

“Calciosome,” a Cytoplasmic Organelle: The Inositol 1,4,5-trisphosphate-Sensitive Ca^{2+} Store of Nonmuscle Cells?

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Notes:

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(calsequestrin/immunocytochemistry/cell fractionation/PC12 cell line/HL-60 cell line)

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ABSTRACT Calsequestrin (CS) is the protein responsible for the high-capacity, moderate affinity binding of Ca^{2+} within the terminal cisternae of the sarcoplasmic reticulum, believed up to now to be specific for striated muscle. The cells of two nonmuscle lines (HL-60 and PC12) and of two rat tissues (liver and pancreas) are shown here to express a protein that resembles CS in many respects (apparent mass and pH-dependent migration in NaDodSO₄/PAGE; blue staining with StainsAll dye; Ca^{2+} binding ability) and is specifically recognized by affinity-purified antibodies against skeletal muscle CS. In these cells, the CS-like protein is shown by immunofluorescence and immunogold procedures to be localized within peculiar, heretofore unrecognized structures distributed throughout the cytoplasm. These structures appear to be discrete organelles, which we propose to be named “calciosomes.” By cell fractionation (Percoll gradient and free-flow electrophoresis), the CS-like protein of HL-60 cells is shown to copurify with the markers of the inositol 1,4,5-trisphosphate (Ins- P_3)-sensitive Ca^{2+} store, whereas the markers of other organelles (endoplasmic reticulum, Golgi complex, mitochondria, endosomes) and of the plasma membrane do not. Calciosome might thus be the intracellular target of Ins- P_3 —i.e., the source of the Ca^{2+} redistributed to the cytosol following receptor-triggered generation of the messenger.

A variety of cellular functions are regulated by fluctuations of the cytosolic concentration of Ca^{2+} , $[\text{Ca}^{2+}]_i$. In striated muscle fibers, the organelle responsible for the fluctuations of $[\text{Ca}^{2+}]_i$ that underlie the contraction-relaxation cycle, the sarcoplasmic reticulum (SR), is endowed with a Ca^{2+} -binding protein, calsequestrin (CS), characterized by high capacity (>40 sites per mol) and moderate affinity (K_d in the submillimolar range) (1). Because of the specific localization of CS, large amounts of Ca^{2+} can be accumulated within the SR terminal cisternae, without precipitation of Ca^{2+} salts taking place. In nonmuscle cells the release of Ca^{2+} is known to follow activation of a number of receptors coupled to the generation of inositol 1,4,5-trisphosphate (Ins- P_3) (2). The intracellular target of Ins- P_3 has been suggested to be the endoplasmic reticulum (ER), an endomembrane system endowed with a variety of other functions, but a final proof of the Ins- P_3 -sensitive Ca^{2+} storage properties of the ER has never been given (2–5). Rather, evidence incompatible with the straight coincidence of this Ca^{2+} store with ER has been reported (6–8). The present data indicate that the Ins- P_3 target is not the ER but a specific structure that might be a discrete organelle distributed throughout the cytoplasm and characterized by its content of a CS-like protein. We propose that this structure be named the “calciosome.”

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MATERIALS AND METHODS

Cell Fractionation. All solutions used contained a full cocktail of protease inhibitors (6). SR terminal cisternae from rabbit fast-twitch skeletal muscles as well as microsomes from rat pancreas and liver were isolated according to published procedures (9–11). PC12 (rat pheochromocytoma) cell homogenates (Polytron, 7 × 5 cycles, position 7) were first centrifuged at 10,000 × *g* for 15 min. Microsomes were then isolated by centrifuging the supernatant for 60 min at 150,000 × *g*. Intact HL-60 cells were incubated with ¹²⁵I-labeled insulin (¹²⁵I-insulin) in phosphate-buffered saline (PBS) (pH 7.4, 37°C, 2 min) to label the endosomes, washed with PBS (pH 6) (12), incubated for 5 min at 0°C in isotonic NaCl containing 9 mM diisopropyl fluorophosphate, washed twice with NaCl containing 1 mM EGTA, and homogenized by nitrogen cavitation. Homogenates were usually treated with digitonin (15 μM, 10 min, 0°C). Postnuclear supernatants were underlayered by two Percoll suspensions (19% and 33%, made isotonic with KCl/Hepes buffer, pH 7) and centrifuged at 160,000 × *g* for 10 min. The ensuing bands (1–3 from bottom to top) were collected and washed once. Fraction 3 was applied to a VaP22 free-flow electrophoresis apparatus (Bender & Hobein, Munich). The separation buffer contained (mmol/liter, pH 7) triethanolamine, 10; acetic acid, 10; sucrose 250; KCl, 2. Flow of the chamber buffer was 150 ml/hr, and sample injection rate was 1 ml/hr. Protein was recovered in 15 of 99 fractions, which were pooled into three fractions (A–C from anode to cathode). Fraction markers and protein were assayed as in refs. 6 and 13–15.

PAGE, Immunoblot, and ⁴⁵Ca Overlay. Subcellular fractions were suspended in 0.3 M sucrose/5 mM imidazole, pH 7. Slab NaDodSO₄/PAGE was carried out (16) with 5–15% gradient gels stained by either Coomassie blue or StainsAll (1). Proteins blotted onto nitrocellulose sheets (17) were exposed to either chicken or guinea pig anti-muscle CS antibody (Ab), which was revealed by alkaline phosphatase-conjugated anti-chicken IgG or protein A, respectively. Two-dimensional NaDodSO₄/PAGE was carried out by a modification of the procedure of Michalak *et al.* (18) using 10% acrylamide gels in both dimensions. ⁴⁵Ca overlay of blots was carried out as in ref. 17.

Immunocytochemistry. For immunofluorescence, cells grown on coverslips were fixed with 4% formaldehyde (from paraformaldehyde) in 125 mM phosphate buffer (pH 7.4) at 4°C for 1 hr and washed overnight. Thick cryosections of

Abbreviations: Ab, antibody(ies); CS, calsequestrin; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; Ins- P_3 , inositol 1,4,5-trisphosphate; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration.

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similarly fixed tissue were also investigated. Immunodecoration (19) was carried out in a buffer containing 0.3% Triton X-100, 0.45 M NaCl, 20 mM phosphate (pH 7.2), and 1/6 volume of goat serum. Chicken anti-CS Ab or nonimmune IgG was used at a concentration of 15 $\mu\text{g}/\text{ml}$; this was followed by rabbit anti-chicken IgG (1:1000) and finally by rhodamine-coupled goat anti-rabbit IgG (1:80). For immunogold labeling, cell suspensions were centrifuged ($500 \times g$, 5 min). The pellets, as well as small tissue cubes, were fixed with 4% formaldehyde/0.25% glutaraldehyde in 125 mM phosphate buffer (pH 7.4) (1 hr, 4°C), rinsed in PBS, infiltrated with sucrose (0.5–2.3 M), and frozen in Freon 12 cooled with liquid nitrogen. Ultrathin cryosections were cut in a Reichert Ultracut equipped with an LM4 apparatus, collected onto carbon-coated nickel grids, sequentially floated over drops of 2% gelatin/PBS (two times, 5 min) followed by 1% bovine serum albumin/0.15 M glycine/PBS (three times, 10 min), and then immunodecorated (60–180 min, 37°C). In many experiments gold particles coated with either chicken anti-CS Ab or nonimmune IgG were used; in others the sections were first treated with these Ab (15 $\mu\text{g}/\text{ml}$, 60 min, 37°C), then treated with rabbit anti-chicken IgG (60 min, 37°C, 1:1000), and finally treated with protein A-gold (60 min, 37°C). Cryosections exposed to guinea pig anti-CS Ab were decorated with protein A-gold. For CS-secreto granin double immunolabeling, the sections were first decorated with chicken anti-CS Ab coupled to small (5 nm) gold particles and then were exposed to rabbit anti-secreto granin Ab that was finally recognized by protein A-coated, 14-nm gold particles. In between the various steps and at the end of the treatments the sections were washed with bovine serum albumin/glycine/PBS (six times, 10 min, room temperature). Immunodecorated cryosections were processed as recommended by Keller *et al.* (20).

Materials. Anti-CS Ab, raised in hens and guinea pigs by injection of the protein purified from rabbit fast-twitch skeletal muscle, were affinity purified (21). Anti-secreto granin Ab was the kind gift of W. Huttner (European Molecular Biology Laboratory, Heidelberg). Rabbit anti-chicken IgG was purchased from Nordic (Tiburg, The Netherlands) and rhodamine-coupled goat anti-rabbit IgG was from Cappel (Cochranville, PA). Other chemicals were analytical or highest available grade.

RESULTS

Structural and Immunological Characterization of Nonmuscle CS-like Proteins. Muscle CS is known to stain blue with the cationic carbocyanine dye StainsAll (1). In PAGE gels of nonmuscle cell and tissue homogenates, only a few blue bands were revealed, one of which had an apparent molecular weight close to muscle CS (M_r 65,000).

Similar to CS, (i) this blue band (not shown in figures) was enriched in the microsomal fractions (fraction 3 of HL-60) and absent from the final supernatants; (ii) this blue band was extracted in soluble form by alkali treatment (Tris/EDTA or sodium carbonate) of the enriched fractions, suggesting an intraluminal localization; and (iii) its electrophoretic mobility was markedly pH-dependent, as revealed by crossed PAGE carried out by sequential runs at pH 7 and 8.3. In muscle CS, this property is due to an extended structure, which becomes more asymmetrical at alkaline pH. The CS-like blue band of microsomes (fraction 3 of HL-60) was by far the predominant Ca^{2+} -binding protein, as revealed by ^{45}Ca overlay (31). The similarity between muscle CS and nonmuscle CS-like proteins was further substantiated by immunoblot results (Fig. 1). Affinity-purified anti-CS Ab decorated not only their antigen (lane A) but also one band of similar migration from nonmuscle cells: HL-60 (fraction 3, lane B), PC12, and rat pancreas and liver micro-

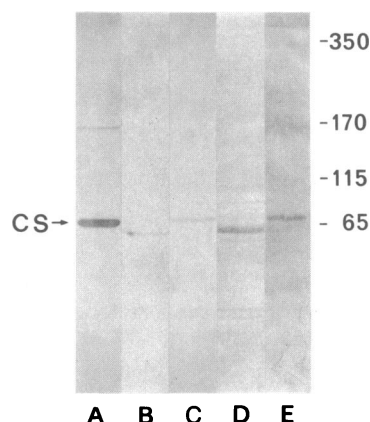


FIG. 1. Immunological evidence for CS-like protein(s) in HL-60, PC12, and rat pancreas and liver cells. Immunoblots with affinity-purified chicken anti-skeletal muscle CS Ab show immunoreactivity with CS of skeletal muscle terminal cisternae (lane A, 15 μg of total protein) and cross-reactivity with one band of fraction 3 from HL-60 and microsomes from PC12 and from rat pancreas and liver (lanes B, C, D, and E, respectively; 150 μg of protein each). Alignment of gels was made based on migration of standards. Molecular weights are given as $M_r \times 10^{-3}$.

somes (lanes C–E). The immunodecorated bands coincided with the blue bands of the StainsAll pattern.

Immunocytochemistry. Immunofluorescence of cultured cells and thick tissue sections previously permeabilized with Triton X-100 revealed a specific pattern distributed over the whole cytoplasm due to a large number of small, discrete spots. In contrast, nuclei were negative. In cultured cells (Fig. 2 A and B for PC12 cells) the spots appeared better resolved toward the cell periphery. With nonimmune chicken IgG (Fig. 2C) or anti-CS Ab previously adsorbed with the antigen (1:1 ratio; incubation, 12 hr, 4°C; not shown), fluorescence was very low and uniform over nucleus and cytoplasm. High-resolution immunogold studies were carried out on ultrathin cryosections. In both cell lines and rat tissues (Fig. 2 D–J and Fig. 3, respectively), labeling was restricted to one type of structure, distinct from readily recognizable organelles such as nuclei, nuclear envelope (Fig. 2F), Golgi complex (Fig. 2 F and J), ER (Fig. 2F; Fig. 3 A, B, and D–H), mitochondria (Fig. 3 D and H), large secretory granules (Fig. 3F), and lysosomes and multivesicular bodies (not shown). With chicken Ab the background and the labeling of controls was negligible (<5 gold particles per μm^2). With the guinea pig Ab, however, the signal-to-background ratio was not as good, but the general trend was as with chicken Ab. In the cryosections, the limiting membrane of CS-positive structures was sometime difficult to appreciate and less precisely resolved with respect to adjacent membranes (ER, granules, plasma membrane). The diameter of CS-positive structures ranged between 50 and 250 nm, and their shape was variable, from spherical (vesicles to small vacuoles, Fig. 2J; Fig. 3 A, D, and F) to pleiomorphic (Fig. 2 E and G; Fig. 3G). Some labeled structures appeared empty or containing material of moderate density (Fig. 2 D, G, and J; Fig. 3 A–D). Others, however, showed a dense content (Fig. 2 D, E, and H; Fig. 3E), similar to small secretory granules, such as those in PC12. Double-label experiments were therefore carried out in PC12, using Ab against a known secretory protein, secreto granin (22). The results (Fig. 2I) showed the distributions of the CS-like protein and secreto granin to be completely different. In PC12 and HL-60 (Fig. 2 D–F and G–J), CS-positive structures often appeared individually distributed (but some were adjacent to ER or Golgi cisternae) to unidentified structures and even to the plasmalemma.

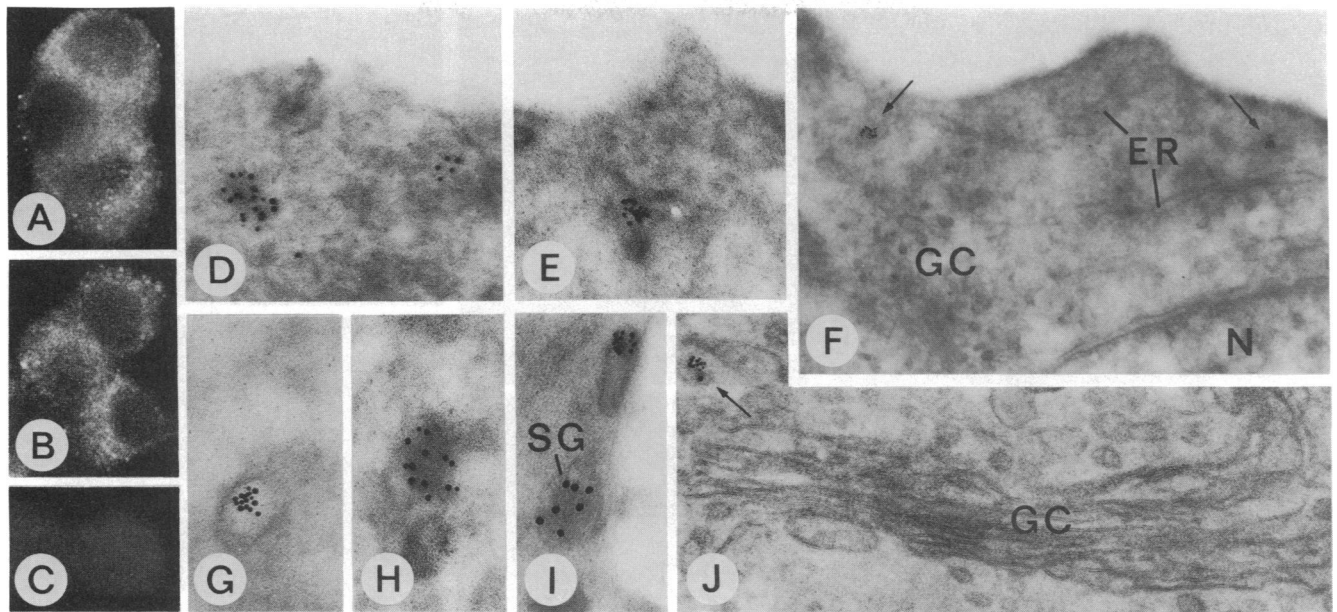


FIG. 2. Immunocytochemistry of HL-60 and PC12 cells with anti-CS Ab. (A–C) Immunofluorescence in PC12 cells. With anti-CS Ab (A and B), a granular fluorescence distributed over the entire cytoplasm is resolved into small spots over the nucleus and at the cell edge. With nonimmune IgG (C), labeling is very low and uniform over nucleus and cytoplasm. ($\times 150$.) (D–F) Immunogold labeling with anti-CS Ab of ultrathin frozen sections of HL-60. (G–J) The same as in D–F, except for PC12 cells. CS-positive structures (calciosomes, arrows in F and J) appear as small, often irregular vacuoles of variable density. The section in I is doubly labeled, with large gold decorating secretogranin in a secretion granule (SG). Nuclei (N), ER, Golgi complex (GC), and the cytosol are unlabeled. (D, E, and H, $\times 96,000$; F, $\times 40,000$; G and I, $\times 80,000$; J, $\times 60,000$.)

Countings over pictures chosen at random (28 and 49 cellular profiles for HL-60 and PC12) showed the average concentration (number per unit area) in the cytoplasm to be 0.1 and 0.08 per μm^2 , respectively. Assuming each CS-positive structure to be a discrete organelle (see *Discussion*), the total number in a PC12 cell [for which a morphometric study is available (23)] can be calculated to be around 5000. In rat hepatocytes (Fig. 3 A–E) and pancreatic acinar cells (Fig. 3 F–H) many CS-positive structures were in the cytoplasmic region occupied primarily by ER cisternae. Close apposition to a cisterna was very common (Fig. 3 A, B, D, E, and G), but membrane continuity was never revealed. Other CS-positive structures appeared apposed to mitochondria (Fig. 3 D and H), and some were independently distributed, particularly in the region beneath the plasma membrane (Fig. 3C).

Subcellular Fractionation of HL-60 Cells. Clues to the functional role of the CS-positive structures were sought in HL-60 cells, where ATP-dependent Ca^{2+} uptake and Ins-P_3 -dependent Ca^{2+} release are relatively stable with time. Experiments were carried out under conditions known to minimize proteolysis artifacts (6). Pretreatment of the homogenate with digitonin improves the separation of the markers for the Ca^{2+} store from those of other organelles. Of the three fractions obtained by the Percoll gradient centrifugation of digitonin-treated HL-60 cell homogenates, the lightest (fraction 3) showed the highest Ca^{2+} uptake and Ins-P_3 -dependent release activities. In contrast, other markers were enriched in the heavier fractions 1 and 2 (Fig. 4A). Statistical analysis (least-square regression, $n = 24$) revealed a good correlation ($r = 0.91$) between the Ca^{2+} uptake and release responses, a significant, albeit weak, correlation ($r = 0.62$) between Ins-P_3 -induced Ca^{2+} release and the endosomal marker (^{125}I -insulin bound to the cells *in vivo*), and no correlation with the markers for the plasma membrane (alkaline phosphatase, $r = -0.26$), granules (β -glucuronidase, $r = -0.22$), Golgi complex (galactosyl transferase, $r = 0.38$), and ER [sulfatase C (6, 14, 24) $r = -0.15$]. The distribution of a second ER marker, glucose-6-phosphatase, resembled that of sulfatase C ($r = 0.91$),

whereas the mitochondrial marker, cytochrome *c* oxidase, was recovered primarily in fractions 1 and 2 ($55 \pm 10\%$ and $36 \pm 13\%$, respectively, mean $\pm$ SEM, $n = 4$). To further improve the purification of the Ins-P_3 -responsive, Ca^{2+} sequestering compartment, particularly with respect to endosomes, fraction 3 was further resolved by free-flow electrophoresis, a technique especially suited for the separation of subcellular structures with similar buoyant densities (25). Fractions 1, 2, and 3 of Fig. 4B are pooled free-flow electrophoresis fractions corresponding to an anode-side shoulder, a central peak, and a cathode-side shoulder, respectively. Around 60% of the Ca^{2+} pump and release activities were recovered in the central peak, whereas ^{125}I -insulin was distributed in roughly equal amounts in the three fractions. The lack of correlation between Ins-P_3 response and ^{125}I -insulin was confirmed by statistical analysis ($r = 0.18$; $n = 18$). Distribution of the CS-like protein was investigated in parallel with that of the other markers. Fig. 4C shows that the blue band (Fig. 4C, lane 3), also known to bind Ca^{2+} and to be decorated by anti-CS Ab (Fig. 1, lane B), was enriched in the Ca^{2+} -pumping, Ins-P_3 -responsive fraction 3 obtained by the Percoll gradient (Fig. 4A, fraction 3) and was practically undetectable in fractions 1 and 2 from the same gradients (compare Fig. 4A, fractions 1 and 2, with Fig. 4C, lanes 1 and 2). Likewise, the free-flow electrophoresis fraction 2 contained greater concentrations of the CS-like protein compared to fractions 1 and 3 (not shown).

DISCUSSION

A protein that shares structural and immunological features with skeletal muscle CS is expressed in each of the four cell types we have investigated: two cell lines and two rat tissues. CS was already known to be not a single protein but a family, whose members, however, were believed to be expressed in striated muscles only (1, 26). Our results demonstrate that expression of CS-like proteins is widespread and possibly general. Another Ca^{2+} -binding protein, endoplasmic reticulum chaperone, which might be ubiquitous, has been recently

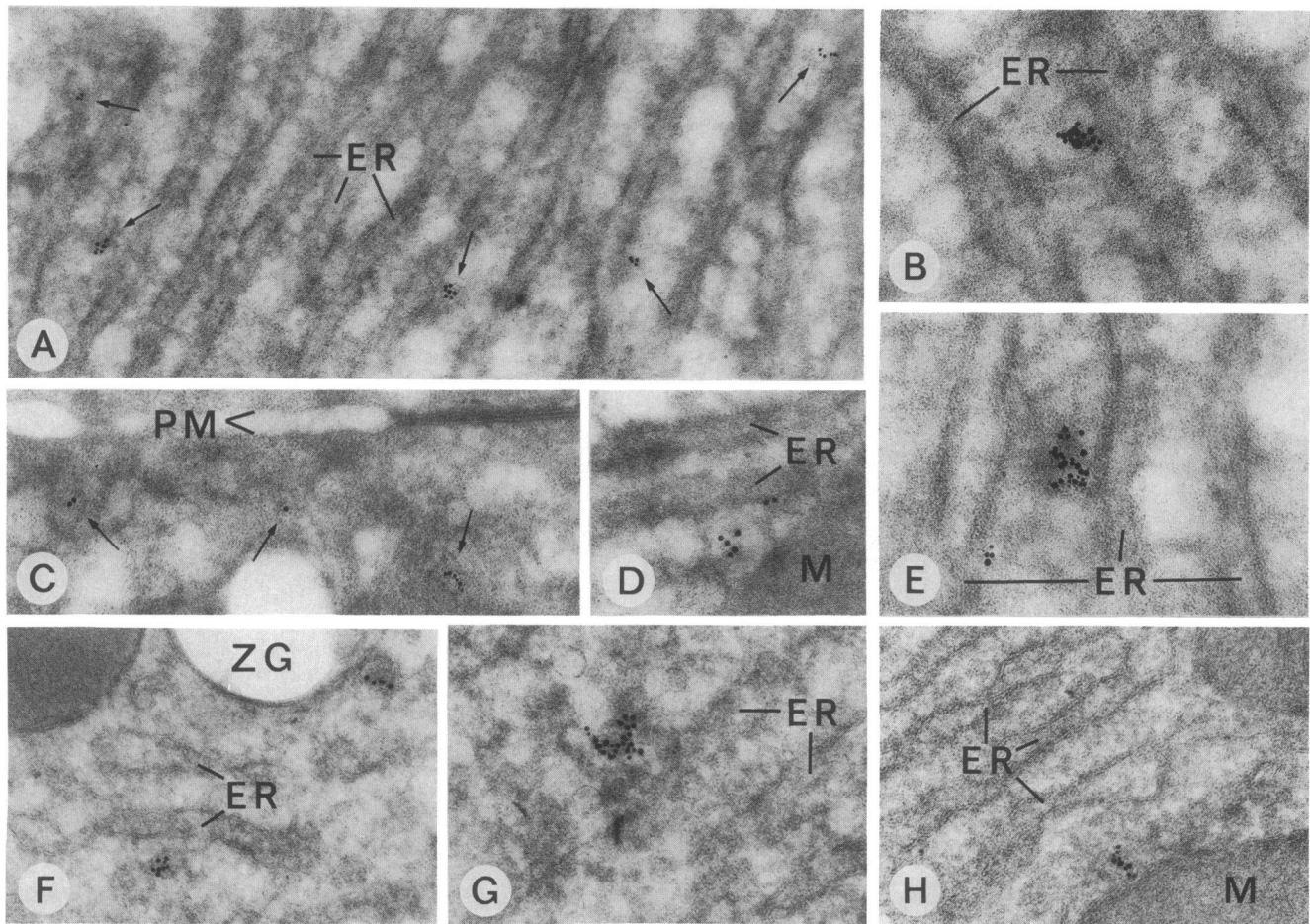


FIG. 3. Immunogold labeling of ultrathin frozen sections of rat hepatocytes (A-E) and pancreatic acinar cells (F-H). CS-positive structures (calciosomes, arrows in A and C) resembling those in cultured cells (Fig. 2) are often closely adjacent to ER cisternae and/or mitochondria (M) and zymogen granules (ZG), which are unlabeled. No membrane continuities between calciosomes and other organelles are seen. PM, plasma membrane. (A, $\times 70,000$; B, D, and E, $\times 100,000$; C, $\times 55,000$; F and H, $\times 75,000$; G, $\times 85,000$.)

described (27). Because of its much greater size, different distribution (in the ER), and vastly different degree of expression in various cell types, endoplasmic reticulum appears to have nothing to do with our CS-like proteins.

In nonmuscle cells, CS-like protein(s) was found to be exclusively localized within structures of peculiar morphology distributed throughout the cytoplasm. Many such structures appeared as isolated, discrete organelles, especially in

cultured cells. The proximity of other such structures with ER cisternae, especially in pancreas and liver, raises, however, the question of their possible continuity with the ER. Indeed, in muscle fibers, CS is concentrated in terminal cisternae, which are continuous with the rest of the SR. In our opinion, the analogy with the muscle might not hold in nonmuscle cells because (i) continuities were never detected, (ii) the appearance of the two membranes is quite

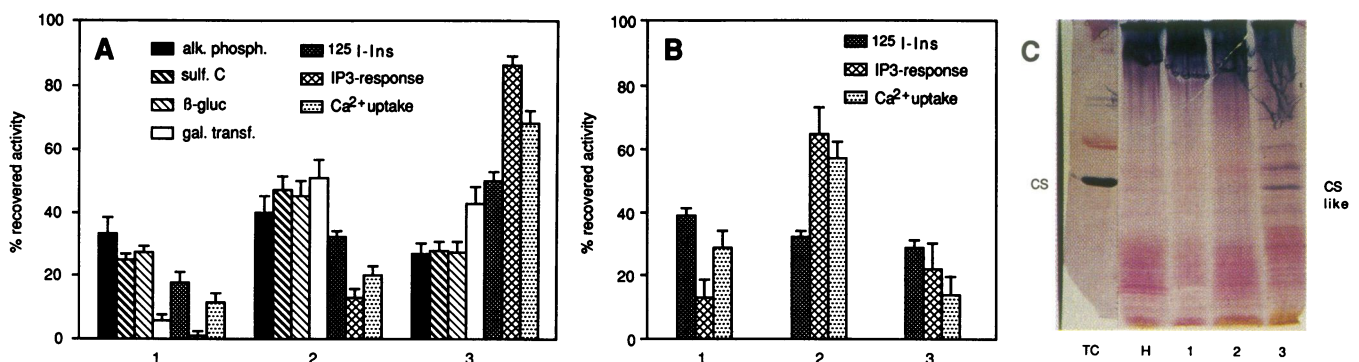


FIG. 4. Distribution of Ca^{2+} uptake, Ins-P_3 response, various organelle markers, and CS-like protein among subcellular fractions from HL-60 cell homogenates treated with digitonin. (A and C) Distribution of markers in fractions 1-3 obtained by Percoll gradient centrifugation. (B) Further free-flow electrophoresis of fraction 3 (fractions 1-3). Results in A and B are means $\pm$ SEM ($n = 8$ and $n = 6$, respectively) expressed as percentages of recovered activities. ^{125}I -Ins, ^{125}I -insulin; alk. phosph., alkaline phosphatase; sulf. C, sulfatase C; β -gluc, β -glucuronidase; gal. transf., galactosyl transferase. The PAGE slab shown in C was stained by the StainsAll procedure. Lanes labeled TC and H refer to skeletal muscle terminal cisternae and HL-60 total homogenate, respectively. The positions of CS and the CS-like protein are indicated.

different, and (iii) the ER is endowed with a variety of functions, whereas the whole SR is primarily devoted to Ca^{2+} transport. Therefore, we feel justified in considering our CS-positive structures as discrete organelles and propose for them the name of calciosome. Clearly, this conclusion does apply to the "mature" form of this entity, not necessarily to its biogenesis. Future work will tell whether ontogeny of the calciosome implies a transient connection to the ER or other subcellular structures.

As the name implies, we believe the CS-like-containing organelle to be involved in the regulation of Ca^{2+} homeostasis. The fractionation results on HL-60 cells represent an indication of the calciosome as the intracellular target of Ins-P_3 . Based on studies mainly in the liver and pancreas, mitochondria and the plasma membrane had already been excluded from playing the role of Ins-P_3 target, which was attributed to the ER (2–5, 28). Results in neutrophils and liver argue, however, against this last hypothesis (6, 8). The results in HL-60 cells appear inconsistent with the involvement of the ER as well as other organelles (Golgi complex, granules, endosomes) that up to now had not been even considered. In view of the structure of the calciosome, and of its ER association in liver and pancreas, the previous cell fractionation data (4) could have been due to contamination of ER (microsome) fractions with calciosomes. The binding properties of CS (high capacity, moderate affinity) appear exquisitely suited for the storage of the rapidly exchanging Ca^{2+} pool mobilized by Ins-P_3 . The concentration of CS-like proteins in HL-60 and PC12 has been estimated (by microdensitometry of StainsAll-stained gels) to be around 0.1–0.2% of the total cell protein. If we assume the Ca^{2+} -binding properties of CS-like proteins to be similar to those of muscle CS, the total Ca^{2+} capacity of the calciosome pool can be estimated to be around 0.2–0.4 mmol/liter of cell volume. Amounts of this order appear sufficient to sustain the rapid, intracellularly derived $[\text{Ca}^{2+}]_i$ transients triggered by Ins-P_3 (29, 30). Clearly, for a final demonstration of the functional role of the calciosome further work is needed—in particular, the purification of the CS-like proteins and of the calciosome itself.

Note Added in Proof. Recent double-labeling experiments carried out in liver cells by the use of anti-CS and anti-cytochrome P-450 antibodies have shown that the calciosome membrane is not endowed with the latter enzyme, a typical marker of ER membranes.

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