Immunocytochemistry of Calciosomes in Liver and Pancreas

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Abstract. Calciosomes are small cytoplasmic vacuoles identified in various nonmuscle cell types by their content of protein(s) similar to calsequestrin (CS), the Ca^{2+} storage protein of the muscle sarcoplasmic reticulum (SR). These entities have been interpreted as the "primitive" counterpart of the SR, and suggested to be the organelle target of inositol-1,4,5-triphosphate action (Volpe, P., K. H. Krause, S. Hashimoto, F. Zorzato, T. Pozzan, J. Meldolesi, and D. P. Lew. *Proc. Natl. Acad. Sci. USA.* 85:1091-1095). Immunoperoxidase and immunogold experiments carried out in both thick and ultrathin cryosections of rat hepatocytes and pancreatic acinar cells by using antimuscle CS antibodies revealed a specific labeling widely distributed in the entire cytoplasm, while nuclei were negative. Individual calciosomes appeared as small (105 nm) membrane-bound vacuoles intermingled with, and often apposed to ER cisternae and mitochondria. Other calciosomes were scattered in the Golgi area, in between zymogen granules and beneath the plasma membrane. The cumulative volume of the CS-positive organelles was measured to account for the 0.8 and 0.45% of the cytoplasm in liver and pancreas cells, respectively. The real total volume of the calciosome compartment is expected to be approximately twice as large. In hepatocytes, structures similar to CS-positive calciosomes were decorated by antibodies against the Ca^{2+} ATPase of muscle SR, while ER cisternae were not. By dual labeling, colocalization was revealed in 53.6% of the organelles, with 37.6% positive for the ATPase only. CS appeared preferentially confined to the content, and the Ca^{2+} ATPase to the contour of the organelle. The results suggested a partial segregation of the two antigens, reminiscent of their well-known segregation in muscle SR. Additional dual-label experiments demonstrated that hepatic calciosomes express neither two ER markers (cytochrome-P450 and NADH-cytochrome b_5 reductase) nor the endolysosome marker, luminal acidity (revealed by 3-[2,4-dinitroanilino]-3′-amino-*N*-methyl dipropylamine). Calciosomes appear as unique cytological entities, ideally equipped to play a role in the rapid-scale control of the cytosolic-free Ca^{2+} in nonmuscle cells.

CYTOPLASMIC Ca^{2+} storage organelles capable of both high affinity uptake and rapid triggered release of Ca^{2+} are believed to be ubiquitous among eukaryotic cells (3, 9). So far, however, the intracellular structure responsible for these activities has been identified with certainty only in striated muscle fibers, where a specific, highly developed tubular network, the sarcoplasmic reticulum (SR)[1], accounts for the $[Ca^{2+}]_i$ fluctuations that underly the contraction-relaxation cycle. The SR is endowed with two major proteins: a Ca^{2+} ATPase, which accounts for over 80% of its membrane proteins, and is responsible for the high affinity Ca^{2+} uptake; and calsequestrin (CS), a high capacity, low affinity Ca^{2+}-binding protein, responsible for Ca^{2+} storage. Ca^{2+} release from the SR is due to a channel activated by T tubule depolarization (7, 8, 12–14, 18). A tubular network similar to the SR was described, but not yet characterized in detail, in smooth muscle cells (31). In all other cells the structure responsible for the rapid control of $[Ca^{2+}]_i$ was proposed to be the ER, or at least a part of this endomembrane system (3, 25, 32, 33, 36). Indeed, microsomal fractions isolated from different sources were found to accumulate Ca^{2+} in an ATP-dependent fashion, and to release it when treated with inositol-1,4,5-triphosphate (Ins-P_3) (25, 27, 32, 36). The latter is a second messenger responsible for the receptor-triggered intracellular release of Ca^{2+} (3). Up to now, however, no conclusive demonstration has been given that the Ca^{2+} uptake, storage and release properties of microsomes reside in the ER-derived elements, and not in other components of the fraction. Moreover, the

1. *Abbreviations used in this paper*: Ab(s), antibody(ies); CS, calsequestrin; DAMP, 3-(2,4-dinitroanilino)-3′-amino-*N*-methyl-dipropylamine; Ins-P_3, inositolo-1,4,5-triphosphate; SR, sarcoplasmic reticulum.

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molecular machinery responsible for these activities has not been fully characterized.

During the past year, proteins that bear immunological and/or biochemical resemblance to CS have been shown to be expressed not only in striated but also in smooth muscle fibers (37), and in a variety of nonmuscle tissues, blood cells, oocytes and cultured cell lines (11, 23, 34). In liver and pancreas, as well as in two cell lines (HL60 human promyelocytic leukemia and PC12 rat pheochromocytoma), the CS-like proteins were shown by high resolution immunocytochemistry to reside in a population of small vacuoles distributed throughout the cytoplasm (34). Subcellular fractionation of HL60 cells suggested that these new structures, and not the ER, might constitute the intracellular target organelle of Ins-P_3 action. Because of their content of a CS-like protein, and their hypothesized involvement in the regulation of Ca^{2+} homeostasis, the new structures were given the name of calciosomes and suggested to be a simplified, nonmuscle version of the SR (34). If these were indeed the case, calciosomes might be expected to express not only a CS-like protein, but also other SR-like components, and to exclude components and activities typical of different structures, such as the ER. At the present time, answers to these questions cannot be given by biochemical analyses because pure calciosome fractions have not been isolated yet. Well-characterized antibodies (Abs) addressed to both the SR Ca^{2+} ATPase and components of other organelles are, however, available. The characterization of the calciosome was therefore pursued at the cytochemical level by single and dual immunolabeling studies of cryosections of the rat liver and pancreatic tissues.

Materials and Methods

Male Sprague-Dawley rats were perfused-fixed with either 4% formaldehyde (freshly prepared from paraformaldehyde) or a mixture of 2.5% formaldehyde together with 0.25% glutaraldehyde in 125 mM phosphate buffer pH 7.4. Small fragments of liver and pancreas tissue were excised, and fixation was continued by immersion for another 60 min at 4°C. The samples were then extensively washed in the buffer, infiltrated with sucrose (0.5-2.3 M), and frozen in freon 12 cooled with liquid nitrogen.

Immunolabeling

Thick (1 μm) and ultrathin (0.1 μm) cryosections were cut in a Reichart Ultrocut equipped with a FC4 apparatus (Reichart Scientific Instruments, Div. Warner-Lambert Technologies, Inc., Buffalo, NY). For immunoperoxidase experiments at the light microscope level, subsequent thick tissue sections were treated with either anti-CS, anti-Ca^{2+} ATPase, or nonimmune IgG (10 μg/ml) dissolved in a buffer containing 0.3% Triton X-100, 0.45 M NaCl, 1:6 vol of goat serum, and 20 mM phosphate, pH 7.2. In the experiments in which chicken Abs were used, they were revealed by rabbit anti-chicken IgG (1:500) followed by peroxidase-coupled goat anti-rabbit IgG (1:80). Other experimental conditions were as described in reference 20.

Immunolabeling at the electron microscope level was carried out with 5- and 14-nm gold particles prepared and purified by sucrose gradient centrifugation as described by Slot and Geuze (3). Ultrathin cryosections were 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 directly coated with chicken IgG (either anti-CS or nonimmune) were used; in others the sections were first treated with chicken Abs (15 μg/ml, 60 min, 37°C), then treated with rabbit anti-chicken IgG (60 min, 37°C, 1:1,000), and finally with protein A-gold (60 min, 37°C). Additional experiments were carried out with other anti-CS Abs: guinea pig anti-rabbit CS, or rabbit anti-human CS Abs. Bound Abs were revealed by small gold particles coated with protein A.

For double label experiments, the ultrathin cryosections were first treated with either anti-cytochrome-P450 or anti-NADH-cytochrome b_5 reductase Abs that were revealed by protein A-coated large (14 nm) gold particles, and then dually decorated with chicken anti-CS Abs coupled to small (5 nm) gold particles. For double labeling with anti-Ca^{2+} ATPase and anti-CS Abs (both raised in chicken), the same sequence was followed, but the sections reacted with the anti-Ca^{2+} ATPase Abs were treated with a rabbit anti-chicken IgG (1:500), and then immunodecorated with the protein A gold particles. Before the subsequent application of the anti-CS-coated small gold particles the sections were extensively quenched with nonimmune chicken IgG (100 μg/ml, 3 h). Parallel experiments with small gold particles coated with nonimmune chicken Abs under these conditions revealed a very low background of small gold particles. Dual Ca^{2+} ATPase-CS-labeling experiments were also carried out by the use of the rabbit anti-human CS Abs, with similar, although less satisfactory, results compared with those obtained with chicken anti-CS Abs.

To reveal acidic compartments, the DAMP (3-[2,4-dinitroanilino]-3'-amino-*N*-methyl-dipropylamine) technique was used in freshly excised, unfixed liver fragments processed as described in reference 24. DAMP trapped by glutaraldehyde fixation was revealed by mouse anti-dinitrophenol Abs followed in sequence by rabbit anti-mouse IgG Abs and protein A-coated large gold particles (24). The sections were then doubly decorated by the chicken anti-Cs Ab-coated small gold particles.

In all high resolution immunolabeling experiments, 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; reference 34). Immunodecorated cryosections were processed as recommended by Keller et al. (17).

Background

The background labeling was systematically evaluated for all Abs used by counting the density (number/unit area) of gold particles in control sections (reacted with homologous nonimmune Abs) and over subcellular structures, such as nuclei, that appeared negative for the various antigens investigated. In the case of CS the background was found to be very low, particularly in the experiments with chicken anti-CS Abs directly coupled to small gold particles (0.22 particles/μm^2). Under the conditions of the experiments presented, background with anti-Ca^{2+} ATPase, cytochrome-P450, and DAMP was estimated to be 1.25, 1.5, and 0.5 particles/μm^2, respectively.

Materials

Anti-CS and anti-Ca^{2+} ATPase Abs, raised by injecting the purified rabbit fast twitch muscle SR proteins in hens, were affinity purified and characterized as reported in references 11 and 34. Other anti-CS Abs, obtained by injecting the rabbit protein into guinea pigs, and the human protein into rabbits, were similarly affinity purified and characterized. Affinity purified anti-rat cytochrome-P450 Abs were obtained from Drs. Y. Tashiro and R. Masaki (38), and anti-rat NADH-cytochrome b_5 reductase Abs from Dr. N. Borgese (6), DAMP and the anti-dinitrophenol Abs (monoclonal mouse IgG) from Dr. R. G. W. Anderson, University of Texas, Dallas, TX. Rabbit anti-mouse and anti-chicken IgG were purchased from Nordic Immunology (Tilburg, The Netherlands), and peroxidase-coupled goat anti-rabbit IgG from Cappel Laboratories, Inc. (Cochranville, PA). Other chemicals were analytical or the highest grade available.

Results

Light Microscopy

Immunoperoxidase labeling of subsequent 1-μm-thick cryosections pairs was used to compare the overall distribution of CS and Ca^{2+} ATPase immunoreactivities. With nonimmune (control) IgG, the response was uniformly negative over nucleus and cytoplasm, but a surface reaction was observed in the pancreatic acinar cells (not shown). With anti-CS and anti-Ca^{2+} ATPase, on the other hand, distinct, specific signals were revealed over the cytoplasm of liver cells whereas nuclei were consistently negative (Fig. 1, *A* and *B*). The general pattern of immunolabeling observed for two antigens appeared the same, i.e., a granular response widely,

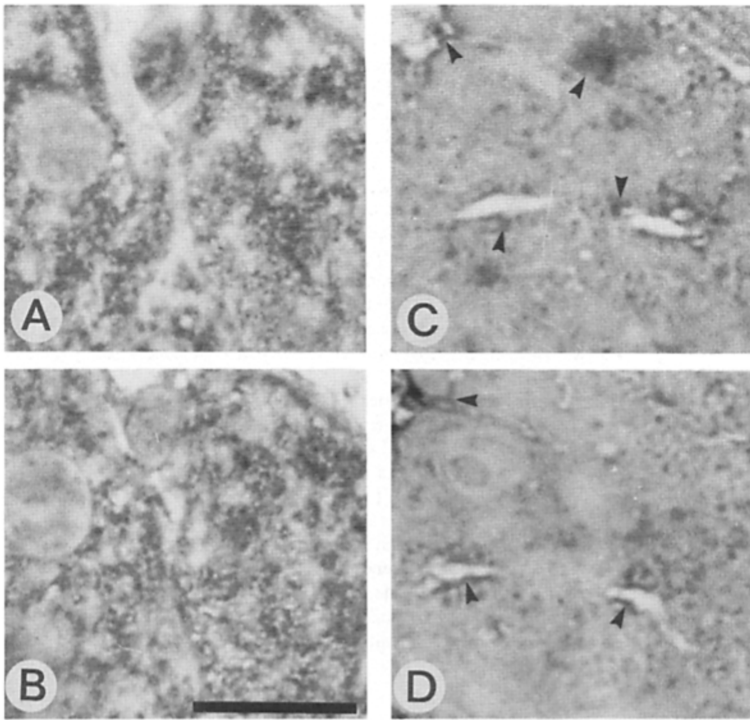

Figure 1. Immunoperoxidase of subsequent 1-μm-thick cryosections of hepatic (*A* and *B*) and pancreatic acinar (*C* and *D*) cells decorated with anti-CS (*A* and *C*) and anti-Ca^{2+} ATPase (*B* and *D*) Abs. In hepatocytes, an intense, diffuse granular labeling of the cytoplasm is evident, with the same general pattern of distribution for the two antigens. In pancreatic acinar cells immunolabeling was also widespread, but much weaker than in the liver. Arrowheads in *C* and *D* point to unspecific surface peroxidase spots, which were visible in control pancreas preparations as well. Bar, 1 μm.

although not uniformly, distributed throughout the cytoplasm: beneath the plasma membrane, around nuclei, and in the other cytoplasmic areas as well. In subsequent section pairs, the labeling ratio for CS and Ca^{2+} ATPase was remarkably constant, whereas the labeling intensity varied somewhat from cell to cell, with no apparent correlation with the localization in the hepatic lobule. Negative hepatocytes were never observed, however. In the acinar cells of the pancreas (Fig. 1, *C* and *D*), investigated under conditions strictly parallel to those used for the liver, the labeling for both CS and Ca^{2+} ATPase was distinctly weaker than in hepatocytes, but the overall pattern appeared also widely distributed. In particular, the specific labeling was present both towards the base and in the apical cytoplasm.

Electron Microscopy

Hepatocyte Immunolabeling with Anti-CS and Anti-ATPase Abs. Figs. 2 and 3 illustrate the appearance and distribution of rat hepatocyte calciosomes, as revealed in ultrathin cryosections by anti-muscle CS Abs (small gold particles), used alone (Fig. 2) or in combination with anti-muscle Ca^{2+} ATPase Abs (large gold, Fig. 3). In agreement with our previous results (17), CS immunolabeling was found to be restricted to a population of vesicles and small vacuoles of moderate electron density, with profile diameters ranging from 35 to 150 nm (average 105 nm). Labeling over the nucleus and the recognizable organelles of the cytoplasm (mitochondria, ER and Golgi cisternae, dense bodies) was close to the background, estimated in control preparations (0.22 particles/μm^2). In many cases (Fig. 2, *A–D*) the limiting membrane of the calciosomes was well recognized, although the familiar, triple-layered structure was less frequently resolved than in other, surrounding membranes (ER, mitochondria). In other cryosections, however, calciosome membranes were less easily discerned. In many such cases, however, the contour could still be identified by the different texture of the organelle with respect to the surrounding cytoplasm (Fig. 2, *A* and *B*). The shape of many calciosome profiles was round or ovoidal. Others, however, were more irregular in their outline. In all cases CS immunolabeling was preferentially located over the calciosome content and not over the limiting membrane (Figs. 2 and 3).

Within the hepatocytes, calciosomes were distributed throughout the cytoplasm, interdispersed with, and often proximal to the other organelles. In the regions occupied by ER stacks, calciosomes were seen in between adjacent cisternae, often closely apposed (Fig. 2, *A* and *B*; Fig. 3, *A*, *C*, and *D*), or apparently connected by means of a filamentous material (Fig. 2, *C* and *D*), to the cytosolic surface. Elsewhere in the cell, calciosomes appeared often apposed to other organelles, for example mitochondria (Fig. 2 *A*), or sandwiched in between a mitochondrion and an ER cisterna (Fig. 2 *D*). In the cytoplasmic rim beneath the plasma membrane calciosomes were seen isolated or adjacent to tubular structures. Apposition of calciosomes to the plasma membrane was not common, especially at the biliary pole (not shown). Quite a number of calciosomes were present in the Golgi area, often located at the *trans*-Golgi (Fig. 3 *B*) or intermingled with other, CS-negative vesicles, lateral to the cisternal stacks.

When the liver cryosections were immunodecorated by antimuscle Ca^{2+}ATPase Abs only (not shown in Figs.), the overall distribution pattern appeared analogous to that observed with anti-CS Abs. In particular, labeling was most often over vesicles and small vacuoles similar in size, shape, and distribution to calciosomes, while other structures such as nuclei, ER cisternae, and plasma membrane were labeled

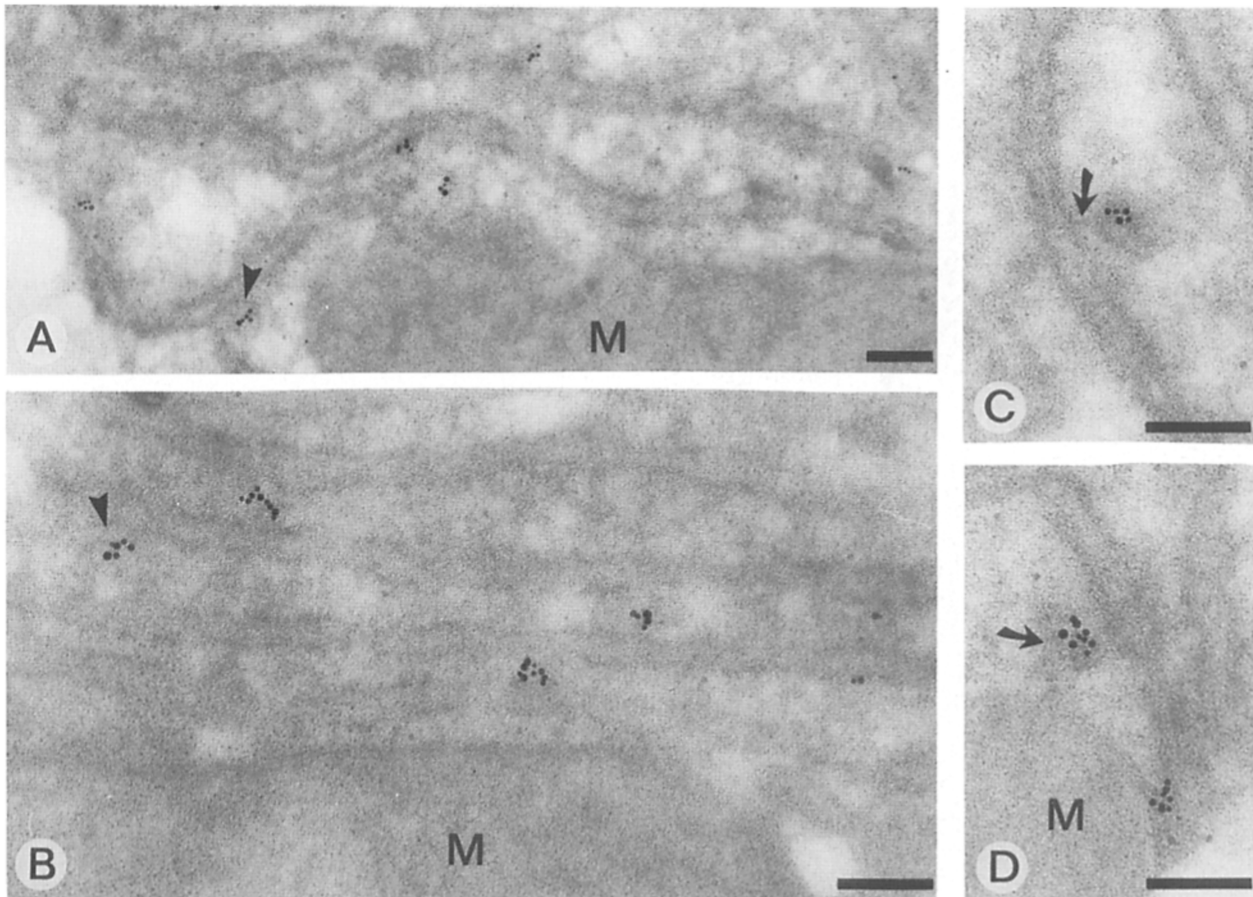

Figure 2. Ultrathin liver cryosections immunolabeled for CS. The various panels illustrate the shape and distribution of calciosomes. Notice the frequent apposition to both ER cisternae (*A–D*) and mitochondria (*A* and *D*), which at least in some cases appears mediated by thin filaments (*arrows* in *C* and *D*). Arrowheads in *A* and *B* point to a few calciosomes with readily discernable limiting membrane. *M*, mitochondrion. Bar, 0.1 μm.

very little. Ca^{2+}ATPase and CS labeling were not identical, however. In fact, with the anti-ATPase Abs the gold particles were mostly aligned at the periphery of the labeled organelles, as was to be expected for a membrane antigen. Moreover, labeling of mitochondria (but not of ER and nuclei) was higher than the background, suggesting a cross-reactivity with a mitochondrial antigen.

The similarity of the results obtained with anti-Ca^{2+}ATPase and anti-CS Abs suggested that both these Abs recognize the same organelle. This possibility was substantiated by the results of dual-label experiments, illustrated in Fig. 3. Most CS-positive calciosomes localized in all regions of the cell were in fact markedly labeled by anti-ATPase Abs as well. In these specimens, the two labels often exhibited the distribution previously described in the samples labeled by either one of the Abs separately: small gold particles addressed to CS preferentially decorated the content, whereas the large particles addressed to the ATPase were often arranged in short rows and preferentially located at the organelle rim (Fig. 3, *C* and *D*). In a few cases a partial topological segregation of the two labels was observed, with the CS and ATPase immunodecorations concentrated towards opposite edges of one calciosome. In addition to the calciosomes immunodecorated by both Abs, other organelles positive for only one antigen were also seen in the dually labeled sections (Fig. 3). The frequency of these structures was different: rare for CS, much more abundant for ATPase. The relative proportion of the variously labeled organelles was established by countings carried out on a total of 30 prints of dually labeled sections taken at random (494 organelles). In this population, dually labeled calciosomes were 53.6%, those labeled only for ATPase, 37.6%, and those for CS, 8.8%. The same group of dually labeled prints was used to estimate the relative volume of calciosomes in the liver cytoplasm. CS-positive organelles (with and without Ca^{2+}-ATPase immunolabeling) were found to account for 0.80%, and those positive for ATPase only for 0.55% of the total cytoplasmic volume.

Hepatocyte Dual Immunolabeling for CS and ER or Acidity Markers

In these experiments, the immunolocalization of CS was compared with that of other antigens which are bona fide markers of other organelles. A major effort was made to characterize the relationship between calciosomes and ER. In the liver, ER membranes contain cytochrome-P450, which is a predominant protein (38), as well as another enzyme,

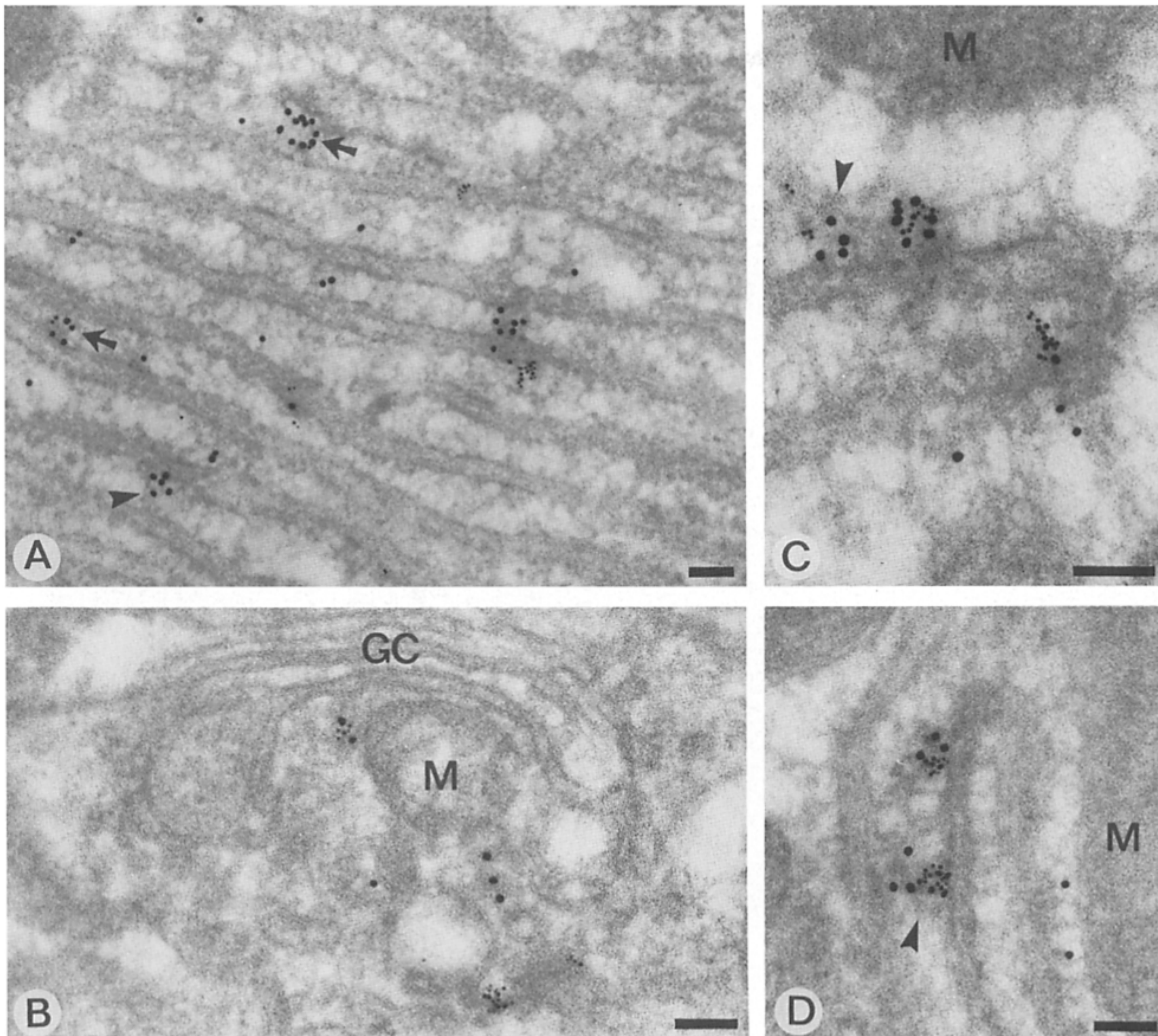

Figure 3. Dual CS (small gold)- Ca^{2+} ATPase (large gold) immunolabeling of liver ultrathin cryosections. The structures (calciosomes) labeled for either one or for both antigens appear quite similar in the ER cisternae-rich region of the cell (*A*, *C*, and *D*) as well as at the *trans*-Golgi (*B*). Notice the preferential labeling of the profile periphery for the Ca^{2+} ATPase (up to semicontinuous rows of gold particles, *arrows* in *A*) and the content labeling for CS. Arrowheads in *A*, *C*, and *D* point to calciosomes with readily discernable limiting membrane. *M*, mitochondrion; *GC*, Golgi complex. Bar, 0.1 μm.

NADH-cytochrome b_5 reductase, which is present at a smaller concentration. The latter enzyme is known to be localized also in the outer mitochondrial membrane (5). The results obtained with anti-cytochrome-P450 Abs are illustrated in Fig. 4, *A* and *B*. In agreeement with previous findings (38), this protein was immunolocalized exclusively to the ER, with only background level of labeling over other structures (nuclei, Golgi cisternae, mitochondria). Most calciosomes, identified by CS immunodecoration, were not labeled by anti-cytochrome-P450 Abs (Fig. 4, *A* and *B*). Results similar to those with anti-cytochrome-P450 Abs were also obtained for the other ER membrane protein, NADH-cytochrome b_5 reductase, which however gave a weaker signal and was immunolocalized also at the outer mitochondrial membrane (data not shown).

To quantitate the data obtained with anti-cytochrome-P450 Abs, immunogold particles decorating ER and calciosomes were counted in a group of 25 randomly selected printed pictures, and the results normalized to a membrane basis (number of particles/μm of membrane profile length). The degree of ER labeling (1.25 per μm; total analyzed membrane length: 890 μm) was found to exceed by a factor of almost 10 the labeling of calciosomes (0.145 per μm; analyzed membrane length: 95 μm). This low level labeling of calciosomes for cytochrome-P450 can be entirely accounted for by signal spreading from the adjacent, much more heavily labeled ER cisternae (22).

To investigate whether calciosomes have a low internal pH, small hepatic tissue fragments were first treated with DAMP, a weak amine which accumulates preferentially within acidic compartments where it can be cross-linked to protein by glutaraldehyde fixation, and finally revealed by

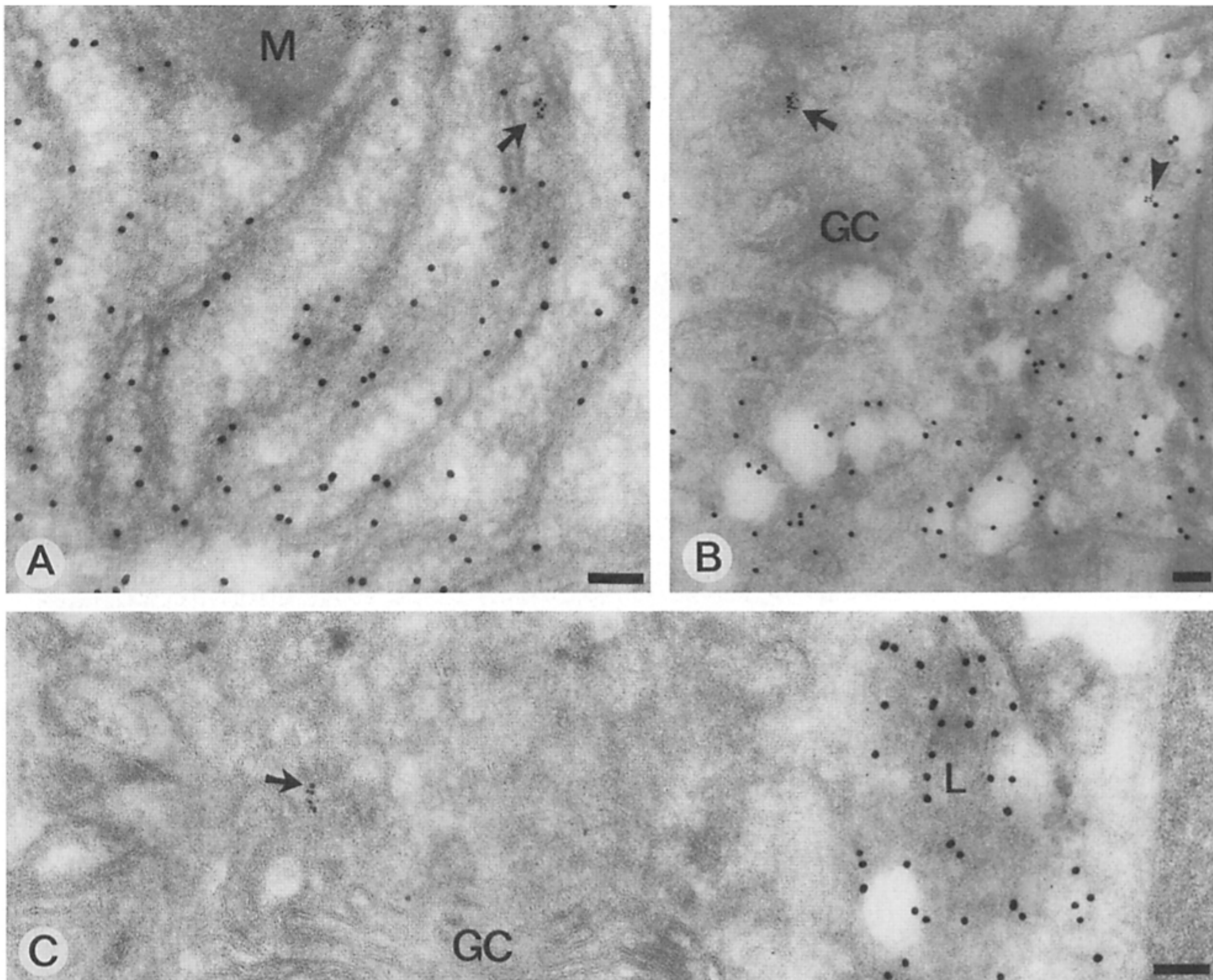

Figure 4. Dual immunolabeling of liver ultrathin cryosections for CS (small gold) and ER (*A* and *B*) or endolysosome (*C*) markers (large gold). (*A* and *B*) Cryosections double labeled for CS and cytochrome P450. The latter immunoreactivity (large gold particles) is shown to be confined to the ER cisternae (*A*) and tubules (*B*), while the Golgi complex (*GC*) and mitochondria (*M*) are unlabeled. The calciosomes, (marked by the small gold particles of CS immunoreactivity, *arrows* in *A* and *B*), are unlabeled for cytochrome-P450. The small labeling with large gold particles of a few calciosomes (such as the one indicated by an *arrowhead* in *B*) could be attributed to signal spreading from adjacent ER cisternae. (*C*) A large dense body (*L*) heavily labeled for DAMP (large gold), while in the Golgi area (*GC*) a calciosome labeled for CS (small gold, arrow) is DAMP negative. Bar, 0.1 μm.

anti-dinitrophenol Abs (24). As can be seen in Fig. 4 *C*, DAMP immunolabeling was confined to heterogeneous dense bodies, while CS-positive calciosomes were negative. Colocalization of the two antigens was observed only once within a recognizable autophagocytic vacuole (not shown).

Pancreatic Acinar Cell Immunolabeling

In the pancreas, cytochrome-P450 is not expressed at a high level, and immunolabeling for CS and, especially, Ca^{2+}ATPase was too weak to yield an adequate signal under dual-labeling conditions. Therefore, only single CS-labeling data will be presented here. Vesicular structures, morphologically very similar to those already described in the liver and distributed throughout the cytoplasm, were found to be decorated by anti-CS Abs (Fig. 5). Compared with the liver, calciosomes were less frequently encountered in pancreatic acinar cells (measured volume of CS-positive organelles = 0.45% of the cytoplasm). Pancreatic calciosomes were often localized in between adjacent ER cisternae (Fig. 5 *A*) and in the Golgi area (Fig. 5 *B*). In the apical region of the cytoplasm calciosomes were intermingled with (Fig. 5 *D*), and sometimes adjacent to zymogen granules. Juxta-plasmalemma calciosomes were seen also in the pancreas, both at the basolateral (Fig. 5 *C*), and less frequently at the lumenal region.

Discussion

The existence of a new cytoplasmic organelle, the calciosome, was recently proposed on the basis of the specific localization of protein(s) immunologically similar to muscle CS within a population of small vacuoles (34). The present results in liver cells demonstrate that the specificity of calciosomes is not confined to their content of CS-like protein(s),

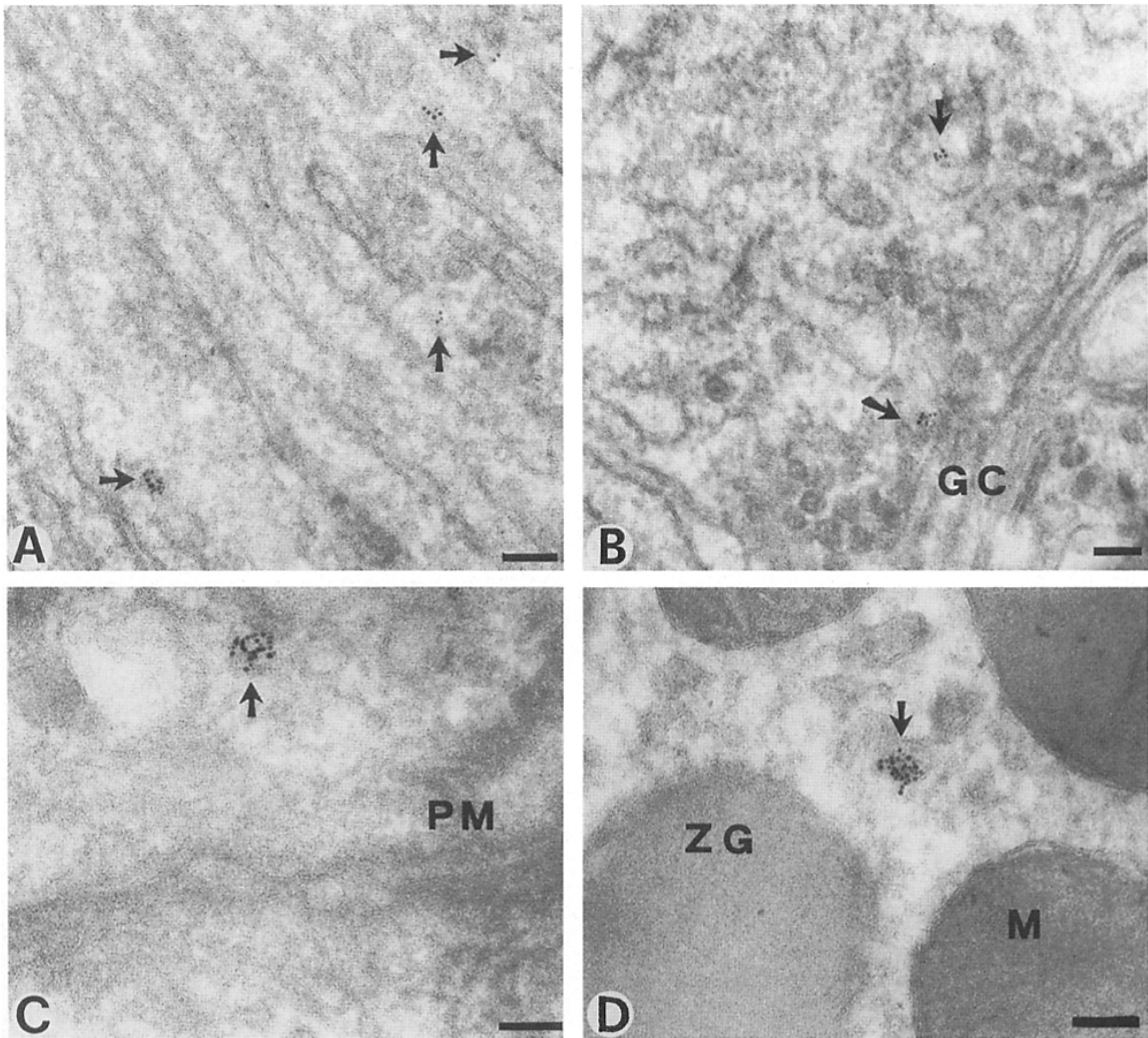

Figure 5. Calciosomes in pancreatic acinar cells. Various calciosomes labeled for CS (*arrows*) are visible in between ER cisternae (*A*); in the area surrounding the cisternal stack of the Golgi complex (*GC, B*); at a short distance from the lateral plasmalemma (*PM, C*); and in between zymogen granules (*ZG*) and mitochondria (*M*), towards the cell apex (*D*). Bar, 0.1 μm.

but extends also to the limiting membrane, where a Ca^{2+}-ATPase, immunologically similar to the muscle SR enzyme, was found to be exclusively located. In addition, calciosomes are shown here not to express markers of other cytoplasmic organelles: cytochrome-P450 and NADH-cytochrome b_5 reductase for the ER; luminal acidity for endolysosomes.

Distribution and Structure of Calciosomes

The distribution of calciosomes, revealed at both the light and electron microscope level by the parallel distribution of CS and Ca^{2+}ATPase immunoreactivities, appeared widespread throughout the cytoplasm. Even in the acinar cells of the pancreas, which are distinctly polarized, calciosomes were present both at the base of the cell, which contains primarily ER cisternae, and at the apex, where zymogen granules are concentrated. A widespread distribution of calciosomes (or CS immunofluorescence) has been recently observed also in a variety of other cell types (PC12, HL60 [34], neurons, blood neutrophils, platelets, chromaffin cells, B cells of the pancreas; unpublished observations). It may therefore be concluded that calciosomes differ from many other organelles in having no marked preference for any of the various cytoplasmic regions.

Individual calciosomes appeared as small, discrete vacuolar profiles. In many cryosections the profiles of these organelles appeared delimited by a discernable membrane, which in a few, usually large calciosomes exhibited the familiar triple-layered appearance. In other cases, however, the limiting membrane was difficult to resolve precisely. The reason for this might be severalfold. First, because of their small size, calciosome profiles included in the ultrathin cryosections were very often sectioned tangentially, and their membranes blurred for geometrical reasons, whereas for

other larger structures (ER cisternae, mitochondria, zymogen granules) this was much less frequently the case. Second, the success of membrane staining was admittedly variable among different cryosections, not only for calciosomes but also for the other organelles and the plasma membrane. Third, even in the same cryosection, the resolution of different membranes was variable, presumably because of their different composition. Thus, the appearance of zymogen granule membranes was most frequently triple-layered, followed by mitochondria, and then by ER and Golgi cisternae, with calciosome and Golgi vesicle membranes as the least well defined in the spectrum. In this respect it is worth mentioning that in ultrathin cryosections of striated muscle fibers, the SR membranes (i.e., the membranes that share the Ca^{2+} ATPase and possibly other components with the calciosome membranes), were often found to be more poorly resolved with respect to mitochondrial membranes (unpublished observations).

In double-label experiments carried out in hepatocytes by the use of both anti-CS and anti-Ca^{2+}ATPase Abs, positive organelles were codecorated in >50% of the cases. In the same experiments, however, a small group of calciosomes were labeled for CS only, and a much larger group of organelles (>35% of the total) for the Ca^{2+}ATPase only. Since the general appearance and distribution of single and dual-labeled organelles was similar, we are convinced that all of them were indeed calciosomes. Technical reasons could play some role in causing the different immunodecoration of these organelle, is reminiscent of the muscle SR, where CS is conly a fraction of the antigen molecules exposed at the section surface (15). The marked disproportion between profiles labeled only for Ca^{2+} ATPase vs. CS suggests however that a partial segregation of the two antigens occurs in the calciosomes. This interpretation, which is also supported by images showing CS immunolabeling confined to one pole of the organelle is reminiscent of the muscle SR, where CS is concentrated within the terminal cisternae, while the Ca^{2+} ATPase is distributed to the longitudinal cisternae as well (8, 14).

The cumulative volume of CS-positive calciosomes measured in liver and pancreatic cells was found to be 0.80 and 0.45% of the cytoplasmic volume, respectively. These values are certainly underestimates of the true calciosome compartment both because of the existence of profiles positive for the Ca^{2+} ATPase only, as discussed above, and because of the small size of the organelles, whose average diameter (105 nm) is close to the thickness of ultrathin cryosections. A considerable number of the calciosomes are expected, therefore, to be exposed only at one face of ultrathin cryosections, and half of these to remain unlabeled because only one face was immunodecorated. We can conclude therefore that the calciosome compartment, although distinctly smaller than the "classical" membrane-bound compartments (ER, mitochondria, granules, Golgi complex) is not insignificant in pancreas and, especially in liver cells.

Interactions of Calciosomes

So far, the immunocytochemical study of calciosomes has been limited to the use of cryosections for technical reasons. Indeed, any procedures which imply the resin embedding of the samples before immunodecoration was found to cause the loss of the CS immunoreactivity. Unfortunately, serial cryosections cannot be cut, and this has prevented a detailed study of calciosome interactions. At present we do not know for sure whether calciosomes are discrete organelles, or are arranged in a network, as it has recently been recognized for peroxisomes (19). The same uncertainty concerns the interactions with other organelles. In both the pancreas and liver many calciosomes were found to lie in close apposition to ER cisternae, suggesting a luminal continuity between the two structures. However, calciosomes were seen not only in the ER-rich regions, but also in the Golgi region and other areas. This argument is particularly cogent for the apical area of pancreatic acinar cells, which is primarily occupied by zymogen granules. Moreover, close apposition of calciosomes was seen not only with the ER, but also with other structures (mitochondria, zymogen granules, Golgi cisternae), and in no case was a clear luminal continuity with any of these structures observed. Finally, the specific markers of calciosomes and ER appeared completely segregated (Ca^{2+}-ATPase and CS on one side; cytochrome-P450 and NADH-cytochrome b_5 reductase on the other). To our knowledge, the existence of highly heterogeneous subcompartments has never been demonstrated for the liver ER where both rough and smooth-surfaced portions possess a number of common membrane components and luminal proteins (1, 21). The relationship between ER and calciosomes in the liver might on the contrary resemble that between ER and SR in striated muscle. In the mature fibers these two structures are known to be distinct both in composition and structural organization (8, 14). Of the SR components, Ca^{2+}ATPase is directly transferred from the ER shortly after completion of its synthesis (10) while CS, which is a glycoprotein, is partially processed in the Golgi complex before reaching its final destination within the SR lumen (16, 28).

Another interaction of calciosomes that might be of importance is with the plasma membrane. In both hepatocytes and pancreatic acinar cells some calciosomes were seen adjacent to the plasma membrane. In no case, however, were images suggestive of a functional coupling (gap junctions?) of the two adjacent membranes ever observed, as hypothesized in a model proposed to account for the interconnection of Ca^{2+} transport operations at the plasma membrane and intracellular organelles (26). Moreover calciosomes were observed not only near the basolateral plasmalemma, where receptors are located, but also (although less frequently) near the lumenal plasmalemma.

A final feature of the calciosome was the lack of an acidic internal milieu, as revealed by the DAMP experiments. This property readily differentiates calciosomes from endosomes and lysosomes. It should also be added that a H^+/Ca^{2+} exchange was recently suggested to be among the mechanisms responsible for the Ca^{2+} accumulation within a "microsomal" store in exocrine cells (29).

On the Calcium Function

Calciosomes have been observed in all cell types investigated so far (>10). They might, therefore, be ubiquitous and, in view of their endowment with a high capacity Ca^{2+}-binding protein and, at least in liver, with a high affinity Ca^{2+}ATPase, they are expected to play a general role in Ca^{2+} homeostasis. Previous subcellular fractionation experiments in HL60 cells revealed that high affinity Ca^{2+} uptake and

Ins-P_3-triggered Ca^{2+} release remain associated with CS, the marker of calciosomes, whereas they tend to dissociate from the markers of other cytoplasmic organelles (34). Based on these findings, the Ins-P_3-sensitive store was proposed to coincide with the calciosome (34). The present results add no direct support to this hypothesis, although the demonstrated colocalization of CS with a high affinity Ca^{2+} ATPase in liver calciosomes appears consistent with the identification of the latter with the Ins-P_3 target organelle. In fact, the low resting $[Ca^{2+}]_i$ of nonmuscle cells is believed to be due to high affinity Ca^{2+} pumping into the same organelle that releases Ca^{2+} in response to Ins-P_3 (3, 4, 25, 36). The final identification of the physiological role of calciosomes will require additional evidence, which can only be obtained through the isolation and detailed biochemical characterization of the organelle.

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