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Differential distribution of calcium stores in Paramecium cells. Occurrence of a subplasmalemmal store with a calsequestrin-like protein

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Calcium – calreticulin – calsequestrin – exocytosis – Paramecium

We have analyzed in Paramecium cells the occurrence and intracellular distribution of the high capacity/low affinity calcium-binding proteins, calsequestrin (CS) and calreticulin (CR) using antibodies against CS from rat skeletal muscle and against CR from rat liver, respectively. As revealed by Western blots, a CS-like protein isolated by affinity chromatography from Paramecium cells comigrated with CS isolated from rat skeletal muscle. The immunoreactivity of this 53 kDa protein band was blocked when the antibodies had been preadsorbed with purified rat CS. A band of identical molecular size was shown to bind ^{45}Ca in overlays. By immunofluorescence and immunogold labeling this CS-like protein was localized selectively to the extended subplasmalemmal calcium stores, the "alveolar sacs", which cover almost the entire cell surface. Concomitantly the 53 kDa ^{45}Ca-binding band became increasingly intense in overlays as we increasingly enriched alveolar sacs. Antibodies against rat CR react with a 61 kDa band but do not cross-react with CS-like protein in Paramecium. These antibodies selectively stained intracellular reticular structures, identified bona fide as endoplasmic reticulum.

Abbreviations. ABs Antibodies. – BSA Bovine serum albumin. – $[Ca^{2+}]_i$ Intracellular free Ca^{2+}-concentration. – CaBP Ca-binding protein. – CR Calreticulin. – CS Calsequestrin. – DEAE Diethylaminoethyl. – $DiOC_{18}$ 3,3'-Dilinoleyloxacarbocyanine. – DTT Dithiothreitol. – EDTA Ethylenediaminetetraacetic acid. – EGTA Ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid. – EM Electron microscopy. – ER Endoplasmic reticulum. – FITC Fluorescein isothiocyanate. – m Molecular mass. – Mops 3-(N-morpholino)propanesulfonic acid. – PAGE Polyacrylamide gel electrophoresis. – PBS Phosphate-buffered saline. – Pipes Piperazine-N,N'-bis(2-ethanesulfonic acid). – PLT "Progressive lowering of temperature" (method). – POX Peroxidase (horseradish). – SDS Sodium dodecyl sulfate. – SR Sarcoplasmic reticulum. – TBS Tris-buffered saline. – Tris Tris(hydroxymethyl)aminomethane.

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Introduction

Ca^{2+} as a ubiquitous second messenger regulates different cell functions, e.g., muscle contraction [15, 17], ciliary beat and exocytosis [6, 31, 32, 44, 48]. An increase in free cytosolic Ca^{2+}-concentration, $[Ca^{2+}]_i$, may be achieved by an influx from the extracellular medium and/or by mobilization from intracellular stores [3, 11, 31, 32, 39].

Calcium stores may contain different high capacity/low affinity calcium binding proteins (CaBPs), most abundantly calsequestrin (CS) in the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) of smooth and skeletal muscle, respectively [8, 36] or calreticulin (CR = CaBP3, calregulin) in the ER of smooth muscle and of most non-muscle cells [5, 34–36, 51, 56]. In Purkinje cells of chicken, calcium stores may also encompass some special organelles containing CS [53, 57–59], as is the case with some egg cells [27] and some lower [16] and higher plant cells [24].

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gives an apparent molecular mass (m) of 55 (heart) or 53 to 63 kDa (skeletal muscle) for CS, as summarized in [12, 36]. Thus, its molecular mass is in the range of that of CR (m = 53–63 kDa), depending on the source [12, 33, 36, 40, 50]. Both CaBPs, CS and CR, are products of distinct genes and can be differently modified posttranslationally [30, 36, 48].

In Paramecium the regulation of Ca^{2+}-dependent processes is not yet fully understood. Ciliary voltage-dependent Ca^{2+}-channels are irrelevant for exocytosis [47]. So far, only the vast, flat subplasmalemmal compartments, the "alveolar sacs", have been identified as calcium stores with a Ca^{2+}-ATPase (pump) [26, 52]. The ER as another potential Ca-store is scattered throughout the cell [1].

CaBPs in alveolar sacs deserved particular interest for the following reasons. (*a*) They closely surround preformed sites of trichocyst exocytosis [1, 46]. (*b*) Polyamines can trigger exocytosis in the absence of extracellular Ca^{2+} [21, 22], although this depends on increased $[Ca^{2+}]_i$ [28, 46], so that alveolar sacs

may regulate trichocyst exocytosis [13, 22]. (*c*) Alveolar sacs not only share some ultrastructural [46], but also some functional characteristics with muscle SR, e.g., with regard to visible connections between these sacs and the cell membrane [46] and the mode of activation of Ca^{2+}-release [26].

In the present study we used antibodies (ABs) generated against rat CS and against rat CR, respectively, to study the potential existence of these CaBPs in Paramecium.

Recently, subplasmalemmal calcium stores are increasingly considered to be an important source of Ca^{2+} during stimulus-secretion coupling in a variety of cell types [3, 11].

Materials and methods

Cell cultures

Paramecium tetraurelia wildtype cells (strain 7S) were grown to stationary phase in a medium prepared from dry salad, supplemented with 0.005% w/v stigmasterol (Sigma, St. Louis, MO/USA), monoxenically inoculated with Enterobacter aerogenes.

Preparation of cell homogenates

Cells were concentrated to 1/10 of volume and washed twice in Pipes buffer (5 mM Pipes [piperazine-N,N'-bis{2-ethanesulfonic acid}], 1 mM KCl, 1 mM $CaCl_2$, adjusted to pH 7.0 with NaOH). Pelleted cells were suspended in Tris (tris[hydroxymethyl]aminomethane)-maleate buffer (20 mM, with 3 mM EDTA (ethylenediaminotetraacetic acid) pH 7.2), washed twice in the same buffer (100*g* and 150*g*, 2 min each). Pellets were homogenized in a buffer containing 20 mM Tris-maleate, 3 mM EDTA, 250 mM sucrose, adjusted to pH 7.0. The buffer contained a cocktail of protease inhibitors (0.02 mg/ml leupeptin, 0.1 mg/ml TAME (Na-p-tosyl-L-arginine methylester), 0.05 mg/ml PMSF (phenylmethylsulfonyl fluoride), 0.01 µg/ml pepstatin A and 2.5 µl/ml aprotinin; all from Sigma). Cells were homogenized by ~30 hand strokes in a glass homogenizer with a Teflon pestle.

Cell fractionation

We isolated cell cortices according to Lumpert et al. [28] and alveolar sacs according to Stelly et al. [52] as modified in [26].

Extraction of CS-like protein

Microsomal fractions of homogenized Paramecium (also containing alveolar sacs) were prepared from 3000*g* supernatants by centrifugation, 100000*g* 1 h, at 4 °C. Pelleted microsomes were opened in 0.1 M $NaCO_3$ buffer, pH 11 by gentle rotation at 4 °C for 30 min. After adjusting to pH 7.2, membranes were spun down at 100000*g* for 30 min, and the released proteins were prepurified on a DEAE (diethylaminoethyl) Sepharose column by elution with increasing NaCl concentration (0–0.5 M). Pooled fractions, containing CS-like immunoreactivity, from the DEAE column were dialyzed against a buffer containing 10 mM Mops (3-[N-morpholino]propanesulfonic acid), 0.1 mM EGTA (ethylene glycol-bis[β-aminoethyl ether]N,N,N',N'-tetraacetic acid), 500 mM NaCl and 1 mM DTT (dithiothreitol), adjusted to pH 7.0, and loaded onto a phenyl-Sepharose column [7]. After washing, material was eluted with the same buffer supplemented with 20 mM $CaCl_2$. Eluted fractions were tested with anti-CS-ABs on Western blots by the ECL (enhanced chemiluminescence) technique. Positive fractions were pooled for eventual affinity purification.

An IgG fraction of anti-CS ABs was immobilized on protein-A Sepharose. IgGs were bound to CNBr-Sepharose and the column was washed with citric acid. From Paramecium CS-like protein was extracted first with citric acid (0.2 M, pH 3) and then additionally with glycine (0.2 M, pH 2.8). Samples were concentrated on Amicon 30 after pH adjustment, and about 0.1 µg of CS-like protein from each fraction was analyzed by SDS-PAGE and immunoblotting with anti-CS ABs.

Extraction of CR-like protein

The method described by Nguyen Van et al. [39] was used to extract 100000*g* pellets from Paramecium homogenates with 0.1 M Na_2CO_3, pH 11.4, which is a standard method to obtain CR.

Protein determinations

We used a BCA (bicinchoninic acid) kit from Pierce (Oud Beyerland/The Netherlands). Calibration was performed with bovine serum albumin (BSA).

PAGE, antibodies and Western blots

Samples were dissolved at 90 °C for 3 min in sample buffer (62.5 mM Tris-HCl, pH 7.2, 1% w/v SDS, 10% v/v glycerol and 0.25% w/v DTT) and applied to 10% w/v polyacrylamide gels. Protein applied per lane usually was up to 0.1 to 0.3 µg for CR and CS, 0.5 to 1.0 µg for SR and up to 20 to 40 µg for Paramecium samples.

ABs used were prepared in rabbits, either against CS (isolated from rat skeletal muscle) or against CR (isolated from rat liver), (see [39]). For detection we used second ABs from goat, conjugated to fluorescein isothiocyanate (FITC) or to horseradish peroxidase (POX) from Dianova (Hamburg/Germany) or adsorbed to colloidal gold of 10 nm diameter (Au_{10} from Aurion, delivered by BioTrend, Köln/Germany).

Nitrocellulose blots were produced with a BioRad mini-transblot unit by applying 100 V for 90 min. Blots were washed 4 × 5 min in Tris-buffered saline (TBS), then 6 × 5 min in TBS with 5% w/v defatted milk powder and 2 × 5 min in TBS. Blots were incubated overnight at 4 °C, either with anti-CR-ABs or with anti-CS-ABs, diluted 1:500 or 1:1000, respectively, in a solution of TBS with 0.25% w/v milk powder and washed 6 × 5 min in the same solution. The second AB (anti-rabbit-POX) was applied at a dilution of 1:2000 in the same solution for 1 h at room temperature, followed by 3 × 5 min washes in the same solution and 6 × 5 min in TBS.

^{45}Ca overlays

Paramecium homogenates, isolated cell cortex fragments and purified alveolar sac fractions, together with rat CS, were electrophoresed as follows. Samples were boiled for 3 min in 0.4 M Tris-HCl, 1% w/v SDS, 0.5% w/v DTT, 20% v/v glycerol, pH 8.0, alkylated with 2% iodoacetamide for 30 min and subjected to electrophoresis on linear 10 to 20% polyacrylamide gels using a discontinuous buffer system [55]. Proteins were transferred onto nitrocellulose (NC)-membranes, type BA-85 from Schleicher and Schuell (Dassel/Germany) by application of 1 mA/cm² for 1 h [25].

Subsequently we made ^{45}Ca overlays according to Maruyama et al. [29] as modified by Hincke [18]. NC-membranes containing transferred proteins were soaked three times, 30 min each, at room temperature in a 10 mM imidazole-HCl buffer containing 60 mM KCl, 5 mM $MgCl_2$ and 0.5 mM DTT, pH 6.8. Then membranes were incubated with 50 ml of the same buffer but supplemented with 10 µg ^{45}Ca (0.1 MBq/ml) for 20 min. Unbound ^{45}Ca was removed by incubation, twice for 2 min, in 5% ethanol. Membranes were blotted to dryness between filter paper and exposed to Kodak X-Omat film for 6 days at −70 °C.

Immunofluorescence microscopy

Cells were washed twice in 5 mM Pipes buffer, pH 7.0, containing 1 mM KCl and 1 mM $CaCl_2$ and twice in 5 mM Pipes buffer, pH 7.0, containing 10 mM $MgCl_2$ and 3 mM EGTA, at room temperature. Cell suspensions were injected with a pipette into a tenfold excess of a 0 °C fixative composed of 4% w/v formaldehyde in 5 mM Pipes buffer, pH 7.0, with 20 mM $MgCl_2$ and 3 mM EGTA (to reduce trichocyst release). While the samples were allowed to warm up to 22 °C during 30 min fixation, we added 1% v/v Triton X-100 10 min after the beginning of fixation. Cells were washed 2 × 5 min in the same solution (without formaldehyde) with 1% Triton X-100 added, then twice in Dulbecco's phosphate-buffered saline (PBS), 2 × 15 min in this solution with 50 mM glycine added and 30 min in this solution with 1% w/v BSA added. The anti-CS-AB or anti-CR-AB was applied in a dilution of 1:30 or up to 1:10 in Dulbecco's PBS (+1% BSA) for 1 h.

After 4 × 15 min washes in PBS + BSA, FITC-conjugated anti-rabbit-ABs, diluted 1:70, were applied for 1 h, followed by 3 to 5 × 10 min washes in PBS. Samples were vigorously shaken during all incubation and washing steps. Mowiol (Hoechst, Frankfurt a.M./Germany) was added to samples as an anti-fading agent [41]. Fluorescence microscopy was performed as in [37].

$DiOC_{18}$ staining and confocal laser scanning microscopy

$DiOC_{18}$ from Molecular Probes Inc. (Eugene, OR/USA) was applied by microinjection as a saturated solution in soybean oil. The volume injected was ~ 10% of the cell volume [20]. After 15 min equilibration, samples were evaluated with an Odyssey-LSM-C system (Tracor/Noran, Bruchsal/Germany) equipped with an Ar-laser (100 mW output), in combination with a Zeiss (Oberkochen/Germany) Axiovert microscope and the 2D/3D image processing System Image I.

Preembedding POX-labeling

Cells were fixed and permeabilized as indicated above. After the first AB, a POX-conjugated second AB was applied. We used 3,3'-diaminobenzidine as a substrate, followed by osmication and uranyl and lead staining (see [37]).

Postembedding immunogold labeling

Cells were fixed as for fluorescence labeling, but without permeabilization. After washing in Pipes buffer, cells were pelleted (5 min, 150g, 0 °C) and processed by the PLT ("progressive lowering of temperature") method [9]. After 30 min in 4% formaldehyde in Pipes buffer (0 °C), increasing methanol concentrations were used for different times and at decreasing temperatures as follows: 30% v/v, 30 min, 0 °C; 50%, 1 h, 0 °C → − 20 °C; 70%, 1 h, − 20 °C → − 35 °C; 95%, 1 h, − 35 °C → − 50 °C; 100%, 30 min, − 50 °C → − 60 °C; 100% overnight at − 60 °C. Samples were embedded in Lowicryl K11M (Chemische Werke Lowi, Waldkraiburg/Germany) composed of 19 g of monomer, 1 g of crosslinker and 0.15 g of initiator. Samples were impregnated at − 60 °C, for 1 h each, with K11M + methanol (first 1 part + 2 parts, then 1 + 1 part and finally 2 parts + 1 part). Pure K11M medium was applied overnight, also at − 60 °C, and UV polymerization was done as indicated previously [4, 37].

Ultrathin sections on Ni-grids were washed in Dulbecco's PBS, first 4 × 5 min with 50 mM glycine and then 6 × 5 min with 0.8% BSA and 0.5% fish gelatine added (wash solution, WS). ABs were applied, 1:10 to 1:30 diluted in WS, followed by 6 × 5 min WS, incubation for 1 h with anti-rabbit IgG-coated 10 nm colloidal gold particles (Au_{10}) diluted 1:30 in PBS and 6 × 5 min PBS wash. Sections were postfixed in 1% v/v glutaraldehyde (Merck, Darmstadt/Germany) in PBS, washed 4 × 5 min with double-distilled water, stained for 5 min with 4% unbuffered uranyl acetate, washed and stained for 5 min in Pb-citrate, pH 12.0, and thoroughly rinsed.

Results

Calsequestrin has been shown previously [7] to behave on phenyl-Sepharose differently compared to other CaBPs in that it binds in the presence of 0.1 mM EGTA and 0.5 M NaCl and can be eluted in the presence of 10 mM Ca^{2+}. When using this method for the analysis of an extract prepared from Paramecium, a protein was eluted from phenyl-Sepharose with Ca^{2+}. This protein exhibited a similar apparent molecular mass as CS from rat skeletal muscle and it reacted with the AB against CS from rat skeletal muscle (Fig. 1). This immunoreaction was abolished after preadsorption of the AB with purified rat skeletal muscle CS (Fig. 2). Anti-CR-ABs recognize CR from rat liver, but they do not bind to the Paramecium protein isolated by phenyl-Sepharose chromatography (Fig. 3) which, thus, reacted specifically with anti-CS-ABs (cf. Fig. 1).

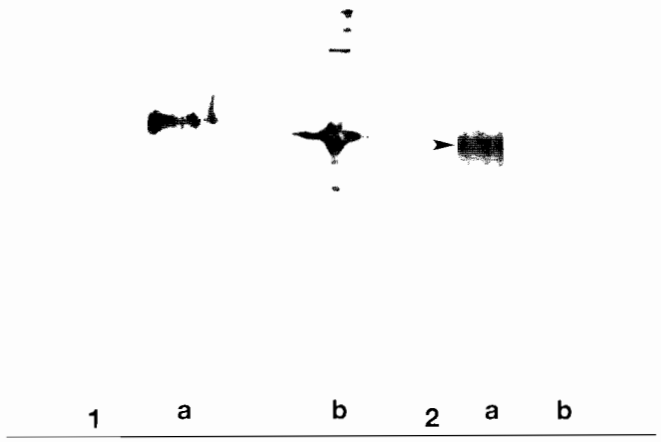


Fig. 1. Isolation of CS-like protein (53 kDa) from Paramecium by AB-affinity chromatography and identification on Western blots using anti-CS-ABs. 10% gel covering about 135 to 15 kDa. – *Lane a:* CS-like protein from Paramecium, eluted with citric acid and glycine. (*lane b*) original rat CS with similar electrophoretic mobility. Note widely different sensitivity with 0.1 or 0.01 µg protein, respectively, in *lanes a* and *b*.

Fig. 2. Specificity of CS recognition by anti-CS-ABs in Paramecium. Western blots (10% gel covering about 180–10 kDa), 20 µg protein per lane. Paramecium homogenates analyzed with anti-CS-ABs used before (*lane a*) or after (*lane b*) adsorption of antibodies with purified CS from rat skeletal muscle. Note the disappearance of reactive band (53 kDa, *arrowhead*) in (*lane b*).

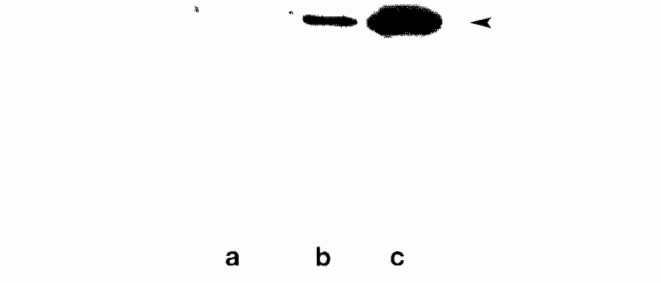


Fig. 3. Anti-CR-ABs (1:1000) do not bind to CS-like protein in Paramecium cells. Western blot (10% gel covering about 170–20 kDa), 0.1 µg protein per lane, using anti-CR-ABs. – *Lane a:* CS-like protein purified from Paramecium. – *Lane b:* Crude extract from rat skeletal muscle, (*lane c*) purified CR from rat liver. Note absence of a reaction with the CS-like protein from Paramecium (*lane a*), whereas CR from rat skeletal muscle is recognized (*lane b*). Also note that the strongest labeling (61 kDa, *arrowhead*) occurs with purified CR (*lane c*).

Next we tested by ^{45}Ca overlays which cell fractions would contain a CaBP of the same size as the anti-CS-AB reactive band. In Figure 4 we compared rat CS with Paramecium homogenate, isolated cell cortex fragments [28] and purified alveolar sacs. In this sequence the relative contribution of internal ER components would decrease, while that of peripheral calcium stores increases. Figure 4 shows a concomitant increase of a CaBP of about the size of original CS, when proceeding (from right to left) from homogenates to cortices and alveolar sacs.

ABs directed against rat liver CR were used to test an extract from the 100 000*g* sediment of a Paramecium homogenate prepared with 0.1 M Na_2CO_3, pH 11.4. Clearly the AB detects a single protein band of similar size (Fig. 5). The AB against CR does not cross-react with CS.

From all these data, which we could confirm with other batches of ABs (data not shown), we conclude that Paramecium contains a CS-like and a CR-like protein, respectively.

The ER-specific affinity stain, $DiOC_{18}$ [54], in confocal laser scanning microscopy images (Fig. 6) clearly labels two structural elements, (*a*) the outlines of ~ 1 to 2 µm large surface fields (compatible with alveolar sacs) and (*b*) irregularly shaped internal tubular structures (compatible with ER) which sometimes closely approach the cell surface.

Immunofluorescence labeling with anti-CS-ABs or anti-CR-ABs, respectively, yielded a differential distribution (Figs. 7, 8). Anti-CS-AB labeling was almost exclusively associated with the cell periphery showing a patchy (Fig. 7a) or crenulated pattern (Fig. 7b) in superficial or median views, respectively. This clearly correlates with the localization of the antigen to alveolar sacs (see blow) and this finding could be reproduced with different sets of ABs (data not shown). Any unspecific binding of ABs to the outer cell surface was excluded by controls with non-permeabilized cells (not shown). Anti-CR-ABs selectively labeled an extensive internal reticular structure, probably including outlines of nuclei, whereas the cell periphery remained unlabeled (Fig. 8).

At the EM level different protocols had to be used since both antigens were sensitive to preparation conditions. Anti-CS-ABs selectively labeled alveolar sacs, as shown by application of the colloidal gold method (Fig. 9) to Lowicryl K11M sections. Alveolar sacs were the only structures labeled with anti-CS-ABs. Their outlines are recognized by AB-gold decoration. Labeling was most intense where alveolar sacs were tangentially cut, so that more of the CS-like antigen was exposed. Again this was reproduced with another set of ABs by postembedding labeling or, using preembedding labeling, with second AB-POX, though ultrastructural preservation was poor in this case (data not shown). In summary, EM analysis showed much more clearly homogeneous distribution of CS-like protein, which in immunofluorescence looked more patchy, possibly due to structural rearrangements. Extracellular regions and cell regions below alveolar sacs always contain no or only little background label. This also holds true for the ER. When the anti-CS-AB was omitted, background labeling was minimal (not shown). To demonstrate the distribution of the CR-like antigen(s) at the EM level (Fig. 10), the best method was to apply formaldehyde fixation and permeabilization, followed by incubation with anti-CR-ABs and a POX-conjugated second AB under vigorous shaking. Though ultrastructural preservation was only moderate, cytoplasmic regions (often with vesicles) known to contain abundant ER were labeled, while other structures, like trichocysts, were largely devoid of label. Again controls without the first AB remained negative (not shown).

In summary, at the light microscope and at the EM level, anti-CS-ABs labeled alveolar sacs, while anti-CR-ABs labeled internal ER-type structures. These staining patterns were mutually exclusive.

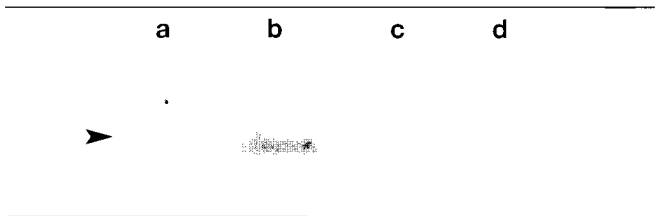


Fig. 4. ^{45}Ca binding assay showing 53 kDa CaBP enrichment in alveolar sacs. Semidry blot from a linear 10 to 20% gel. – *Lane a:* Rat muscle CS (4.5 µg protein), (*lane b*) purified alveolar sacs, (*lane c*) isolated cell cortex fragments, (*lane d*) whole cell homogenate; *lanes b* to *d* are from Paramecium, 150 µg protein applied per lane. The m-values determined were ~ 53 kDa for *lanes a* and *b*. The other Paramecium samples (*lanes c, d*), with less enriched alveolar sacs, did not or only weakly allow recognition of this ^{45}Ca binding band.

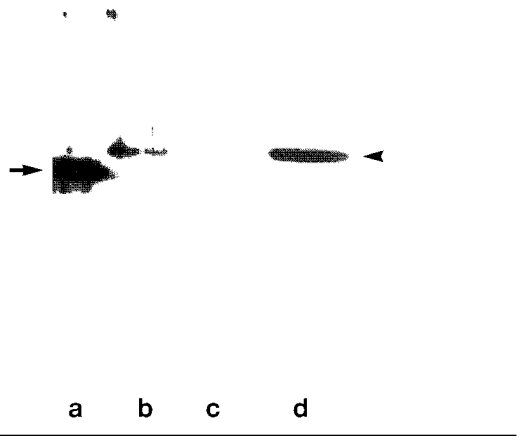


Fig. 5. Anti-CR-ABs (1:1000) recognize a CR-like protein in Paramecium cells. Western blot from a 10% gel covering about 170 to 20 kDa. – *Lane a* contained purified CR, 0.08 µg protein. *Lane b* was loaded with a Na_2CO_3 extract from a Paramecium homogenate (see Materials and methods), 25 µg protein. Note the occurrence of one band in the Paramecium sample, showing slightly slower mobility (m ~ 61 kDa, *arrowhead, lane b*) than its equivalent from pig (m ~ 55 kDa, *arrow, lane a*). – *Lane c:* Control showing absence of cross-reactivity of anti-CR-ABs with rat CS, but selective reactivity with rat CR (*lane d*).

Discussion

Occurrence of CS- and CR-like proteins in Paramecium

We have shown the occurrence of a protein which has an apparent molecular mass like that of CS and which cross-reacts with anti-(rat, SR)CS-ABs on Western blots prepared

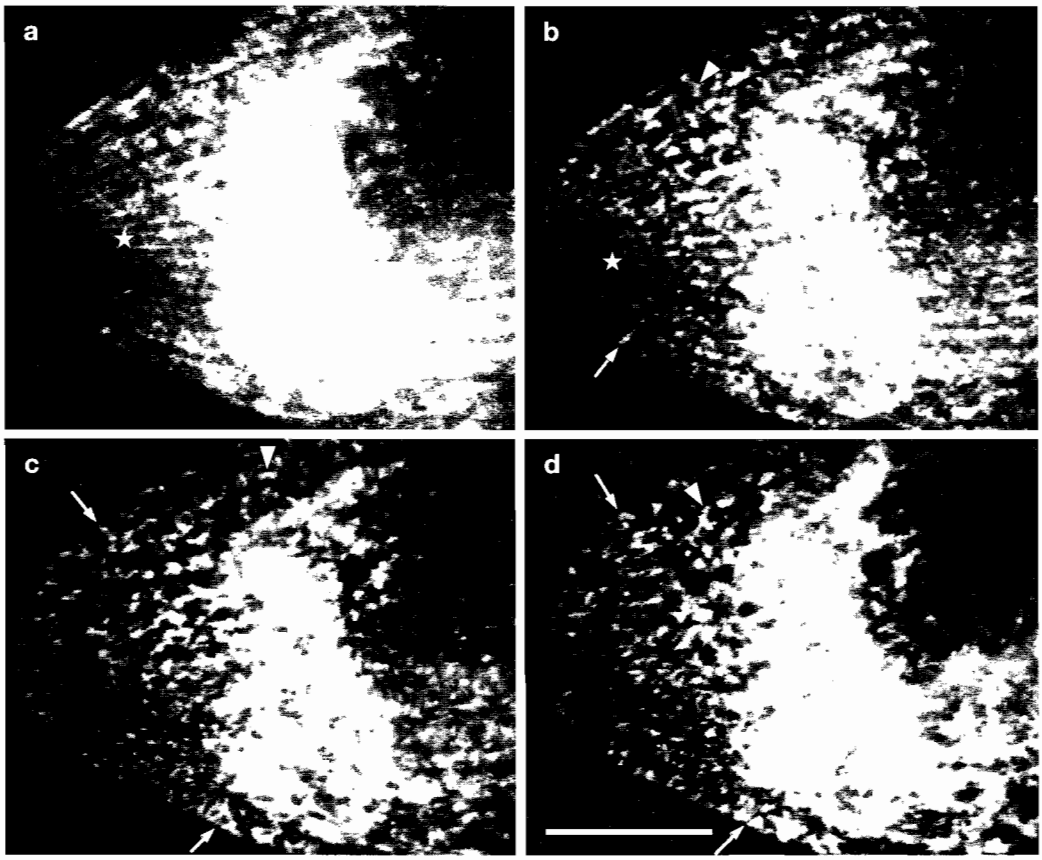


Fig. 6. Identification of ER-type structures. Paramecium cell injected with $DiOC_{18}$ and analyzed by confocal laser scanning microscopy on planes proceeding from the surface (**a**) by 5 µm steps (**b–d**). Note that the cell (slightly flattened under the cover glass) shows heavy surface staining in (**a**), with rectangular surface fields at the left (*asterisk*), presumably outlines of alveolar sacs. In (**b**), these are still visible, now closer to the cell pole (*asterisk*), while heavily stained irregularly shaped tubular structures (*arrowheads*) emerge with progressing focal planes (**b–d**). Some of these tubular ER-type structures closely approach the cell surface (*arrows*). - Bar 10 µm.

from Paramecium homogenates. Another reactive protein was detected with anti-(rat, ER)CR-ABs. We showed that the CS-like protein from Paramecium behaved in the same way as skeletal muscle CS during phenyl-Sepharose chromatography. The reaction of anti-CS-ABs with the Paramecium protein could be abolished by preincubation of the ABs with purified rat CS. ABs could also be successfully used for isolation specifically of this protein from Paramecium. The isolated protein band corresponded to the ^{45}Ca binding band enriched in alveolar sacs where we also achieved immunolocalization on the EM level (see below). As the molecular identification of the proteins reacting with anti-CS- and anti-CR-ABs, respectively, is still missing, we designate these antigens in Paramecium as CS- and CR-like proteins, respectively.

Identification of ER-type structures and selective immunolabeling

In Paramecium, the fluorescent stain $DiOC_{18}$, largely assumed to label ER-type structures [54], outlines the circumference of about 1 to 2 µm large units under the cell surface and, in addition, reticular structures inside the cell. Based on the established cell anatomy [1], the first structure can correspond only to alveolar sacs and the second to the ER. While anti-CS-ABs exclusively label alveolar sacs, anti-CR-AB labeling colocalizes with internal structures whose distribution is similar to that of the ER (including the nuclear envelope). This labeling pattern was corroborated by EM analysis.

Functional aspects pertinent to Paramecium cells

The differential distribution of different ER-type calcium stores is strikingly pronounced in Paramecium. Alveolar sacs are closely attached to the cell membrane, covering almost all of the cell surface. Every trichocyst docking site is intimately surrounded by the borders of adjacent alveolar sacs [2, 42, 45], thus suggesting a role in exocytosis regulation. In fact, alveolar sacs have been identified as calcium stores endowed with a calmodulin-independent Ca^{2+}-pump [52]. This was corroborated by Ca-imaging at the EM level, by electron spectroscopic imaging, in conjunction with quenched-flow processing [22]. By the same methodology we also showed that during synchronous (80 ms) exocytosis subplasmalemmal $[Ca^{2+}]_i$ increases, while exocytotic membrane fusion occurs also after complete chelation of extracellular Ca^{2+} [22].

What might be the relevance of two widely different types of calcium stores, one at the cell periphery and one inside the cell? Microsomes derived mainly from ER are known to store calcium in another ciliate, Tetrahymena [38]. However, it would be difficult to imagine how in Paramecium Ca^{2+} could be delivered by the internal ER during synchronous trichocyst exocytosis, e.g., within 30 to 80 ms after polyamine stimula-

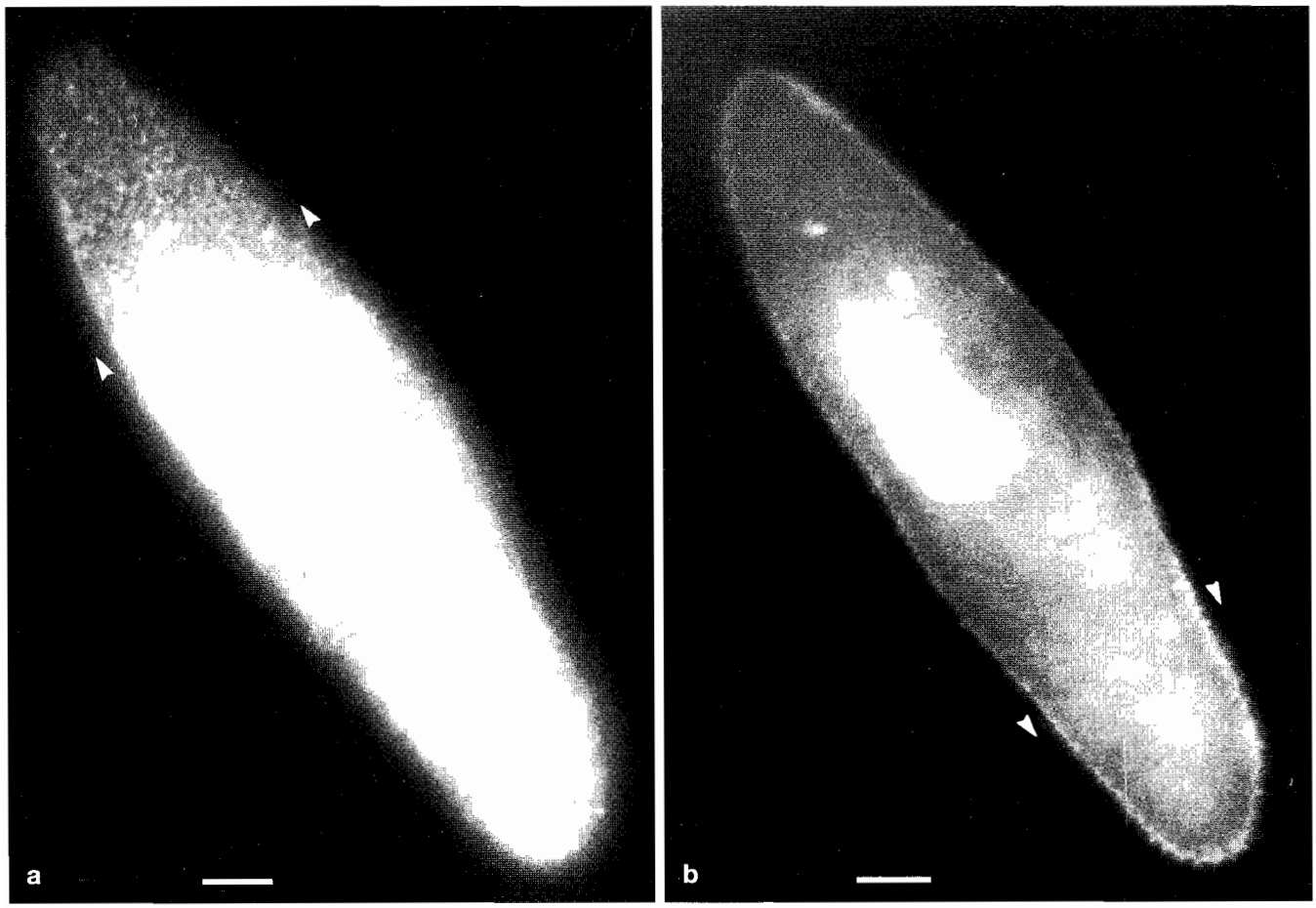
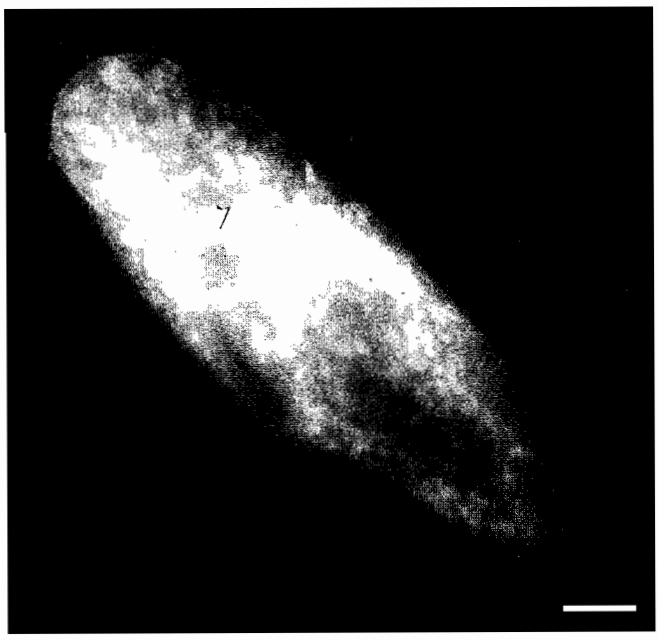
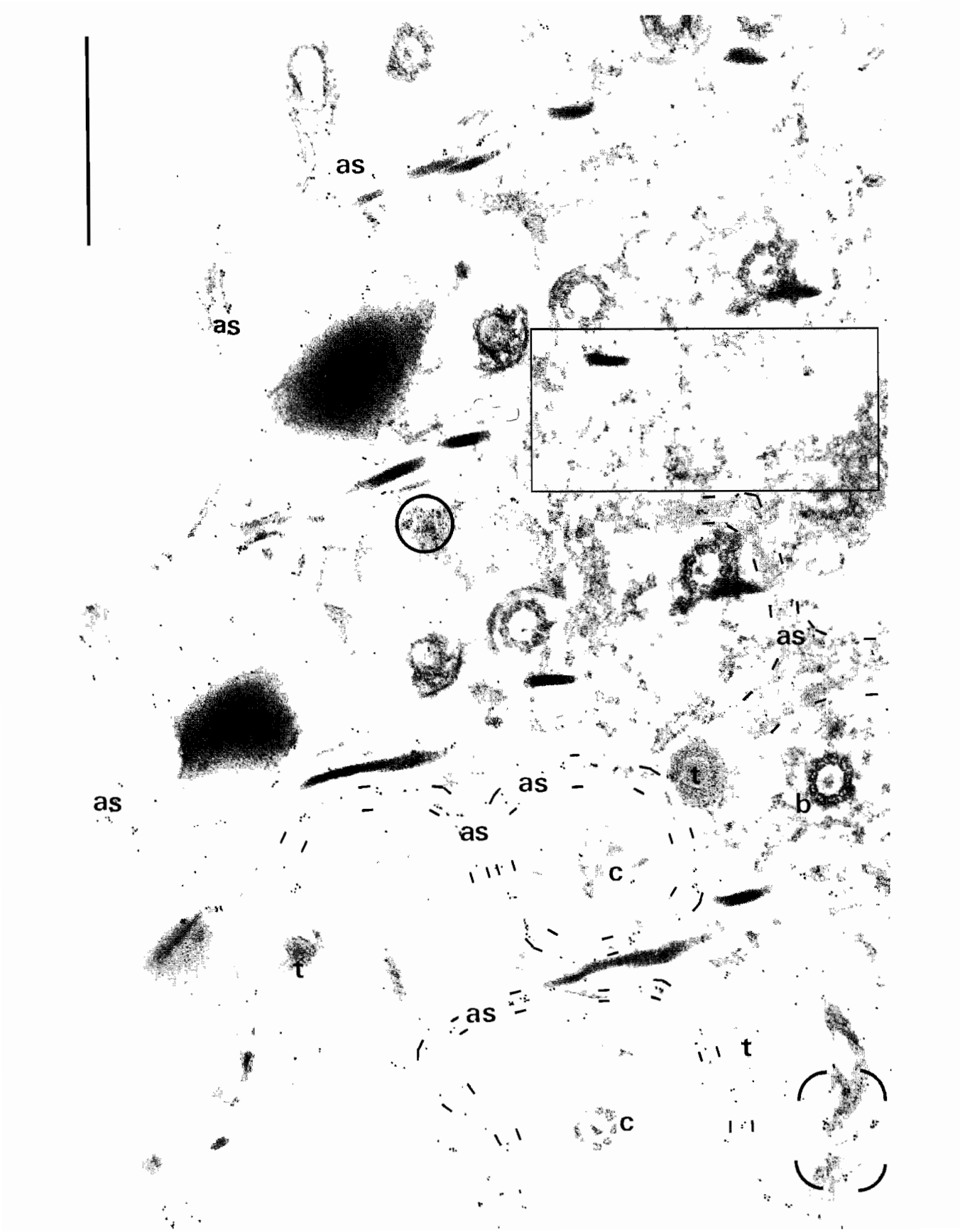


Fig. 7. CS-like protein is selectively detectable in the cell cortex. Immunofluorescence staining with anti-CS-ABs, focusing on the cell surface (**a**) and in a median plane (**b**). Note spotty appearance in (**a**) which, on the top left (from *arrowheads* up), allows to recognize the outlines of surface fields (bona fide alveolar sacs). In (**b**), cortical labeling is frequently of crenulated appearance (from *arrowheads* down), corresponding to the size and arrangement of surface fields with alveolar sacs. Note that intracellular organelles are not labeled, with the exception of a large field (probably an oral cavity which notoriously traps a variety of ABs). – Bars 10 μm.



◀ **Fig. 8.** CR-like protein(s) occur deeper inside cells. Immunofluorescence staining with anti-CR-ABs in a median focal plane. Note staining of diffusely arranged organelles, probably ER, including outlines of a nucleus (*arrowhead*). – Bar 10 μm.

Fig. 9. EM immunolocalization of CS-like protein(s) in alveolar sacs. Anti-CS-ABs, followed by Au-labeled second ABs, applied to Lowicryl K11M sections from freeze-substituted cells. Note gold decoration of the outlines of alveolar sacs (e.g., in narrow regions between the interrupted lines). This is most clearly visible where sacs are tangentially sectioned, so they appear dark (*encircled areas*). Alveolar sacs (as) are located at the border of depressions of the cell surface which may contain cilia (c) or basal bodies (b), respectively, alternating with trichocysts (t). Label frequently adheres to the borders of alveolar sacs, while label is absent from embedding medium (e.g., top left) and largely also from deeper cell layers (e.g., in the framed area). – Bar 1 μm.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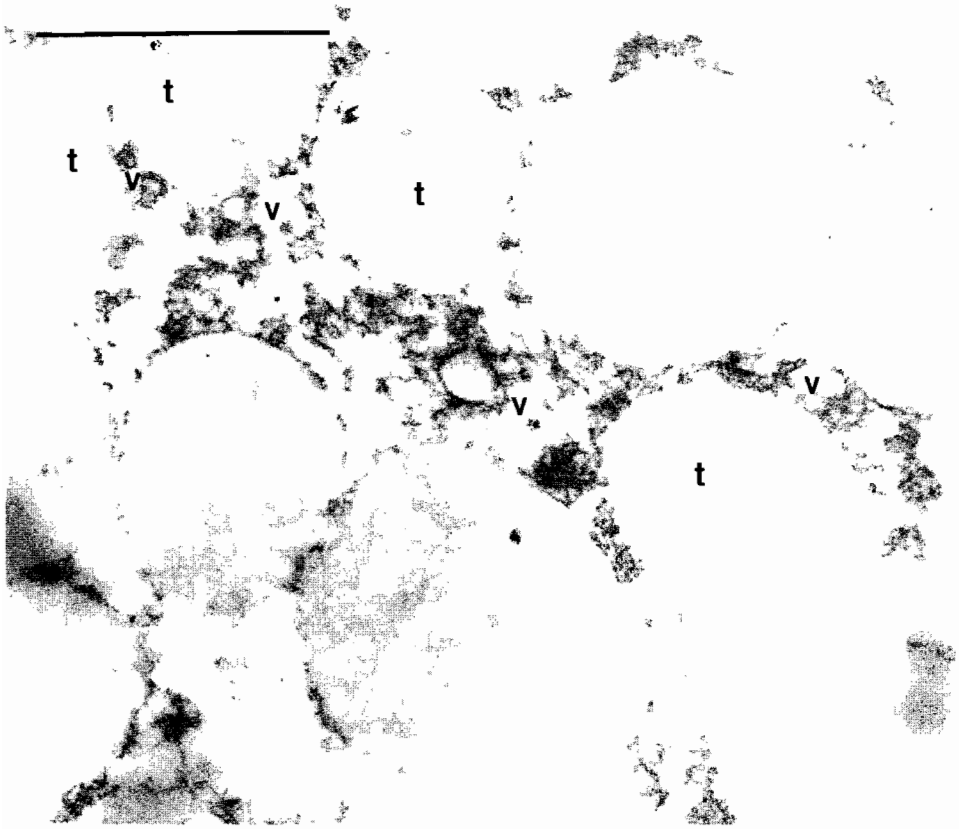

Fig. 10. Anti-CR-ABs, followed by second ABs-POX, applied to permeabilized cells. Note labeling of diffusely arranged, partly vesicular structures (v) between unlabeled, cross-cut trichocysts (t). - Bar 1 µm.

tion [21]. Internal ER-type structures, on the other hand, may serve as calcium stores for some other functions. By analogy with gland cells [10], this may include intracellular traffic and calcium homeostasis. Internal ER may, thus, be a sink for rapid sequestration of Ca^{2+} after the massive flush occurring during aminoethyldextran stimulation [23], as we found by fluorochrome analysis (N. Klauke, H. Plattner, submitted).

Another crucial aspect for future investigations is the unknown biogenesis of alveolar sacs and the sorting of specific CaBPs to specific calcium storing compartments. Most remarkably alveolar sacs of another ciliate, Coleps, contain Ca-phosphate [14].

Comparison with other secretory cells

By analytical EM methods, calcium has been shown to occur in millimolar concentrations not only in the SR [19], but also in the ER of different mammalian cells [5, 51]. Similar estimates of total calcium concentrations may hold true for the cortex of Paramecium [49], i.e., its alveolar sacs.

Since monospecific ABs against CS and CR have allowed to sequence them and, thus, to reveal CR as the prevailing CaBP in calcium stores of non-muscle cells [34–36, 56], we now envisage a similar approach. Simultaneous occurrence of different types of stores, with either CS or CR, was observed only occasionally [43, 48]. In no case could a situation be found which would be as clear-cut as in Paramecium cells, i.e., with a CS-type store tightly coupled to the cell membrane and a CR-type compartment deeper inside the cell.

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References

[1] Allen, R. D.: Cytology. In: H. D. Gortz (ed.): Paramecium. pp. 4–40. Springer-Verlag, Berlin, Heidelberg, New York 1988.

[2] Beisson, J., M. Lefort-Tran, M. Pouphile, M. Rossignol, B. Satir: 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 (1976).

[3] Berridge, M. J.: Capacitative calcium entry. Biochem. J. **312,** 1–11 (1995).

[4] Bittermann, A. G., G. Knoll, A. Németh, H. Plattner: Quantitative immuno-gold labelling and ultrastructural preservation after cryofixation (combined with different freeze-substitution and embedding protocols) and after chemical fixation and cryosectioning. Analysis of the secretory organelle matrix of Paramecium trichocysts. Histochemistry **97,** 421–429 (1992).

[5] Buchanan, R. A., R. D. Leapmen, M. F. O'Connell, T. S. Reese, S. B. Andrews: Quantitative scanning transmission electron microscopy of ultrathin cryosections: subcellular organelles in rapidly frozen liver and cerebellar cortex. J. Struct. Biol. **110,** 244–255 (1993).

[6] Burgoyne, R. D., A. Morgan: Regulated exocytosis. Biochem. J **293,** 305–316 (1993).

[7] Cala, S. E., L. R. Jones: Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca^{2+}-dependent elution from phenyl-Sepharose. J. Biol. Chem. **258,** 11932–11936 (1983).

[8] Campbell, K. P., D. H. MacLennan, A. O. Jorgensen: Staining of the Ca^{2+}-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanide dye "stains all". J. Biol. Chem. **258,** 11267–11273 (1983).
[9] Carlemalm, E., W. Villiger: Low temperature embedding. In: G. R. Bullock, P. Petrusz (eds.): Techniques in Immunocytochemistry. Vol. 4, pp. 29–45. Academic Press, London 1989.
[10] Cheek, T. R., A. J. O'Sullivan, R. B. Moreton, M. J. Berridge, R. D. Burgoyne: The caffeine-sensitive Ca^{2+} store in bovine adrenal chromaffin cells: an estimation of its role in triggering secretion and Ca^{2+} homeostasis. FEBS Lett. **266,** 91–95 (1990).
[11] Clapham, D. E.: Calcium signaling. Cell **80,** 259–268 (1995).
[12] Damiani, E., A. Margreth: Characterization study of the ryanodine receptor and of calsequestrin isoforms of mammalian skeletal muscles in relation to fibre types. J. Muscle Res. Cell Motil. **15,** 86–101 (1994).
[13] Erxleben, C., H. Plattner: Ca^{2+} release from subplasmalemmal stores as a primary event during exocytosis in Paramecium cells. J. Cell Biol. **127,** 935–945 (1994).
[14] Fauré-Fermiet, E., J. André, M. C. Ganier: Calcification tégumentaire chez les ciliés du genre Coleps Nitzsch. J. Microsc. (Paris) **7,** 693–704 (1968).
[15] Fleischer, S., M. Inui: Biochemistry and biophysics of excitation-coupling. Annu. Rev. Biophys. Biophys. Chem. **18,** 333–364 (1989).
[16] Franceschi, V. R., X. Li, D. Zhang, T. W. Okita: Calsequestrin-like calcium-binding protein is expressed in calcium-accumulating cells of Pistia stratiotes. Proc. Natl. Acad. Sci. USA **90,** 6986–6990 (1993).
[17] Franzini-Armstrong, C., A. O. Jorgensen: Structure and development of E-C coupling units in skeletal muscle. Annu. Rev. Physiol. **56,** 509–534 (1994).
[18] Hincke, M. T.: Conditions for improved adsorption of calmodulin to nitrocellulose: detection by ^{45}Ca binding. Electrophoresis **9,** 303–306 (1988).
[19] Jorgensen, A. O., R. Broderick, A. P. Somlyo, A. V. Somlyo: Two structurally distinct calcium storage sites in rat cardiac sarcoplasmic reticulum: an electron microprobe analysis study. Circ. Res. **63,** 1060–1069 (1988).
[20] Kersken, H., J. Vilmart-Seuwen, M. Momayezi, H. Plattner: Filamentous actin in Paramecium cells: mapping by phalloidin affinity labeling in vivo and in vitro. J. Histochem. Cytochem. **34,** 443–454 (1986).
[21] Knoll, G., C. Braun, H. Plattner: 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 (1991).
[22] Knoll, G., A. Grässle, C. Braun, W. Probst, B. Höhne-Zell, H. Plattner: A calcium influx is neither strictly associated with nor necessary for exocytotic membrane fusion in Paramecium cells. Cell Calcium **14,** 189–199 (1993).
[23] Knoll, G., D. Kerboeuf, H. Plattner: A rapid calcium influx during exocytosis in Paramecium cells is followed by a rise in cyclic GMP within 1 s. FEBS Lett. **304,** 265–268 (1992).
[24] Krause, K. H., M. Chou, M. A. Thomas, R. D. Sjolund, K. P. Campbell: Plant cells contain calsequestrin. J. Biol. Chem. **264,** 4269–4272 (1989).
[25] Kyhse-Andersen, J.: Multi-chamber apparatus for preparative isoelectric focusing. Electrophoresis **10,** 6–10 (1989).
[26] Länge, S., N. Klauke, H. Plattner: 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 (1995).
[27] Lebeche, D., B. Kaminer: Characterization of a calsequestrin-like protein from sea-urchin eggs. Biochem. J. **287,** 741–747 (1992).
[28] Lumpert, C. J., H. Kersken, H. Plattner: Cell surface complexes ('cortices') isolated from Paramecium tetraurelia cells as a model system for analysing exocytosis in vitro in conjunction with microinjection studies. Biochem. J. **269,** 639–645 (1990).
[29] Maruyama, K., T. Mikawa, S. Ebashi: Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. J. Biochem. **95,** 511–519 (1984).
[30] Matsuoka, K., K. Seta, Y. Yamakawa, T. Okuyama, T. Shinoda, T. Isobe: Covalent structure of bovine brain calreticulin. Biochem. J. **298,** 435–442 (1994).
[31] Meldolesi, J., E. Clementi, C. Fasolato, D. Zacchetti, T. Pozzan: Ca^{2+} influx following receptor activation. Trends Pharmacol. Sci. **12,** 289–292 (1991).
[32] Meldolesi, J., L. Madeddu, T. Pozzan: Intracellular Ca^{2+} storage organelles in non-muscle cells: heterogeneity and functional assignment. Biochim. Biophys. Acta **1055,** 130–140 (1990).
[33] Menegazzi, P., F. Guzzo, B. Baldan, P. Mariani, S. Treves: Purification of calreticulin-like protein(s) from spinach leaves. Biochem. Biophys. Res. Commun. **190,** 1130–1135 (1993).
[34] Michalak, M., S. Baksh, M. Opas: Identification and immunolocalization of calreticulin in pancreatic cells: No evidence for "calciosomes". Exp. Cell Res. **197,** 91–99 (1991).
[35] Michalak, M., R. E. Milner, K. Burns, M. Opas: Calreticulin. Biochem. J. **285,** 681–692 (1992).
[36] Milner, R. E., K. S. Famulski, M. Michalak: Calcium binding proteins in the sarcoplasmic/endoplasmic reticulum of muscle and non-muscle cells. Mol. Cell. Biochem. **112,** 1–13 (1992).
[37] Momayezi, M., A. W. Habermann, J. J. Sokolova, R. Kissmehl, H. Plattner: Ultrastructural and antigenic preservation of a delicate structure by cryopreparation: identification and immunogold localization during biogenesis of a secretory component (membrane-matrix connection) in Paramecium trichocysts. J. Histochem. Cytochem. **41,** 1669–1677 (1992).
[38] Muto, Y., Y. Nozawa: Ca^{2+} transport studied with arsenazo III in Tetrahymena microsomes. Effect of calcium ionophore A23187 and trifluoperazine. Biochim. Biophys. Acta **815,** 410–416 (1985).
[39] Nguyen Van, P., F. Peter, H.-D. Söling: Four intracisternal calcium-binding glycoproteins from rat liver microsomes with high affinity for calcium. No indication for calsequestrin-like proteins in inositol 1,4,5-trisphosphate-sensitive calcium sequestering rat liver vesicles. J. Biol. Chem. **264,** 17494–17501 (1989).
[40] Nigam, S. K., T. Towers: Subcellular distribution of calcium-binding proteins and a calcium-ATPase in canine pancreas. J. Cell Biol. **111,** 197–200 (1990).
[41] Osborn, M., K. Weber: Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. In: L. Wilson (ed.): Methods in Cell Biology. Vol. 24, pp. 98–132. Academic Press, New York 1982.
[42] Pape, R., H. Plattner: Secretory organelle docking at the cell membrane of Paramecium cells: dedocking and synchronized redocking of trichocysts. Exp. Cell Res. **191,** 263–272 (1990).
[43] Parys, J. B., S. M. McPherson, L. Mathews, K. P. Campbell, F. J. Longo: Presence of inositol 1,4,5-trisphosphate receptor, calreticulin, and calsequestrin in eggs of sea urchins and Xenopus laevis. Dev. Biol. **161,** 466–476 (1994).
[44] Plattner, H.: Regulation of membrane fusion during exocytosis. Int. Rev. Cytol. **119,** 197–286 (1989).
[45] Plattner, H., G. Knoll, R. Pape: Synchronization of different steps of the secretory cycle in Paramecium tetraurelia: trichocyst exocytosis, exocytosis-coupled endocytosis, and intracellular transport. In: H. Plattner (ed.): Membrane Traffic in Protozoa, pp. 123–148. JAI Press Inc. Greenwich (CT), London 1993.
[46] Plattner, H., C. J. Lumpert, G. Knoll, R. Kissmehl, B. Höhne, M. Momayezi, R. Glas-Albrecht: Stimulus-secretion coupling in Paramecium cells. (Review Article.) Eur. J. Cell Biol. **55,** 3–16 (1991).
[47] Plattner, H., H. Matt, H. Kersken, B. Haacke, R. Stürzl: Synchronous exocytosis in Paramecium cells. I. A novel approach. Exp. Cell Res. **151,** 6–13 (1984).
[48] Pozzan, T., R. Rizzuto, P. Volpe, J. Meldolesi: Molecular and cellular physiology of intracellular calcium stores. Physiol. Rev. **74,** 595–636 (1994).
[49] Schmitz, M., K. Zierold: X-ray microanalysis of ion changes during fast processes of cells, as exemplified by trichocyst exocytosis of Paramecium caudatum. In: H. Plattner (ed.): Electron Microscopy of

Subcellular Dynamics. pp. 325–339. CRC Press Inc. Boca Raton (FL) 1989.
[50] Smith, M. J., G. L. E. Koch: Multiple zones in the sequence of calreticulin (CRP55, calregulin, HACBP), a major calcium binding ER/SR protein. EMBO J. **8,** 3581–3586 (1989).
[51] Somlyo, A. P., M. Bond, A. V. Somlyo: Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly in vivo. Nature **314,** 622–625 (1985).
[52] Stelly, N., J. P. Mauger, G. Kéryer, M. Claret, A. Adoutte: Cortical alveoli of Paramecium: a vast submembranous calcium storage compartment. J. Cell Biol. **113,** 103–112 (1991).
[53] Takei, K., H. Stukenbrok, A. Metcalf, G. A. Mignery, T. C. Südhof, P. Volpe, P. De Camilli: Ca^{2+} stores in Purkinje neurons: endoplasmic reticulum subcompartments demonstrated by the heterogeneous distribution of the $InsP_3$ receptor, Ca^{2+}-ATPase, and calsequestrin. J. Neurosci. **12,** 489–505 (1992).
[54] Terasaki, M.: Probes for the endoplasmic reticulum: In: W. T. Mason (ed.): Fluorescent and Luminescent Probes for Biological Activity. pp. 120–123. Academic Press. London, San Diego, New York 1993.
[55] Treptau, T., R. Kissmehl, J.-D. Wissmann, H. Plattner: A 63 kDa phosphoprotein undergoing rapid dephosphorylation during exocytosis in Paramecium cells shares biochemical characteristics with phosphoglucomutase. Biochem. J. **309,** 557–567 (1995).
[56] Treves, S., M. De Mattei, M. Lanfredi, A. Villa, N. M. Green, D. H. MacLennan, J. Meldolesi, T. Pozzan: Calreticulin is a candidate for a calsequestrin-like function in Ca^{2+}-storage compartments (calciosomes) of liver and brain. Biochem. J. **271,** 473–480 (1990).
[57] Villa, A., P. Podini, D. O. Clegg, T. Pozzan, J. Meldolesi: Intracellular Ca^{2+} stores in chicken Purkinje neurons: differential distribution of the low affinity-high capacity Ca^{2+} binding protein, calsequestrin, of Ca^{2+} ATPase and of the ER lumenal protein, Bip. J. Cell Biol. **113,** 778–791 (1991).
[58] Volpe, P., L. Gorza, M. Brini, R. Sacchetto, S. Ausoni, D. O. Clegg: Expression of the calsequestrin gene in chicken cerebellum Purkinje neurons. Biochem. J. **294,** 487–490 (1993).
[59] Volpe, P., A. Villa, E. Damiani, A. H. Sharp, P. Podini, S. H. Snyder, J. Meldolesi: Heterogeneity of microsomal Ca^{2+} stores in chicken Purkinje neurons. EMBO J. **10,** 3183–3189 (1991).