J. exp. Biol. 139, 181–193 (1988)
Printed in Great Britain © The Company of Biologists Limited 1988

THE Ins(1,4,5)P_3-SENSITIVE Ca^{2+} STORE OF NON-MUSCLE CELLS: ENDOPLASMIC RETICULUM OR CALCIOSOMES?

BY TULLIO POZZAN[1], POMPEO VOLPE[2], FRANCESCO ZORZATO[2], MARIANGELA BRAVIN[2], KARL H. KRAUSE[3], DANIEL P. LEW[3], SADIMATSU HASHIMOTO[4], BERNARDINO BRUNO[4] AND JACOPO MELDOLESI[4]

[1]*Istituto di Patologia Generale, Ferrara, Italy,* [2]*Istituto di Patologia Generale, CNR Units of Mitochrondrial and Muscle Biology and Pathophysiology, Padova, Italy,* [3]*Department of Internal Medicine, Geneva, Switzerland* and [4]*Department of Pharmacology, Institute San Raffaele, Milano, Italy*

Summary

The binding of a number of extracellular ligands (hormones, growth factors, neurotransmitters etc.) to their plasma membrane receptors causes hydrolysis of phosphatidylinositol bisphosphate to initiate the formation of two second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol, DAG. DAG has been shown to activate protein kinase C, whereas Ins(1,4,5)P_3 induces the release of Ca^{2+} from an intracellular pool. This rapidly mobilizable, Ins(1,4,5)P_3-sensitive Ca^{2+} store has until now been identified as the endoplasmic reticulum, ER. We demonstrate that this is untenable and provide evidence for the existence of an unrecognized organelle, the 'calciosome'. This conclusion is based on the following experimental evidence. (1) There is no correlation between the abundance of ER and the amount Ins(1,4,5)P_3-sensitive Ca^{2+} release. (2) There is no correlation between ER markers and those for the Ca^{2+} store [Ins(1,4,5)P_3 binding and sensitivity, Ca^{2+} uptake]. (3) A protein similar to striated muscle calsequestrin, CS, has been identified in microsomal fractions from a number of tissues; it copurifies with markers of the Ca^{2+} store, but not with those of ER. (4) Subcellular localization of the CS-like protein by electron microscopy reveals that in all cells so far analysed this protein is localized in small, membrane-enclosed structures, calciosomes, which are also stained by an anti-Ca^{2+}-ATPase antibody. Calciosomes appear to be morphologically distinct from any other known cell organelle. (5) Although they stain different portions of the calciosomes (membrane and lumen, respectively), anti-Ca^{2+}-ATPase and anti-CS antibodies do not recognize any antigen in ER cysternae; antibodies directed against known components of ER do not bind to calciosomes.

Introduction

The calcium content of typical mammalian cells is of the order of 1–3 mmol l^{-1}

Key words: Ins(1,4,5)P_3, Ca^{2+}, calsequestrin, endoplasmic reticulum.

of cell water (i.e. similar to that of the extracellular fluid). However, although the free calcium concentration in the external milieu is about $1\,mmol\,l^{-1}$, that in the cytoplasm is four orders of magnitude lower (i.e. around $100\,nmol\,l^{-1}$). Little information is available on the total calcium content of the cytoplasm, whether it is bound to soluble proteins, or to membranes. Although it is likely that large variations exist among different cell types, depending on the amount of Ca^{2+}-binding proteins and on the surface area and phospholipid composition of cellular membranes, it is reasonable to assume that total cytoplasmic Ca^{2+} represents no more than 10–20% of cellular calcium content (Baker, 1972). Thus the vast majority of calcium in the cell must be sequestered within intracellular structures. Various methods have been used to determine the calcium content of different organelles: (i) *in situ* measurements with electron microscopic techniques (Ca^{2+}-oxalate precipitate; Constantin, Franzini-Armstrong & Podolski, 1965) and, more recently, electron probe microanalysis (Bond, Kitizawa, Somlyo & Somlyo, 1984; Kowarsky, Shuman, Somlyo & Somlyo, 1985), (ii) separation by differential centrifugation (Tischler, Hecht & Williamson, 1977), (iii) isotopic methods using ^{45}Ca (Hodgkin & Keynes, 1957), (iv) indirect approaches ultilizing the Ca^{2+}-sensitivity of a number of enzymes (MacCormack & Denton, 1979). All subcellular structures appear to contain calcium to variable extents, but only a few are known to accumulate Ca^{2+} against an electrochemical gradient. Among these only sarcoplasmic reticulum, SR, in striated muscles and a vesicular organelle (identified by some investigators as endoplasmic reticulum, ER in the other cell types), appear to be able to release Ca^{2+} rapidly into the cytoplasm upon cell activation. In this report we discuss mainly the characteristics of the Ca^{2+} store of non-muscle cells which can rapidly release or take up Ca^{2+} into/from the cytoplasm. We will provide experimental evidence which indicates that this Ca^{2+} store is a previously unrecognized and unique organelle, that we have named the calciosome. We suggest that calciosomes represent the functional equivalent of SR in non-muscle cells.

For all experimental details see Volpe *et al.* (1988).

Slowly exchangeable intracellular Ca^{2+} pools

From a physiological point of view the primary difference between different cellular Ca^{2+} storage compartments is their capacity rapidly to exchange trapped Ca^{2+} with the cytoplasm. We will thus assign Ca^{2+} stores into 'rapidly' and 'slowly' exchangeable pools. Mitochondria, secretory granules, lysosomes etc. belong to the second group, whereas SR is the prototype of the first.

In endocrine and exocrine cells, large amounts of Ca^{2+} are contained in secretory granules (Clemente & Meldolesi, 1975). For example, zymogen granules have been found to contain up to $20–30\,nm\,mg^{-1}$ granule protein of Ca^{2+} (Clemente & Meldolesi, 1975), but it is likely that the Ca^{2+} contained in these structures is either bound to proteins or somehow complexed to them, so that its exchange with the cytoplasm is probably extremely slow. No evidence has so far

been provided as to the capacity of secretory granules actively to accumulate Ca^{2+}, and it is not known how such amounts of calcium can be accumulated. One possible explanation is that part of the Ca^{2+} ends up in secretory granules as a consequence of the continuous membrane traffic to and from the plasma membrane. According to this hypothesis, Ca^{2+} present in the extracellular medium remains trapped in the endocytotic vesicles and, through them, is eventually transported to other intracellular vesicles. Thus, unless a Ca^{2+} extrusion mechanism is involved, it would be expected that all structures directly or indirectly linked to the membrane traffic should contain at least the amount of Ca^{2+} typical of the extracellular milieu. Surprisingly, no thorough investigation of this problem appears to have been carried out, although some reports describing Ca^{2+}-ATPases in Golgi vesicles (Virk, Kirk & Shears, 1985) and lysosomes (Klempner, 1985) have recently appeared.

Mitochondria have long been thought to be the major intracellular Ca^{2+} store of non-muscle cells. Undoubtedly, mitochondria *in vitro* have the capacity to accumulate large quantities of Ca^{2+}, but the most recent investigations carried out in intact healthy cells indicate that the Ca^{2+} content of mitochondria *in situ* is much lower than would be expected (Somlyo, Bond & Somlyo, 1985). This is probably due to the low affinity of the mitochondrial Ca^{2+} uptake system, whose K_m for Ca^{2+} is in the $10\,\mu mol\,l^{-1}$ range (Crompton, Spiegel, Salzmann & Carafoli, 1976) (i.e. about 100-fold higher than the basal cytosolic free calcium concentration, $[Ca^{2+}]_i$). However, in activated cells (where $[Ca^{2+}]_i$ may rise well above $1\,\mu mol\,l^{-1}$) it is possible that mitochondria participate in cellular Ca^{2+} buffering. This role of mitochondria might become even more important in pathological conditions. There is another important consideration that makes unlikely a primary role for mitochondria in the changes of $[Ca^{2+}]_i$ that occur during cell activation: the two efflux pathways from mitochondria (the Na^+-dependent and the Na^+-independent Ca^{2+} efflux) operate very slowly (Rizzuto, Bernardi, Favaron & Azzone, 1987) under physiological conditions. This is incompatible with the rapid release of stored Ca^{2+} that follows stimulation of surface receptors.

As mentioned above, little is known about the regulation of Ca^{2+} content in Golgi vesicles, lysosomes, endosomes and related structures, and even less is known about the nucleus. Williams, Fogarty, Tsien & Fay (1985) have recently reported that in smooth muscle cells under resting conditions the Ca^{2+} concentration in the nucleus is two- to three-fold higher than that in the cytoplasm, but this observation has not been confirmed by other investigators.

Rapidly exchangeable intracellular Ca^{2+} stores

By far the best known Ca^{2+} store of this kind is SR, a specialized membrane network characteristic of striated muscle fibres. Morphologically no similar structure can be recognized in smooth muscles. In all other cell types a vesicular organelle capable of rapidly accumulating and releasing Ca^{2+} (i.e. functionally similar to SR) is thought to exist, but its identity is far from clear. Although we

devote most of this contribution to discussing the properties, and the identification, of the Ca^{2+} store of non-muscle cells, we will next briefly summarize a few characteristics of SR relevant for the discussion of its functional counterpart in non-muscle cells.

Striated muscle sarcoplasmic reticulum

The first detailed morphological description (at the electron microscopic level) of skeletal muscle SR was made by Porter & Palade (1957). At that time, the physiological role of this membrane network was unknown and the authors concluded their paper by saying 'This (SR) consists of membrane limited vesicles, tubules, and cisternae associated in a continuous reticular structure which forms lace-like sleeves around the myofibrils... The structure of the system relates it to the endoplasmic reticulum of other cell types'. Probably this sentence generated the idea – still largely accepted – that SR is a specialized morphological modification of ER. The discovery of the unique functional properties of SR (Ca^{2+} uptake and release) made a few years later (Hasselbach & Makinose, 1961) did not cast doubts on this belief. Thus, even in recent reviews (see, for example, Carafoli, 1987), 'sarco(endo)plasmic reticulum' is referred to as if the two structures were basically the same organelle, although none of the enzymes and functional characteristics of rough ER, signal recognition particle (SRP) receptors, ribophorin I and II, or of ER as a whole (cytochrome P450, NADH cytochrome *b*5 reductase, glucose-6-phosphatase) have ever been found in muscle SR (Campbell, 1986).

In recent years, it has become clear that the morphological distinction of skeletal muscle SR into longitudinal SR, LSR, and terminal cisternae, TC, is accompanied by biochemical and functional specificities (Campbell, 1986; Costello *et al.* 1986). LSR is characterized by the predominance of a single polypeptide, the Ca^{2+}-ATPase, responsible for the Ca^{2+}-accumulating capacity of this organelle. The Ca^{2+}-ATPase represents more than 80% of all LSR proteins (Costello *et al.* 1986). *In situ*, Ca^{2+} accumulates in the lumen of LSR and is rapidly transported to TC. The TC face the T tubule, an invagination of the plasma membrane, and their lumen is continuous with that of LSR. The TC can be further separated into two functionally distinct regions, one continuous with LSR, the other facing the T tubule, also known as junctional face membrane, JFM. Compared with LSR, TC are characterized by a lower content of Ca^{2+}-ATPase (about 40% of total protein) (Campbell, 1986) and by the presence of a protein named calsequestrin, CS, which represents up to 20–30% of total TC proteins (Campbell, 1986; Costello *et al.* 1986; Jorgensen, Shen, MacLennan & Tokuyasu, 1982; Jorgensen, Shen, Campbell & MacLennan, 1983). The JFM is practically devoid of Ca^{2+}-ATPase molecules (Costello *et al.* 1986). Following T tubule depolarization, Ca^{2+} is released from TC, at the JFM level, where Ca^{2+} channels are presumably localized (Smith, Coronado & Meissner, 1985).

CS is an intraluminal Ca^{2+}-binding glycoprotein (Campbell, 1986; Costello *et al.*

Table 1. *Comparison between some of the functional characteristics of Ca^{2+} transport of sarcoplasmic reticulum and those of the Ca^{2+} store of non-muscle cell*

	Sarcoplasmic reticulum striated fibres	Ca^{2+} store non-muscle cells
Ca^{2+} uptake		
ATP-dependence	Yes	Yes
Vanadate-sensitivity	Low	Low
Anion-dependence	Yes	Yes
Ca^{2+} release induced by		
Ins1,4,5P_3	Yes	Yes
Caffeine	Yes	(Yes)
Ryanodine	Yes	?
Ca^{2+}	Yes	?
Inhibition of Ca^{2+} release by		
Ruthenium red	Yes	No

1986; Jorgensen *et al.* 1982, 1983; Smith *et al.* 1985; MacLennan & Wong, 1971), endowed with the following unique characteristics. (1) It is a high-capacity Ca^{2+}-binding protein: probably the most efficient Ca^{2+}-buffering polypeptide known to date. It contains more than 40 Ca^{2+}-binding sites mol^{-1} of protein (i.e. over 10 times more than calmodulin). (2) At variance with the properties of calmodulin and related proteins, CS is a moderately low-affinity Ca^{2+}-binding protein, its K_D for Ca^{2+} being in the submillimolar range. (3) Although purified CS is a highly hydrophilic protein, *in situ* it appears to be somehow connected to SR membrane.

CS has three essential functions. (1) It prevents the rise of intraluminal Ca^{2+} concentration above the limit of Ca^{2+} salt precipitation. (2) It reduces the gradient against which the Ca^{2+}-ATPase has to pump. (3) Being strategically located in the TC (from which Ca^{2+} release takes place under physiological conditions), it concentrates Ca^{2+} at the release site.

It is beyond the purpose of this contribution to discuss in detail the functional characteristics of SR, but we have summarized in Table 1 the main similarities between SR and the Ca^{2+} storage compartment of non-muscle cells (for further details see Carafoli, 1987; Campbell, 1986).

The Ins(1,4,5)P_3-sensitive Ca^{2+} store of non-muscle cells

The capacity of a microsomal fraction to accumulate Ca^{2+} in an ATP-dependent manner had been appreciated since the early 1970s (Moore, Chen, Knapp & Landon, 1975). The introduction of selective techniques to permeabilize the plasma membrane (Murphy, Coll, Rich & Williamson, 1980) confirmed the existence *in situ* of a non-mitochondrial, high-affinity Ca^{2+} store. At the time, however, its importance for cell activation escaped the attention of most investigators. In fact at that time the interests of 'calcium fanatics' were focused on

the role of mitochondria. It was thus surprising when R. Y. Tsien, T. J. Rink, P. Arslan and one of us, TP (Pozzan, Arslan, Tsien & Rink, 1982), using the newly developed intracellular Ca^{2+} indicator quin2, demonstrated that, in mouse B lymphocytes, cross-linking of surface immunoglobulins caused an almost immediate release of Ca^{2+} from a 'non-mitochondrial store'. After that initial observation, it took a couple of years to demonstrate that in many other cellular models the source of rapidly mobilizable Ca^{2+} was not the mitochondrion, but some unidentified vesicular structure (for recent reviews see Carafoli, 1987; Berridge, 1987). Late in 1983 Streb *et al.* (Streb, Irvine, Berridge & Shultz, 1983) demonstrated that the Ins(1,4,5)P_3 [generated upon hydrolysis of phosphatidylinositol bisphosphate (PtdInsP_2) by a receptor-regulated phospholipase C] was the second messenger causing Ca^{2+} release from the non-mitochondrial store. A few months later Prentki *et al.* (1984) demonstrated, by cellular fractionation, that the Ins(1,4,5)P_3-sensitive Ca^{2+} store co-purified with markers of ER. In a more detailed study Bayerdorrfer *et al.* (1984) concluded that, at least in the exocrine pancreas, the Ins(1,4,5)P_3-sensitive Ca^{2+} store could be identified as rough ER. These initial observations were soon confirmed by many other laboratories in all systems investigated and to date there is a general consensus as to the role of Ins(1,4,5)P_3 as the chemical mediator linking the stimulation of surface receptors to Ca^{2+} release from non-mitochondrial stores. However, little progress has been made in the identification and purification of this Ca^{2+} store.

A critical re-evaluation of the data (Streb *et al.* 1983; Prentki *et al.* 1984; Bayerdorrfer *et al.* 1984) which led to the initial suggestion that the ER was the Ins(1,4,5)P_3-sensitive Ca^{2+} store shows that the Ins(1,4,5)P_3-sensitivity was simply enriched in a microsomal fraction, which still contained Golgi vesicles, endosomes, plasma membrane (and other organelle)-derived vesicles. Until very recently no further purification was even attempted. Even then (1983), however, there were a number of circumstantial reasons to doubt that the ER, as a whole, could indeed be the Ca^{2+} store. (1) Among different cell types the size of the agonist-mobilizable Ca^{2+} store is rather constant (200–300 μmol l^{-1} of cell water). This is at variance with the amount of readily recognizable ER cysternae which varies enormously among different cell types. In neutrophils, for example, it is difficult to identify classical ER structures even though these cells are among those in which Ca^{2+} mobilization is quantitatively most important (Pozzan & Treves, 1988). However, the exocrine pancreas has the same or smaller amounts of Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Bruzzone, Pozzan & Wollheim, 1986), whereas the cytoplasm is packed with ER cysternae. (2) The SR (which is regarded as the prototype of a Ca^{2+} store) does not contain any of the ER enzyme markers (Campbell, 1986). (3) The specific activity of the Ca^{2+}-ATPase of the microsomal Ca^{2+} store is about two orders of magnitude lower than that of SR, suggesting either a rather poor efficiency of Ca^{2+} pumping or the existence of a subfraction of ER enriched in Ca^{2+}-ATPase.

In a number of informal discussions with colleagues we often ended by hypothesizing that this microsomal Ca^{2+} store had to be a subfraction of the ER.

Recently, more compelling evidence has militated against the idea that the ER, smooth or rough, is the Ins(1,4,5)P_3-sensitive Ca^{2+} store. (1) Binding experiments with ^{3}H-labelled Ins(1,4,5)P_3 revealed a much higher concentration of specific sites in subcellular fractions enriched in plasma membrane, rather than ER, markers (Guillemette *et al.* 1987). (2) Microinjection of Ins(1,4,5)P_3 in intact *Limulus* photoreceptors (giant cells with well-defined topological localization of organelles) loaded with aequorin showed that when the micropipette was positioned in the region where most ER cysternae are concentrated (the so-called A lobe) it caused almost no rise of intracellular calcium concentration $[Ca^{2+}]_i$. On the contrary, when the microinjection was made in the R lobe region (that contains little or no ER), Ins(1,4,5)P_3 caused a much larger rise in $[Ca^{2+}]_i$ (Paine & Fein, 1987). (3) In neutrophils, Krause & Lew (1987) isolated a subcellular fraction enriched in Ca^{2+}-pumping activity which was clearly separated from the bulk of ER. Starting from these observations we hypothesized that the Ca^{2+} store was a unique organelle, which was more similar to SR than to ER. The problem was to identify a marker distinct from the Ca^{2+}-pumping activity and Ins(1,4,5)P_3-sensitivity, characteristic of this organelle. Only such a marker would overcome the unconvincing negative correlations mentioned above and clearly distinguish, and eventually isolate, this organelle from other subcellular structures. This marker was discovered while reconsidering the similarities between SR and the microsomal Ca^{2+} store. As discussed above, an essential feature of SR is the presence in the TC lumen of calsequestrin, the moderate-affinity, high-capacity Ca^{2+}-binding protein. Since the problems of Ca^{2+} salt precipitation, reversal of the ATPase and high release rate (mentioned above for SR), also apply to the microsomal Ca^{2+} store, it seemed reasonable to predict that this organelle would contain a protein with characteristics similar to those of calsequestrin. We thus started to investigate, using a variety of approaches (see below), whether a CS-like protein also existed in non-muscle cells and whether the subcellular distribution of this protein correlated with the Ca^{2+}-pumping activity and Ins(1,4,5)P_3-sensitive Ca^{2+} release.

Table 2 shows that in microsomal fractions of liver, pancreas, PC12 and HL60 cells there is a protein which shares a number of properties with true skeletal muscle CS, including similar M_r, metacromatic staining with carbocyamine dye, pH-sensitivity of the electrophoretic mobility, alkali- and detergent-extractability, Ca^{2+}-binding capacity and, most important, immunological cross-reactivity.

A CS-like protein has been found in microsomal fractions of all other cells and tissues so far investigated (i.e. neutrophils, platelets, spermatozoa and brain). The first conclusion of this study is that a CS-like protein, previously considered to be specific for striated muscle, is expressed in many, if not all, mammalian cells. Table 3 shows the distribution, in subcellular fractions of HL60 cells, of the CS-like protein compared with various markers of intracellular organelles, including Ca^{2+} uptake and Ins(1,4,5)P_3-sensitive Ca^{2+} release. This cell type was chosen because in one of our laboratories a thorough subfractionation had already been carried out which demonstrated a lack of correlation between the properties of the

Table 2. *Properties of calsequestrin-like proteins in different cell types: comparison with true skeletal muscle calsequestrin*

	Cell type				
Characteristic	HL60	PC12	Liver	Pancreas	Muscle
Metachromatic staining	+	+	+	−	+
Apparent $M_r \times 10^{-3}$ in SDS–PAGE at pH 8	61	63	63	60	64
pH-sensitivity of electrophoretic mobility	+	ND	+	+	+
Ca^{2+}-binding	+	+	+	+	+
Alkali solubilization	+	+	+	+	+
Immunological cross-reactivity	+	+	+	+	+

For experimental details see Volpe *et al.* (1988).
ND, not determined.

Table 3. *Distribution of specific markers in subcellular fractions of HL60 cells*

Specific marker	Fraction I	Fraction II	Fraction III
Alkaline phosphatase (plasma membrane)	35 %	40 %	25 %
β-Glucuronidase (granules)	25 %	55 %	25 %
Sulphatase C (ER)	30 %	55 %	25 %
Galactosil transferase (Golgi)	10 %	50 %	40 %
[^{125}I]Insulin (endosomes)	15 %	35 %	50 %
Ins1,4,5P_3 response	3 %	15 %	82 %
Ca^{2+} uptake	10 %	20 %	70 %
CS-like protein	−	±	+++

For experimental details see Volpe *et al.* (1988).
The values are expressed as a percentage of the total activity recovered in the gradient; −, undetectable; ±, barely detectable; +++, strongly positive.
ER, endoplasmic reticulum; CS, calsequestrin.

Ca^{2+} store [Ca^{2+}-pumping activity and Ins(1,4,5)P_3-sensitivity] and markers of other organelles.

Recently D. Pittet (Geneva) (unpublished observations) showed that fraction III isolated from HL60 cells is also highly enriched with Ins(1,4,5)P_3-binding capacity (about three-fold compared with the other fractions). In conclusion, not only is a CS-like protein expressed in non-muscle tissues, but its distribution correlates with the markers of the microsomal Ca^{2+} store. Furthermore Damiani *et al.* (1988) have recently demonstrated that liver microsomal fractions are enriched in a CS-like protein and also contain a protein (about 100 000 M_r) which is cross-reactive with SR Ca^{2+}-ATPase. The availability of anti-CS and anti-Ca^{2+}-ATPase antibodies, which cross-react with related proteins of non-muscle tissues,

allowed the subcellular localization of these two markers *in situ*. Immunofluorescence with the anti-ATPase antibody was not entirely satisfactory because of a high nonspecific signal. In contrast with the anti-CS antibody it was possible to demonstrate a specific cytoplasmic staining (the nucleus was negative). The fluorescence at the cell periphery appeared to be due to a large number of discrete fluorescent dots. A much better spatial resolution could be obtained by electron microscopy of ultrathin frozen sections. In this study the antibodies were either directly coupled with gold particles or gold-labelled protein A was added after the antibody. As shown in Fig. 1, anti-CS antibodies exclusively stained small vesicular structures, often localized near the ER cysternae (liver) or the plasma membrane (PC12 cells) (Volpe *et al.* 1988). No other readily recognizable subcellular organelle appeared to be stained by the anti-CS antibody. We calculated that there are about 5000 CS-positive vesicles per PC12 cell (where a thorough morphometric study has been carried out), enough to justify the diffuse staining observed by immunofluorescence. The diameter of these vesicles varies between 50 and 250 nm; their size and distribution is similar in all tissues investigated. Continuity with ER cysternae, or with other known structures, was never observed, although it cannot be excluded. Double staining with anti-CS antibodies and with antibodies against cytochrome P450 (a specific marker of ER), never showed co-localization of these two antigens (S. Hashimoto, B. Bruno, D. P. Lew, T. Pozzan, P. Volpe & J. Meldolesi, in preparation). Similarly, an antibody directed against a protein contained in secretory granules (secretogranin I) did not stain CS-positive vesicles (Volpe *et al.* 1988). However, co-localization of anti-ATPase and anti-CS antibodies was observed in most cases. Even more striking is the observation that the anti-Ca^{2+}-ATPase antibody never stained the ER, suggesting either that this organelle is incapable of an ATP-driven Ca^{2+} accumulation or that (if it possesses a Ca^{2+}-ATPase) it is either very rare or immunologically distinct from that present in the CS-positive structures. As expected, when the vesicles were positive for both antigens, the anti-ATPase antibody decorated the periphery and the anti-CS one the lumen of the vesicle (Volpe *et al.* 1988; S. Hashimoto, B. Bruno, D. P. Lew, T. Pozzan, P. Volpe & J. Meldolesi, in preparation).

Taken together, all the data presented above suggest the existence of a unique subcellular structure, endowed with Ca^{2+}-ATPase and a CS-like protein, which is very similar to the terminal cysternae of striated muscle SR. We have not yet obtained formal proof that this organelle is the Ins(1,4,5)P_3-sensitive Ca^{2+} store, but the correlation between the distribution of the CS-like protein and that of the Ca^{2+} store in HL60 cells reported above strongly supports this possibility. We have proposed the name calciosome for this newly recognized organelle (Volpe *et al.* 1988).

There are two main arguments that can be raised against our proposal. (1) How can we exclude the possibility that calciosomes do not represent a subspecialization of ER? (2) How do we reconcile our findings with the data obtained in Somlyo's laboratory which show that ER contains Ca^{2+} and, even more import-

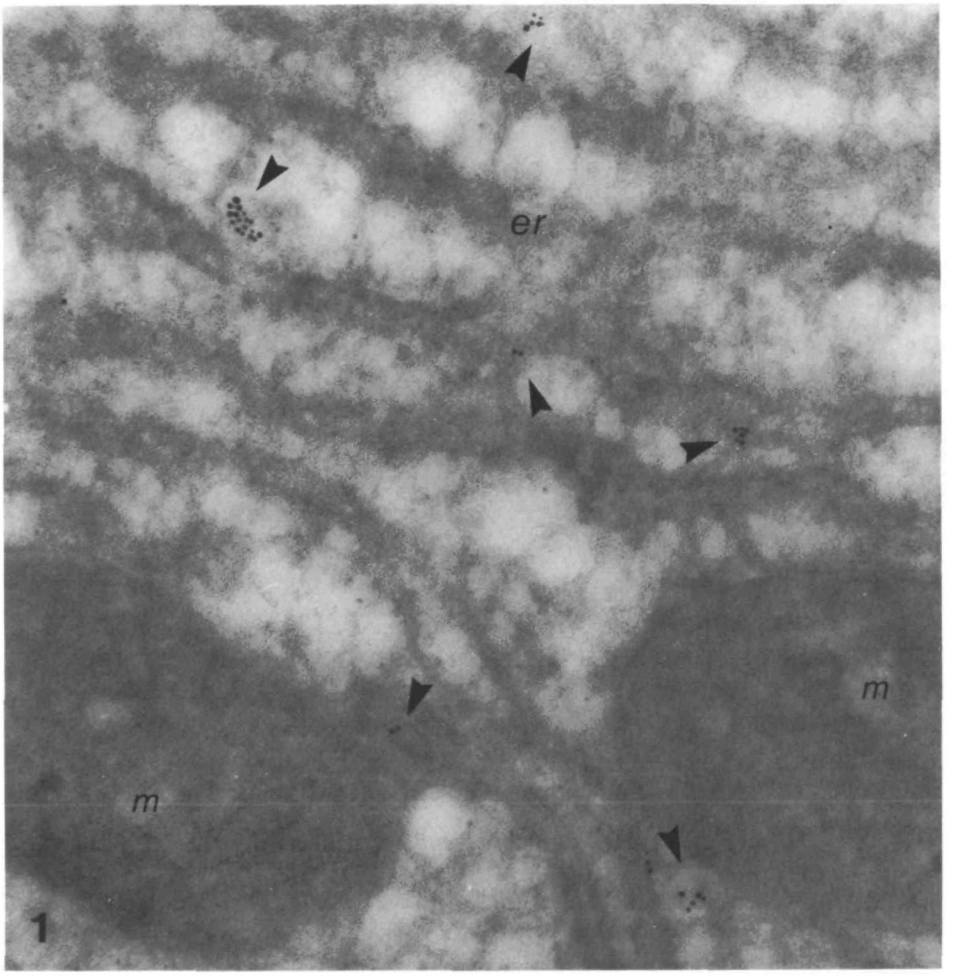

Fig. 1. Immunogold labelling of an ultrathin frozen section of a rat hepatocyte with anti-CS antibodies. Ultrathin cryosections of rat liver tissue were immunodecorated with 6 nm diameter gold particles directly coupled to affinity-purified anti-calsequestrin antibodies raised in chicken by injection of the rabbit muscle antigen. Notice that the immunogold particles label a population of small vacuoles (arrowheads) intermingled with rough endoplasmic reticulum (*er*) cisternae. *m*, mitochondria; ×84 000. For further details see Volpe *et al.* (1988).

ant, that addition of vasopressin to isolated hepatocytes causes a decrease in ER Ca^{2+} content, as measured *in situ* with electron probe microanalysis (Bond, Vadasz, Somlyo & Somlyo, 1987)?

It is impossible at present to give a definitive answer to the first question, since only a three-dimensional reconstruction at the electron microscope level can exclude or demonstrate the physical continuity between ER and calciosomes. This approach is precluded using the present technique of ultrathin frozen sections which cannot be cut in series. However, any immunocytological technique that

implies plastic embedding of the cells has been found to cause the loss of the CS and ATPase immunoreactivity, the only way to date to distinguish calciosomes from other vesicles within the cells. More important, however, is the demonstration that calciosomes are immunologically distinct from ER: in fact anti-CS and anti-Ca^{2+}-ATPase antibodies do not recognize such proteins in ER cysternae and ER markers are not expressed in calciosomes. ER and calciosomes are therefore molecularly and functionally distinct. If a physical continuity between the two were to exist, the difference between the concepts of sub-specialization or diverse organelles would be ultimately only semantic.

The answer to the second question is more straightforward. We have no direct evidence for or against the presence of Ca^{2+} in ER. Our data are thus not in conflict with those of Somlyo's group. In this respect it should be noted that the Ca^{2+} content of ER cysternae in liver (as revealed by electron probe microanalysis) represents less than 25 % of total cellular Ca^{2+}. This allows ample room for any other Ca^{2+} store in the cell. Furthermore, calciosomes in liver are often localized near the ER cysternae and, given the size of the electron probe used in Somlyo's studies, they would be counted as part of the ER. This latter argument also applies to the demonstration that vasopressin mobilizes Ca^{2+} from ER.

The purification and cloning of the CS-like protein and of the Ca^{2+}-ATPase, as well as the purification and study of the biogenesis of calciosomes, are the goals presently pursued in our laboratories.

We are indebted with Mr G. Ronconi, A. Tobaldin and Ms A. Monod for excellent technical assistance, to Mrs F. Mazzari and F. Eccher for secretarial assistance and Dr W. Cantarello (FIDIA Research Laboratories) for helping to assemble bibliographic material. This work was supported in part by grants from the Italian CNR 'Progetto Finalizzato Oncologia' (grant no. 87.01410.44) and from the Italian Ministry of Public Education to TP and JM. MB was supported by a grant from ASSNE (Associazione per lo Sviluppo delle Scienze Neurologiche) Padova, Italy.

References

BAKER, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Progr. Biophys. molec. Biol.* **24**, 177–223.

BAYERDORRFER, E., STREB, H., HAASE, W., IRVINE, R. F. & SHULTZ, I. (1984). Characterization of calcium uptake into rough endoplasmic reticulum of rat pancreas. *J. Membr. Biol.* **81**, 69–82.

BERRIDGE, M. J. (1987). Inositol trisphosphate and diacylglycerol: two interacting second messengers. *A. Rev. Biochem.* **56**, 159–193.

BOND, M., VADASZ, G., SOMLYO, A. V. & SOMLYO, A. P. (1987). Subcellular calcium and magnesium mobilization in rat liver stimulated *in vivo* with vasopressin and glucagon. *J. biol. Chem.* **262**, 15 630–15 636.

BOND, M., KITIZAWA, T., SOMLYO, A. P. & SOMLYO, A. V. (1984). Release and recycling of calcium by the sarcoplasmic reticulum in guinea-pig portal vein smooth muscle. *J. Physiol., Lond.* **355**, 677–695.

RUZZONE, R., POZZAN, T. & WOLLHEIM, C. B. (1986). Caerulein and carbamoylcholine stimulate pancreatic amylase release at resting cytosolic free Ca^{2+}. *Biochem. J.* **235**, 139–143.

CAMPBELL, K. P. (1986). Protein components and their roles in sarcoplasmic reticulum function. In *Sarcoplasmic Reticulum in Muscle Physiology* (ed. M. L. Entman & W. B. Van Winkle), pp. 65–92. Boca Raton, FL: CRC Press.

CARAFOLI, E. (1987). Intracellular calcium homeostasis. *A. Rev. Biochem.* **56**, 399–433.

CLEMENTE, F. & MELDOLESI, J. (1975). Calcium and pancreatic secretion. I. Subcellular distribution of calcium and magnesium in the exocrine pancreas of the guinea pig. *J. Cell Biol.* **65**, 88–102.

CONSTANTIN, L. L., FRANZINI-ARMSTRONG, C. & PODOLSKI, R. J. (1965). Localization of calcium-accumulating structures in striated muscle fibers. *Science* **147**, 158–160.

COSTELLO, B., CHADWICK, C., SAITO, A., MAURER, A. & FLEISHER, S. (1986). Characterization of the junctional face membrane from terminal cisternae of sarcoplasmic reticulum. *J. Cell Biol.* **103**, 741–753.

CROMPTON, M., SPIEGEL, E., SALZMANN, M. & CARAFOLI, E. (1976). A kinetic study of the energy-linked influx of Ca^{2+} into heart mitochondria. *Eur. J. Biochem.* **69**, 429–434.

DAMIANI, E., SPAMER, C., HEILMANN, C., SALVATORI, S. & MARGRETH, A. (1988). Endoplasmic reticulum of rat liver contains two proteins closely related to skeletal sarcoplasmic reticulum Ca-ATPase and calsequestrin. *J. biol. Chem.* **263**, 340–343.

GUILLEMETTE, G., BALLA, T., BANKAL, A. J., SPAT, A. & CATT, K. J. (1987). Intracellular receptors for inositol 1,4,5-trisphosphate in angiotensin II target tissues. *J. biol. Chem.* **262**, 1010–1015.

HASSELBACH, W. & MAKINOSE, M. (1961). Die Calcium Pumpe der Ershlapfungs Grana des Muskels und ihre Abhangigheit von der ATP Splatung. *Biochem. Z.* **333**, 518–528.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol., Lond.* **138**, 253–281.

JORGENSEN, A. O., SHEN, A. C. Y., CAMPBELL, K. P. & MACLENNAN, D. H. (1983). Ultrastructural localization of calsequestrin in rat skeletal muscle by immunoferritin labeling of ultrathin frozen sections. *J. Cell Biol.* **97**, 1573–1581.

JORGENSEN, A. O., SHEN, A. C. Y., MACLENNAN, D. H. & TOKUYASU, K. T. (1982). Ultrastructural localization of the Ca^{2+} + Mg^{2+}-dependent ATPase of sarcoplasmic reticulum in rat skeletal muscle by immunoferritin labeling of ultrathin frozen sections. *J. Cell Biol.* **92**, 409–416.

KLEMPNER, M. (1985). An adenosine triphosphate-dependent calcium uptake pump in human neutrophil lysosomes. *J. clin. Inv.* **76**, 303–310.

KOWARSKI, D., SHUMAN, H., SOMLYO, A. P. & SOMLYO, A. V. (1985). Calcium release by noradrenaline from central sarcoplasmic reticulum in rabbit main pulmonary artery smooth muscle. *J. Physiol., Lond.* **366**, 153–175.

KRAUSE, K. H. & LEW, D. P. (1987). Subcellular distribution of Ca^{2+} pumping sites in human neutrophils. *J. clin. Inv.* **80**, 107–116.

MACCORMACK, J. G. & DENTON, R. M. (1979). The effects of calcium ions and adenine nucleotides on the activity of pig heart 2-oxoglutarate dehydrogenase complex. *Biochem. J.* **180**, 533–544.

MACLENNAN, D. H. & WONG, P. T. S. (1971). Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc. natn. Acad. Sci. U.S.A.* **68**, 1231–1235.

MOORE, L., CHEN, T., KNAPP, H. R., JR & LANDON, E. J. (1975). Energy-dependent calcium sequestration activity in rat liver microsomes. *J. biol. Chem.* **250**, 4562–4568.

MURPHY, E., COLL, K., RICH, T. L. & WILLIAMSON, J. R. (1980). Hormonal effects on calcium homeostasis in isolated hepatocytes. *J. biol. Chem.* **255**, 6600–6608.

PAINE, R. & FEIN, A. (1987). Inositol 1,4,5-trisphosphate releases calcium from specialized sites within *Limulus* photoreceptors. *J. Cell Biol.* **104**, 933–940.

PORTER, K. R. & PALADE, G. E. (1957). Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. *J. biophys. biochem. Cytol.* **3**, 269–298.

POZZAN, T., ARSLAN, P., TSIEN, R. Y. & RINK, T. J. (1982). Anti-immunoglobulin, cytoplasmic free calcium, and capping in B lymphocytes. *J. Cell Biol.* **94**, 335–345.

POZZAN, T. & TREVES, S. (1987). Stimulus–activation coupling in neutrophils: an overview. In *Human Inflammatory Diseases* (ed. G. Marone, L. M. Lichtenstein, M. Condorelli & A. S. Fauci), pp. 263–273. Toronto, Philadelphia: B. C. Decker Inc.

PRENTKI, M., BIDEN, T. J., JANIJC, D., IRVINE, R. F., BERRIDGE, M. J. & WOLLHEIM, C. B.

(1984). Rapid mobilization of Ca^{2+} from rat insulinoma microsomes by inositol-1,4,5-trisphosphate. *Nature, Lond.* **309**, 562–564.

RIZZUTO, R., BERNARDI, P., FAVARON, M. & AZZONE, G. F. (1987). Pathways for Ca^{2+} efflux in heart and liver mitochondria. *Biochem. J.* **246**, 271–277.

SMITH, J. F., CORONADO, R. & MEISSNER, G. (1985). Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature, Lond.* **316**, 446–449.

SOMLYO, A. P., BOND, M. & SOMLYO, A. V. (1985). Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly *in vivo*. *Nature, Lond.* **314**, 622–624.

STREB, H., IRVINE, R. F., BERRIDGE, M. J. & SHULTZ, I. (1983). Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature, Lond.* **306**, 67–69.

TISCHLER, M. E., HECHT, P. & WILLIAMSON, J. R. (1977). Determination of mitochondrial/cytosolic metabolite gradients in isolated rat liver cells by cell disruption. *Archs Biochem. Biophys.* **181**, 278–292.

VIRK, S. S., KIRK, C. J. & SHEARS, S. B. (1985). Ca^{2+} transport and Ca^{2+}-dependent ATP hydrolysis by Golgi vesicles from lactating rat mammary glands. *Biochem. J.* **226**, 741–748.

VOLPE, P., KRAUSE, K. H., HASHIMOTO, S., ZORZATO, F., POZZAN, T., MELDOLESI, J. & LEW, D. P. (1988). Calciosome, a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of non muscle cells? *Proc. natn. Acad. Sci. U.S.A.* **85**, 1091–1095.

WILLIAMS, D. A., FOGARTY, K. E., TSIEN, R. Y. & FAY, F. S. (1985). Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. *Nature, Lond.* **318**, 558–561.