rather different from that routinely proposed in the literature, as we discuss below.

4.3. Spread of Ca K α signals and spill-over phenomena

We observe a rapid spread of calcium within the cilia, resulting in similar Ca K α values along the ciliary shafts. This is in conformance with other EDX analyses, showing spill-over in the opposite direction, i.e., into cilia during secretion stimulation [45], and with the occurrence of ciliary reversal in *d4-500r* cells during exocytosis stimulation [45,46]. Considering this rapid spread of Ca^{2+} in either direction we have averaged in our figures all data points recorded along ciliary shafts. Note that in samples designated as “basal bodies” $\sim 30\%$ of the volume is contributed by tightly attached alveolar sacs.

How to judge Ca^{2+} spill-over during overstimulation? Clearly, during depolarisation, Ca^{2+} is not mobilised from alveolar sacs, but we find that they can take up Ca^{2+} very rapidly. Before stimulation, [Ca] in alveolar sacs is estimated as 43 mM from calibrated data [29], a value well comparable with Ca^{2+} -stores in other cells [47,48]. Overstimulation causes an increase in [Ca] in alveolar sacs and in the outermost cytoplasmic layer of the cell cortex. The extent of increase of Ca K α signals during overstimulation and of the shift occurring between the compartments analysed are in agreement with morphometric data [37], based on the following aspects. (i) Cilia occupy $1950 \mu\text{m}^3$, i.e., 2.6% of the cell volume. Alveolar sacs, which can serve as an internal [Ca] standard, occupy $161 \mu\text{m}^3$. One might estimate from Fig. 3 that during “overstimulation” [Ca] in cilia would approach values of $\sim 30\%$ of those in alveolar sacs. If one adjusts [Ca] values in cilia ($\sim 300 \text{ nm}$ thick) for section thickness (500 nm), values would represent $\sim 50\%$ of [Ca] in alveolar sacs. (ii) Considering that alveolar sacs occupy only 8.3% of the volume represented by cilia, a major fraction of Ca^{2+} must be rapidly dissipated, otherwise values in alveolar sacs would have to rise to much higher levels. In fact, dissipation into the cytosolic compartment must take place already within the first few ciliary beats—either directly or after passing the alveolar sacs. Since [Ca] in the sacs decays within 170 ms to the [Ca] values recorded before stimulation, most of the Ca^{2+} from activated cilia must finally go into the cytosol. (iii) Assuming that after “overstimulation” all Ca^{2+} from cilia would be dissipated into an outermost cytoplasmic layer of $2 \mu\text{m}$ below alveolar sacs (the cortical layer where a detection limit of $<2 \text{ mM}$ is achieved), how does this compare with an estimated value of [Ca] $\sim 20 \text{ mM}$ in cilia at the peak? Considering the ciliary volume of 2.6% of cell volume and a cell surface of $10.7 \times 10^3 \mu\text{m}^2$ [37], the $2 \mu\text{m}$ -cortex layer would

represent 29% of cell volume and dilution would, therefore, reach a value of $\sim 2 \text{ mM}$. Since values above this detection limit value are recorded only in the outermost $1 \mu\text{m}$ -thick cytosolic layer, dilution must proceed rapidly to somewhat deeper layers, all within fractions of a second.

The 15 nm space between the cell membrane and the alveolar sacs membrane cannot be analysed by EDX. Neither one of these two membranes can be assumed to dispose of a pump appropriate for rapid Ca^{2+} extrusion (see below).

4.4. Could a cation antiporter account for a rapid calcium transient in subplasmalemmal stores?

The rapid uptake of Ca^{2+} into alveolar sacs, followed by rapid release that accompanies ciliary reversal, remains to be explained. A quite similar phenomenon occurs during synchronous exocytosis, also analysed by EDX [30]. Considering the slow kinetics of the SERCA-type Ca^{2+} -pump in general [4] and in *Paramecium* in particular [14,28,49] this can impossibly explain the rapid Ca^{2+} -uptake and release during overstimulation of ciliary reversal.

Remarkably, the question of coupling of stores and extracellular medium is unsettled even for rather intensely investigated cells, such as lymphocytes, for which recently Narayanan et al. [50] have postulated a direct mass-action mechanism to explain the rapid capacitative calcium entry into the ER. This would require influx channels in the cell membrane matching with juxtaposed channels in the ER membrane. However, coupling over longer distances, as seen here between cilia and alveolar sacs, may operate by a cation exchanger system. Also using EDX, a cation counter-transport has indeed been found to occur in the sarcoplasmic reticulum of skeletal muscle during tetanic stimulation [51]. This may be comparable to our “overstimulation” conditions. Considering the limited knowledge on biophysical properties of Ca^{2+} -store membranes [52] and the lack of specific antiporter inhibitors [53], our observations may contain important hints for future analyses of cortical calcium stores on a broader scale.

5. Conclusions

Under conditions of “overstimulation” ciliary Ca^{2+} -binding proteins may be unable to bind all of the Ca^{2+} -influx. High $[\text{Ca}^{2+}]$ in cilia may then cause rapid influx into the nearby alveolar sacs in which $[\text{Ca}^{2+}]$, even at high luminal [Ca], is probably sufficiently low due to high capacity/low affinity Ca^{2+} -binding proteins [54], as expected from observations with other systems [48]. Further dissipation would follow the gradient toward deeper cytosolic layers. Since Ca^{2+} -pumps are much too slow it seems reasonable to assume a rapidly acting antiporter system for the processes we describe here. This combined “vacuum cleaner/shower” function can avoid too high local $[\text{Ca}^{2+}]$ over longer times which could be deleterious to the cells.

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