Calreticulin, and Not Calsequestrin, Is the Major Calcium Binding Protein of Smooth Muscle Sarcoplasmic Reticulum and Liver Endoplasmic Reticulum*

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The distribution of calsequestrin and calreticulin in smooth muscle and non-muscle tissues was investigated. Immunobots of endoplasmic reticulum proteins probed with anti-calreticulin and anti-calsequestrin antibodies revealed that only calreticulin is present in the rat liver endoplasmic reticulum. Membrane fractions isolated from uterine smooth muscle, which are enriched in sarcoplasmic reticulum, contain a protein band which is immunoreactive with anti-calreticulin but not with anti-calsequestrin antibodies. The presence of calreticulin in these membrane fractions was further confirmed by \(^{45}\text{Ca}^2+\) overlay and “Stains-All” techniques. Calreticulin was also localized to smooth muscle sarcoplasmic reticulum by the indirect immunofluorescence staining of smooth muscle cells with anti-calreticulin antibodies. Furthermore, both liver and uterine smooth muscle were found to contain high levels of mRNA encoding calreticulin, whereas no mRNA encoding calsequestrin was detected.

We have employed an ammonium sulfate precipitation followed by Mono Q fast protein liquid chromatography, as a method by which calreticulin and calsequestrin can be isolated from whole tissue homogenates, and by which they can be clearly resolved from one another, even where present in the same tissue. Calreticulin was isolated from rabbit and bovine liver, rabbit brain, rabbit and porcine uterus, and bovine pancreas and was identified by its amino-terminal amino acid sequence. Calsequestrin cannot be detected in preparations from whole liver tissue, and only very small amounts of calreticulin are detectable in ammonium sulfate extracts of uterine smooth muscle.

We conclude that calreticulin, and not calsequestrin, is a major \(\text{Ca}^2+\) binding protein in liver endoplasmic reticulum and in uterine smooth muscle sarcoplasmic reticulum. Calreticulin and calsequestrin may perform parallel functions in the lumen of the sarcoplasmic and endoplasmic reticulum.

\(\text{Ca}^2+\) is the primary messenger for contractile activation in both striated and smooth muscle and is also known to be centrally involved in the regulation of a variety of cellular functions in non-muscle tissues. In striated muscle intracellular concentrations of \(\text{Ca}^2+\) are regulated by the proteins of the sarcoplasmic reticulum (SR),¹ the nature and mechanism of action of which have been extensively studied (Michalak, 1985; Inesi, 1985). In smooth muscle cells the SR has also been clearly identified as the intracellular organelle storing \(\text{Ca}^2+\) (Somlyo and Himpens, 1989). In non-muscle cells the ER is responsible for the regulation of cytosolic \(\text{Ca}^2+\) concentrations (Walz and Baumann, 1989), and it is becoming apparent that the movement of \(\text{Ca}^2+\) ions to and from the ER is regulated by a series of proteins which are analogous to the proteins of striated and smooth muscle SR (Walz and Baumann, 1989; Macer and Koch, 1988). For example, recent evidence shows that the \(\text{Ca}^2+\) pumps (\(\text{Ca}^2+,\text{Mg}^{2+}\)-dependent ATPase activity) expressed in smooth muscle SR (Wuytack et al., 1989; Eggermont et al., 1989) and in liver ER (Burk et al., 1989; Heilmann et al., 1989) are similar to those characterized in the SR membranes. The mechanisms of \(\text{Ca}^2+\) release from ER membranes differ from those in striated muscle SR. However, both systems involve high molecular weight, integral membrane proteins, the InP receptor and the ryanodine receptor, respectively, which function as \(\text{Ca}^2+\) channels. The primary structures of the ryanodine receptor (Takeshima et al., 1989; Zorzato et al., 1990) and the InP receptor (Furuchi et al., 1989; Mignery et al., 1990) have recently been reported, revealing that both receptors have significant structural similarities. Interestingly, functional evidence suggests that in smooth muscle SR both types of \(\text{Ca}^2+\) release channel are expressed (van Bremen and Saida, 1989; Somlyo and Himpens, 1988).

\(\text{Ca}^2+\) storage within the SR of striated muscle is enhanced by calsequestrin, a low affinity high capacity \(\text{Ca}^2+\) binding protein which is localized in the lumen of the junctional SR (MacLennan et al., 1983). The nature of \(\text{Ca}^2+\) binding proteins

¹The abbreviations used are: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; EGTA, [ethylenebis(oxyethylenenitritro)]tetraacetic acid; PSS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; InP, inositol 1,4,5-trisphosphate.
in the ER/SR of smooth muscle and non-muscle tissues is currently unclear. In several studies Ca\(^{2+}\) binding proteins in ER membranes from non-muscle tissues have been characterized (Macer and Koch, 1988; Booth and Koch, 1989), but there has been some confusion regarding the identity of these proteins. Skeletal muscle contains, in addition to calsequestrin, a high affinity Ca\(^{2+}\) binding protein (Ostwald and MacLennan, 1974) which is also localized within the lumen of the SR (Michalak et al., 1980). We have recently identified this protein in a variety of non-muscle tissues (Fliegel et al., 1989b). Amino acid sequence of the high affinity Ca\(^{2+}\) binding protein obtained from cDNA and from protein sequence analysis carried out in our laboratory revealed that the same protein has been independently identified in non-muscle tissues by others, but was referred to as calregulin (Waisman et al., 1985), as CRP55 (Macer and Koch, 1988), and as CaBP3 (Van et al., 1989). By consensus, it has now been termed calreticulin (calcium binding protein of the sarcoplasmic/endoplasmic reticulum) in order to eliminate any confusion regarding its identity (Fliegel et al., 1989a).

Calreticulin, in addition to a number of low affinity Ca\(^{2+}\) binding sites (\(K_d \approx 250 \mu\text{M}, B_{\text{max}} = 25 \text{ mol of Ca}^{2+}/\text{mol of protein}\) (MacLennan et al., 1972; Ostwald and MacLennan, 1974) also possesses a single high affinity site (\(K_d = 1 \mu\text{M}, B_{\text{max}} = 1 \text{ mol of Ca}^{2+}/\text{mol of protein}\) (MacLennan et al., 1972; Ostwald and MacLennan, 1974; Waisman et al., 1985). We have recently cloned the skeletal muscle form of calreticulin and have shown that its carboxyl-terminal sequence ends with the KDEL tetrapeptide (Fliegel et al., 1989a), which is an ER retention signal (Pelham, 1988; Andres et al., 1990).

The amino acid sequence of calreticulin indicates that it is highly homologous (identical) with several other proteins. For example, it appears to be identical to the Ro/SS-A autoantigen (McCauliffe et al., 1985), as CRP55 (Macer and Koch, 1988), and as CaBP3 (Van et al., 1989). The autoantibody response to Ro/SS-autoantigens is highly characteristic of Sjogren's syndrome and of some subsets of systemic lupus erythematosus, such as subacute cutaneous lupus erythematosus and neonatal lupus erythematosus. Calreticulin is also highly homologous with the Aplysia californica snail neuronal protein p407 (McCauliffe et al., 1990b; Kennedy et al., 1988) and with the RAL-1 protein from a filarial nematode, Onchocerca volvulus (Unnasch et al., 1988; McCauliffe et al., 1990b). These and other homologies (Fliegel et al., 1989c) suggest that calreticulin is a highly conserved protein, present in a variety of tissues in highly diverse species, where it may perform very basic cellular functions involving Ca\(^{2+}\) binding.

We have suggested that calreticulin may function as a non-muscle analogue of calsequestrin (Fliegel et al., 1989a, 1989b). However, a protein bearing a strong structural and functional relationship to calsequestrin has also been reported to be expressed in liver, pancreas, human neutrophils, and HL-60 cells and has been suggested to be the non-muscle analogue of calsequestrin (Volpe et al., 1988; Hashimoto et al., 1988; Damiani et al., 1988, 1989; Krause et al., 1989b). These latter observations also led to the concept of calsequestrin-rich InsP\(_3\)-sensitive organelles named "calciosomes" (Volpe et al., 1988). In addition, a protein with strong immunological and biochemical resemblance to calsequestrin has recently been reported to be expressed in smooth muscle SR (Wuytack et al., 1987), in plant cells (Krause et al., 1989a), and in the sea urchin (Oberdorf et al., 1988). The precise distribution of calsequestrin and calreticulin in smooth muscle and in non-muscle tissues remains to be clearly established.

In the present study we have investigated the distribution of calsequestrin and calreticulin in smooth muscle and non-muscle tissues using immunological and molecular biological techniques. We have shown that calreticulin is a major Ca\(^{2+}\) binding protein in SR fractions from smooth muscle and in liver ER membranes. Calsequestrin, in contrast, is confined to skeletal and cardiac muscle, and only very small amounts of this protein are detectable in uterine smooth muscle. In this study, we have also developed a method by which calsequestrin and calreticulin can be isolated from whole tissue homogenates and by which they can be clearly resolved from one another, even where present in the same tissue.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Identification of Calreticulin and Calsequestrin in Liver ER and Smooth Muscle SR Fractions—In order to determine whether it is calreticulin and/or calsequestrin which is present in liver ER and smooth muscle SR, first we have isolated ER/ SR vesicles from these tissues. Fig. 1A shows Coomassie Blue staining of proteins, after separation by SDS-PAGE, from isolated rat liver ER (lanes 1 and 2) and from SR-enriched fractions from porcine uterus (lanes 6 and 7). The protein composition of these membranes is compared with purified calreticulin (lane 3) and with purified skeletal muscle and cardiac calsequestrins (lanes 4 and 5, respectively). Immunoblot analysis with antibodies raised against calsequestrin from both cardiac muscle (Fig. 1B) and skeletal muscle (Fig. 1C) showed that neither the rat liver ER nor the uterine smooth muscle SR-enriched membrane fractions contains any immunoactive calsequestrin. These experiments also show that our anti-cardiac and anti-skeletal muscle calsequestrin antibodies cross-react with the skeletal and cardiac muscle forms of calsequestrin, respectively. However, neither antibody cross-reacts with purified calreticulin (Fig. 1, lane 4). The goat anti-calreticulin antibody used in this study is not cross-reactive with skeletal or cardiac muscle calsequestrin (data not shown).

Fig. 1 shows that calreticulin migrates in Laemmli SDS-PAGE as a 60-kDa protein. This is in agreement with the observations of Waisman et al. (1985) and of McCauliffe et al. (1990a) but disagrees with earlier reports that calreticulin is a 55-kDa protein (Ostwald and MacLennan, 1974; Michalak et al., 1980; Michalak and MacLennan, 1980; McMeekin and Koch, 1988). These discrepancies may be the result of the substantial alterations in SDS-PAGE techniques which have occurred since the first estimation of the mobility of the protein (Ostwald and MacLennan, 1974). The actual M, of mature calreticulin, as determined by cDNA cloning, is estimated to be approximately 46,500 (Fliegel et al., 1989a; Smith and Koch, 1989; McCauliffe et al., 1990a).

Fig. 2 shows that both fractions ER I and ER II isolated from rat liver contain calreticulin. These membrane fractions were highly enriched in ER markers (Table 1). The fraction designated ER I corresponds to rough ER, whereas that designated ER II probably contains true smooth ER and some rough ER that lost its ribosomes during membrane purification (Croze and Morre, 1984). Importantly, neither mitochondrial nor plasma membrane fractions isolated from rat liver by this purification procedure contained any immunoactive calsequestrin (data not shown). Fig. 3 shows that calreticulin is also present in SR-enriched membrane fractions isolated from porcine uterus. After sucrose gradient fractionation of uterine
with anti-calsequestrin antibody. Rat liver ER vesicles, smooth muscle ER vesicles, smooth muscle; C, immunostaining with anti-skeletal muscle calsequestrin; C, immunostaining with anti-skeletal muscle calsequestrin: 3,2, 4, 50, 75, 130 kDa. Additional Ca\textsuperscript{2+} binding protein bands were 100–120, 78, and 55 kDa (Fig. 4A, lanes 1 and 2). Importantly, there was no evidence for a Ca\textsuperscript{2+} binding protein migrating with a mobility equivalent to that of either cardiac or skeletal muscle calsequestrin (Fig. 4A, compare lanes 1 and 2 with lanes 3–5). In SR-enriched fractions (SR I and SR II) isolated from uterine smooth muscle, calreticulin was also a major Ca\textsuperscript{2+} binding protein (Fig. 4B, lanes 1 and 2), and again there was no evidence for a Ca\textsuperscript{2+} binding protein migrating in SDS-PAGE with a mobility similar to that of either cardiac or skeletal muscle calsequestrin (Fig. 4B).

We also employed a Ca\textsuperscript{2+} overlay technique to confirm our immunological data and further demonstrated that calreticulin, and not calsequestrin, is the major Ca\textsuperscript{2+} binding protein in both the non-muscle ER and smooth muscle SR membrane fractions described. Four major Ca\textsuperscript{2+} binding proteins were observed in the rat liver ER (Fig. 4A) and a major one (indicated by an arrow) was found to correspond to the SDS-PAGE mobility of purified calreticulin (approximately 60-kDa). Additional Ca\textsuperscript{2+} binding protein bands were 100–120, 78, and 55 kDa (Fig. 4A, lanes 1 and 2). Importantly, there was no evidence for a Ca\textsuperscript{2+} binding protein migrating with a mobility equivalent to that of either cardiac or skeletal muscle calsequestrin (Fig. 4A, compare lanes 1 and 2 with lanes 3–5). In SR-enriched fractions (SR I and SR II) isolated from uterine smooth muscle, calreticulin was also a major Ca\textsuperscript{2+} binding protein (Fig. 4B, lanes 1 and 2), and again there was no evidence for a Ca\textsuperscript{2+} binding protein migrating in SDS-PAGE with a mobility similar to that of either cardiac or skeletal muscle calsequestrin (Fig. 4B).

3. Immunological identification of calreticulin in SR-enriched membrane fractions isolated from uterine smooth muscle. SR I and SR II membrane fractions were isolated from uterine smooth muscle by sucrose gradient centrifugation as described under “Experimental Procedures.” Membrane proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and incubated with goat anti-calreticulin as described under “Experimental Procedures.” Lane 1, SR I; lane 2, SR II; lane 3, calreticulin. Protein amounts were: lanes 1 and 2, 35 µg; lane 3, 2 µg. The arrow indicates calreticulin (CRT).

Smooth muscle microsomes, neither fraction 1 nor the pellet contained any immunoreactive calreticulin (data not shown).

The presence of calreticulin in these membrane preparations was also confirmed by "Stains-All." Fig. 5 shows Stains-All staining of purified membrane fractions and purified proteins. Acidic proteins which bind Ca\textsuperscript{2+}, such as calsequestrin and calreticulin, will stain blue with Stains-All, whereas other proteins stain pink (Campbell et al., 1983; Fliegel et al., 1989b). This method has been used as a tool for the identification of cardiac and skeletal muscle calsequestrins (Campbell et al., 1983; Cala and Jones, 1983). In both the liver ER and uterine smooth muscle SR-enriched membrane fractions, the major blue-stained protein band detected in the 55- to 60-kDa region corresponds to the mobility of purified calreticulin and not to that of skeletal or cardiac muscle calsequestrin. Additional higher molecular weight proteins were found to stain blue with the Stains-All, particularly in the case of the rat liver ER membrane fractions where a number of proteins, M\textsubscript{r} > 200,000, were observed (Fig. 5). Also, in addition to calreticulin, in both membrane preparations a protein band of approximately 120 kDa stained blue with the dye.

**Isolation of Calreticulin from Bovine Liver and Uterine Smooth Muscle—Calsequestrin can be purified from whole tissue homogenates without the need to prepare membrane fractions (Slupsky et al., 1987; Krause et al., 1989a; Krause et al., 1990). We have adopted this procedure, which takes advantage of the high solubility of calsequestrin and calreticulin**.
in solutions of ammonium sulfate (Michalak and MacLennan, 1980), for the isolation of calreticulin. A crude tissue homogenate is extracted with 65% saturated ammonium sulfate which precipitates most proteins. Calsequestrin (Krause et al., 1989a; Slupsky et al., 1987) and calreticulin can then be precipitated from this solution by lowering the pH and simultaneously increasing the concentration of ammonium sulfate. The ammonium sulfate precipitate, which contains both calreticulin and calsequestrin, which are present in the same tissue, is further purified by DEAE-Sepharose chromatography or by FPLC chromatography on a Mono Q column. Fig. 6 illustrates the resolution of calreticulin and calsequestrin obtained on a Mono Q 5/5 FPLC column. Under the conditions of the chromatography used in this study we have found that calreticulin and cardiac calsequestrin elute reproducibly at approximately 0.3 and 0.5 M NaCl, respectively. Mono Q FPLC chromatography is, therefore, an excellent method for the separation of calreticulin and calsequestrin from a variety of tissues.

Fig. 6, A and B, show the separation on a Mono Q FPLC column of proteins in ammonium sulfate pellets prepared from bovine liver and porcine uterus. Analysis of the proteins in these fractions was carried out, after SDS-PAGE, by Coomassie Blue staining and immunoblotting (Figs. 7 and 8). The separation of proteins from a bovine liver ammonium sulfate precipitate showed a protein peak which corresponded to calreticulin (Fig. 7A). However, neither significant protein nor immunologically detectable calsequestrin was present in the "calsequestrin" region (results not shown). The calreticulin, however, was readily identified using specific antibodies against skeletal muscle calreticulin (Fig. 7B). This confirms our earlier observations that calreticulin is present in liver ER and demonstrates that calsequestrin cannot be detected in the liver, in either ER membrane vesicles or in whole tissue preparations.

The separation of proteins in an ammonium sulfate precipitate from porcine uterus smooth muscle is shown in Fig. 6A. A significant peak was observed corresponding to calreticulin. Analysis of these fractions by Coomassie Blue staining and immunoblotting is shown in Fig. 8 (lanes 1-4). Although there was no apparent significant peak corresponding to calsequestrin, we were able to detect very small amounts of a protein, by Coomassie Blue staining, in one fraction (Fig. 8A). This protein reacted with our anti-cardiac calsequestrin antibody (Fig. 8C, lane 6). This result is in keeping with earlier observations by Wuytack et al. (1987) who detected two Ca\(^{2+}\) binding proteins in smooth muscle SR membranes. One of these proteins (M, of 55,000) was immunoreactive with anti-cardiac calsequestrin antisera. The other protein (M, of 63,000), which was present in larger quantities, most likely corresponds to calreticulin. Our results indicate that the level of calsequestrin in smooth muscle from porcine uterus is very low.

**Immunolocalization of Calreticulin and Calsequestrin in Cultured Smooth Muscle Cells**—When cultured smooth muscle cells were stained with affinity-purified anti-calreticulin antibody, heavy intracellular labeling of the SR/ER regions was observed (Fig. 9A). The figure shows that a calreticulin appears to be located in the perinuclear system of membranes corresponding in localization to the SR/ER of the smooth muscle cells. The same smooth muscle cells in culture were also tested for the presence of calsequestrin. No staining was detectable above background or nonspecific binding of antibodies (Fig. 9B), indicating that these cultured smooth muscle cells con-
FIG. 6. FPLC of ammonium sulfate-precipitated calreticulin. Ammonium sulfate pellets containing calreticulin and calsequestrin were prepared from porcine uterus (A) and bovine liver (B) by the procedure described under "Experimental Procedures." Twenty milligrams of protein was applied to an FPLC Mono Q 5/5 anion exchange column followed by elution with a linear (50–750 mM) NaCl gradient. One-milliliter fractions were collected and absorption at 280 nm, and NaCl concentrations were monitored. The positions of calreticulin and calsequestrin peaks are indicated by the arrows and were determined by FPLC chromatography of the purified proteins. CHT, calreticulin; CS, calsequestrin.

FIG. 7. Immunological identification of calreticulin in FPLC fractions of bovine liver. Ammonium sulfate precipitation of bovine liver, followed by FPLC Mono Q column chromatography, was carried out as described under "Experimental Procedures." One-milliliter fractions were collected and calreticulin- and calsequestrin-containing peaks (see Fig. 6B) were analyzed by SDS-PAGE and immunoblotted as described under "Experimental Procedures." A, Coomassie Blue staining; B, immunostaining with anti-calreticulin; lanes 1–4, 20 μl of protein fractions from calreticulin peak (see Fig. 6B). The arrow indicates calreticulin (CRT). The lower molecular weight immunoreactive protein band is a degradation product of calreticulin.

tain very low levels of calsequestrin, confirming our earlier conclusions made on the basis of our biochemical data.

NH₂-terminal Amino Acid Sequence Analysis of Purified Calreticulin—In order to confirm that the proteins identified in rat liver and porcine uterine smooth muscle are indeed calreticulin, we carried out amino-terminal amino acid sequence analysis of the purified proteins. Table 3 shows the amino-terminal amino acid sequences obtained for calreticulins isolated from a variety of different tissues. In addition to the rabbit and bovine liver and porcine uterus, other calreticulins (rabbit brain, uterus, bovine pancreas) were isolated by the procedure described under "Experimental Procedures" and were sequenced in our laboratory. Their amino-terminal amino acid sequences correspond closely to that obtained by us for the rabbit fast-twitch skeletal muscle protein (Fliegel et al., 1989a). Recently other forms of calreticulin have been identified and sequenced or their amino acid sequences deduced from the nucleotide sequence of different cDNA clones. Their amino-terminal sequences are presented in Table 3. These results confirm our earlier observations (Fliegel et al.,
Calreticulin

Calreticulin was identified in these membrane fractions by immunoblotting, 

calreticulin in other tissues (Fliegel et al., 1989a). mRNAs isolated from the same tissues were probed with a cDNA fragment encoding a coding region of a canine cardiac calsequestrin clone. The calsequestrin cDNA hybridized to an mRNA species of 2.2 kilobases in cardiac muscle. With longer exposure, a second band of approximately 2.9 kilobases could be visualized. This is in full agreement with that observed by Scott et al. (1988). However, no hybridization of calsequestrin cDNA was apparent with mRNAs isolated from the liver and from uterine smooth muscle (Fig. 10B).

**DISCUSSION**

In this work we have established that calreticulin, a 60-kDa protein, is a major Ca\(^{2+}\) binding protein in liver ER fractions and in SR-enriched fractions from uterine smooth muscle. Calreticulin was identified in these membrane fractions by immunoblotting, ^{46}Ca\(^{2+}\) overlay, and Stains-All techniques as well as by indirect immunofluorescence staining of cultured smooth muscle cells. Using Northern blot analysis we have also identified mRNA encoding calreticulin in liver (Fliegel et al., 1989a) and uterine smooth muscle. Although our marker enzyme activities indicated some mitochondrial contamination of the SR-enriched fractions from smooth muscle (Table 2), the localization of calreticulin to these mitochondria is very unlikely. This suggestion is based on our immunolocalization data in cultured cells (Fliegel et al., 1989b and Fig. 9). It is also supported by our failure to detect calreticulin in either the liver mitochondrial fraction or in the smooth muscle "mitochondria-enriched" fraction (pellet fraction). The presence of the KDEL ER retention signal at the carboxy-terminal end of calreticulin further supports this contention (Fliegel et al., 1989a).

In this work we have also shown that calsequestrin is not

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Fliegel et al., 1989a; McCauliffe et al., 1990b) that calreticulin, calregulin, CRP55, CaBP3, and the Ro/SS-A autoantigen are all one and the same protein. Calreticulin has a similar molecular weight and amino-terminal amino acid sequence to a number of other previously described proteins: Aplysia p407, a protein whose expression is modulated during long term sensitization (Kennedy et al., 1988), rat fibroblast protein p425 (Kennedy et al., 1988), and murine melanoma protein B50 (Hearing et al., 1986) (Table 3). These may be analogs of calreticulin in different organisms. In fact, the amino acid sequence deduced from the cDNA clone encoding Aplysia p407 protein indicates that it is an Aplysia homolog of calreticulin. It is apparent that all forms of calreticulin are highly homologous, although not identical. Optimal alignment of most of these sequences did not require the introduction of gaps.

**Northern Blot Analysis of Calreticulin and Calsequestrin mRNA**—In order to confirm our immunological observations, we have isolated mRNA from both liver and uterine smooth muscle and tested it for the presence of mRNA encoding calreticulin and calsequestrin. Fig. 10A shows a Northern blot probed with cDNA encoding calreticulin. The cDNA hybridized to mRNAs of 1.9 kilobases in the liver and uterus smooth muscle. This corresponds to the size of mRNA encoding calreticulin found in other tissues (Fliegel et al., 1989a).

![Fig. 9. Localization of calreticulin (A) and calsequestrin (B) in cultured smooth muscle by confocal immunofluorescence microscopy. A, calreticulin is present in smooth muscle cells in a perinuclear system of membranes comprising SR/ER. B, calsequestrin staining, if any, is at the level of background indicating that smooth muscle cells in culture contain very low levels of calsequestrin. Levels of gain and black were set to the same manual level for recording of both images.](image-url)

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**FIG. 8. Immunological identification of calreticulin and calsequestrin in FPLC fractions of porcine uterus.** Ammonium sulfate precipitation of porcine uterus, followed by FPLC Mono Q column chromatography, was carried out as described under "Experimental Procedures." One-milliliter fractions were collected, and calreticulin- and calsequestrin-containing peaks (see Fig. 6A) were analyzed by SDS-PAGE and immunoblotted as described under "Experimental Procedures." A, Coomassie Blue staining; B, immunostaining with anti-calreticulin; C, immunostaining with anti-cardiac calsequestrin; lanes 1–4, 20 \(\mu\)l of protein Mono Q FPLC fractions containing calreticulin (see Fig. 6A); lanes 5 and 6, 20 \(\mu\)l of protein fractions from calsequestrin peak (see Fig. 6A). The short arrows indicate calreticulin. The long arrows indicate cardiac calsequestrin. CRT, calreticulin; CCS, cardiac calsequestrin.

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experimental Procedures." The size of the mRNA hybridizing to the uterine smooth muscle sequestrin and calreticulin and cardiac calsequestrin mRNA was estimated to be 1.9. Calsequestrin was found in liver ER fractions or in SR-enriched muscle, no mRNA encoding calsequestrin was detected in these tissues. These results are in agreement with a previous Northern blot analysis, we showed that although mRNA with ammonium sulfate followed by the Mono Q FPLC chromatography. We propose that calsequestrin is basically confined to skeletal and cardiac muscle and that only very small amounts of this protein are present in uterine smooth muscle. Therefore it has been considered that there may also be a non-muscle equivalent of calsequestrin. Calsequestrin or a calsequestrin-like protein was identified, using immunological methods, by several laboratories in liver, pancreas, human neutrophils, and HL-60 cells (Volpe et al., 1988; Hashimoto et al., 1988; Damiani et al., 1988, 1989; Krause et al., 1989b). This led to the concept of InsP_3-sensitive Ca^{2+} storage organelles in non-muscle cells, which were termed calciosomes (Pozzan et al., 1988; Volpe et al., 1988). These organelles were reported to contain a protein with calsequestrin-like properties which cross-reacted with anti-skeletal muscle calsequestrin antibodies (Hashimoto et al., 1988; Volpe et al., 1988). However, upon purification this protein was determined to have an amino-terminal sequence similar to calreticulin (Collins et al., 1988; Krause et al., 1990) and to be cross-reactive with anti-calregulin/calreticulin antibodies (Collins et al., 1989). Damiani et al. (1988) also reported that a Ca^{2+} binding protein closely related to calsequestrin, which cross-reacted with anti-skeletal calsequestrin antibodies, was apparent in liver ER. However, amino-terminal sequencing of this protein in our laboratory clearly identified it as calreticulin, and further studies of its properties have indicated that it is not a member of the calsequestrin family (Damiani et al., 1989).

The above observations suggest that the calsequestrin or calsequestrin-like protein identified by these authors in non-muscle systems is, in fact, calreticulin. This confusion might be caused by the antibodies used in these studies, which may cross-react with both calsequestrin and calreticulin. Both calsequestrin (Fliegel et al., 1987; Scott et al., 1988) and calreticulin (Fliegel et al., 1989a) contain clusters of acidic residues at their respective carboxyl termini (Fliegel et al., 1989c), and these are predicted to be highly antigenic. It is therefore reasonable to assume that antibodies raised against calsequestrin might cross-react with calreticulin. In our studies we have used an anti-calsequestrin antibody that did not cross-react with calsequestrin and an anti-calsequestrin antibody that did not recognize calreticulin.

We conclude that the calsequestrin or calsequestrin-like protein identified earlier in liver ER and/or in the putative InsP_3-sensitive organelle termed calciosomes is calreticulin. In agreement with Van et al. (1989), however, we find no evidence for calciosomes in the liver by biochemical and immunological analysis of membrane vesicles or by immuno-
cytochemistry. Instead we would like to propose that there might be InsP$_2$-sensitive regions of the ER membrane which are enriched in both calreticulin and the InsP$_3$ receptor. This situation would be similar to the localization of calsequestrin and the ryanodine receptor to the junctional SR membrane (Frazzini-Armstrong et al., 1987).

In conjunction with the studies described above, we also used similar experimental approaches to determine the identity of the Ca$^{2+}$ binding proteins in porcine uterus smooth muscle. We found that calreticulin is readily detected in a mixture of SR proteins by immunoblotting, by $^{40}$Ca$^{2+}$ overlay and by Stains-All staining, but that all three of these techniques indicate that there is no calsequestrin in uterine smooth muscle SR-enriched membrane vesicles. There was also no evidence for the expression of mRNA for calsequestrin in this tissue, in agreement with previous findings in smooth muscle from the aorta (Scott et al., 1988). However, the expression of mRNA for calreticulin was readily detected. Our failure to detect either calsequestrin or mRNA for calsequestrin in these experiments contrasts with the identification of a cardiac isoform of calsequestrin in smooth muscle SR membranes prepared from pig stomach antrum (Wuytack et al., 1987).

Despite these findings, using the Mono Q FPLC column we were able to identify very small quantities of a protein which is immunologically cross-reactive with calsequestrin in whole tissue homogenates from porcine uterus smooth muscle. The apparent presence of calsequestrin in whole tissue homogenates of porcine uterus smooth muscle. The apparent presence of calsequestrin in whole tissue homogenates of porcine uterus contrasts with our observation that it is absent from the isolated SR membranes. Therefore, we have also investigated the distribution of calsequestrin and calreticulin in cultured smooth muscle cells, using immunofluorescence microscopy. The smooth muscle cells in culture showed clear staining with anti-calreticulin antibodies, on a perinuclear membrane system apparently comprising the ER/SR system. The same cells in culture showed negligible staining above background levels with anti-cardiac calsequestrin antibodies, indicating that if calsequestrin is present in these cultured cells it is expressed at very low levels.

Our results indicate that in porcine uterus smooth muscle, calsequestrin is expressed at very low levels, such that it is undetectable in the isolated SR-enriched membrane fractions. In contrast, Wuytack et al. (1987) clearly identified calsequestrin in SR membranes isolated from the smooth muscle of pig stomach antrum. However, despite the identification of calsequestrin, in this study other more prominent extrinsic ER Ca$^{2+}$ binding proteins were also identified, and it is likely that one of these ($M_\text{r}$, 63,000) is calreticulin. Therefore, taken together, these data indicate that calreticulin is the major Ca$^{2+}$ binding protein of smooth muscle SR, but that calsequestrin is also expressed, at varying levels. Different types of smooth muscle might contain different amounts of calsequestrin. The regulation of cellular Ca$^{2+}$ concentrations in smooth muscle is a complex process, the precise mechanisms for which differ depending on the function and location of the smooth muscle being investigated (van Breenen and Saida, 1989). Smooth muscle SR is known to be functionally heterogeneous, since it expresses two different types of Ca$^{2+}$ release channels: the ryanodine-sensitive channel and the InsP$_3$-stimulated channel (van Breenen and Saida, 1989). It is not unreasonable, therefore, to suggest that the dual expression of calsequestrin and calreticulin in smooth muscle SR might be functionally related to the heterogeneity of smooth muscle SR.

In the present study, using a $^{40}$Ca$^{2+}$ overlay technique and Stains-All staining, we have shown that calreticulin is one of the major Ca$^{2+}$ binding proteins in smooth muscle SR and in liver ER. However, we did identify three other prominent Ca$^{2+}$ binding proteins in these membranes, 100-120, 78, and 55 kDa. They most likely correspond to the luminal ER Ca$^{2+}$ binding proteins previously identified in liver (Macier and Koch, 1988; Booth and Koch, 1989). Van et al. (1989) recently identified four intracisternal Ca$^{2+}$ binding proteins in liver ER, 90, 80, 60, and 59 kDa respectively. Amino-terminal amino acid sequencing indicated that the 60-kDa protein (CaBP3) is calreticulin and the 90-kDa protein is GRP94 (Van et al., 1989). The identity of the other proteins is uncertain. The Ca$^{2+}$ binding proteins identified by us in liver ER membranes may correspond to GRP96 (100-120-kDa protein), to immunoglobulin binding protein (BIP) (78-kDa protein), and to protein disulfide isomerase (55-kDa protein). Calreticulin, together with these proteins, may belong to a family of the ER Ca$^{2+}$ binding proteins which are involved in stress responses in the cell. This hypothesis is supported by the identity of calreticulin with the Ro/SS-A autoantigen (McCauliffe et al., 1990a, 1990b) and by similarities between the amino acid sequences of calreticulin and of some stress proteins residing in the lumen of the ER (Fliegel et al., 1989c). This suggests that in addition to being a major Ca$^{2+}$ binding protein in ER/SR membranes, calreticulin may also be important in stress responses in the cell and may play some role in the pathology of rheumatic diseases.

**Acknowledgments**—We are grateful to Dr. Y. Coe (University of Alberta) for providing fresh porcine uterine. We thank Drs. D. Brindley and A. Martin (University of Alberta) for providing both the FPLC system and invaluable help in performing the calreticulin purification experiments using a Mono Q column. We would also like to thank Dr. L. R. Jones (Kramner Institute of Cardiology, Indianapolis, IN) for providing the cDNA clone encoding cardiac muscle calsequestrin. We thank members of the Ontario Laser and Lightwave Research Center for helpful assistance with confocal microscopy.

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6 M. Opas, L. Fliegel, E. Dziak, and M. Michalak, submitted for publication.

Continued on next page.
EXPERIMENTAL PROCEDURES

Materials - Imidazole, Tritc, Peptr and Toln X-100 were purchased from Sigma. 4°C columns were obtained from New England Nuclear. Pronase-conjugated rabbit antigastric tGgs and pronase-conjugated goat anti-rabbit tGgs were from B&K and B&K. B&K Mannheim, respectively. N-methylmaleimide (p-Nyoldinyl) delivered from Bio-Rad, Dimethylsulfone, benzamidine, phenylmethylsulfon fluoride (PMSF). L-(N-methyl)carboxybenzamidine-2-carboxylic acid (MCA), phosphoramidon, eGTA and Oligo(dT) were obtained from B&K. The mixture of the following protease inhibitors made in Q method involves direct extraction of calreticulin and calsequestrin from whole tissue homogenization of myometrium tissue and subsequent membrane fractionation of a slaughterhouse and fresh porcine muscle, respectively. 

Syringe, diluted with supernatant sucrose, performed in the presence of a mixture of the following protease inhibitors made in Q method involves direct extraction of calreticulin and calsequestrin from whole tissue homogenization of myometrium tissue and subsequent membrane fractionation of a slaughterhouse and fresh porcine muscle, respectively.

To prepare any membrane fractions, DEAE-Sephadex chromatography was carried out on a Drake Sephadex column coupled with a hydroxyapatite column chromatography as described by MacLennan (1974). Fractions enriched in calreticulin were concentrated using an Amicon concentrator, dialyzed by passage through a Sephadex G-25 column, and directly applied onto a hydroxyapatite column equilibrated with 10 M potassium phosphate, pH 7.0. Fractions were using a linear gradient of 10 to 300 mM phosphate (Fig. 11). Peaks containing calreticulin were pooled, dialyzed against 10 M ammonium carbonate and freeze-dried. Calreticulin can be purified by this method from a variety of different tissues. Using this procedure we have purified calreticulin from the following tissues: rabbit kidney, pancreas, liver, uterine, skeletal muscle, cardiac muscle, human liver and pancreas, porcine cardiac muscle and cardiac muscle. Recently, Kresse et al. (1986) used the same procedure to isolate calsequestrin from HL-60 cells. NLx-terminal sequence analysis of some of the purified calreticulin is presented in Table 3. 

Protein Separating - SDS-polyacrylamide gel electrophoresis and Analysis of 4Ca2+ Binding - SDS-PAGE was on 12% polyacrylamide gels as described by Laemmli (1970). After gel electrophoresis, gels were stained with either Coomassie Blue or with the cytoskeletal dye "Stains-All." For 4Ca2+ binding analysis, proteins were transferred electrothermally onto nitrocellulose membrane according to the method of Towbin et al. (1979). Identification of the 4Ca2+ binding to membrane vesicles and isolated proteins was carried out as described earlier (Maryanska et al., 1984). Standards were Bio-Rad low range molecular weight protein; phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (51,000), carbonic anhydrase (37,000), soybean trypsin inhibitor (21,500), and L-arginine (14,000), or Bio-Rad prestained markers; phosphorylase b (110,000), bovine serum albumin (66,200), ovalbumin (51,000), carbonic anhydrase (37,000), soybean trypsin inhibitor (21,500) and L-arginine (14,000).

Preparation of Anti-calreticulin and Anti-calsequestrin Antibodies and Immunoblotting - Calreticulin and calsequestrin were purified by ammonium sulfate precipitation as described above. Goat anti-bovine skeletal calreticulin and rabbit anti-bovine calsequestrin antibodies were raised against ammonium sulfate purified proteins separated on SDS-PAGE prior the injections. Goat anti-rabbit skeletal muscle and rabbit anti-bovine cardiac calsequestrin antibodies were also raised against SDS-PAGE purified proteins. Rabbits and goats were immunized with 1.6 mg and 1 mg of protein emulsified in Freund's complete adjuvant, respectively. After 2 weeks and 4 weeks, the immunization was repeated using Freund's incomplete adjuvant. Antibodies were screened by immunoprecipitating proteins after electrophoretic transfer onto a nitrocellulose membrane, according to the method of Towbin et al. (1979). Nitrocellulose membranes were blocked with 5% milk powder in phosphate-buffered saline, and then incubated with antisera as described by Michaelis et al. (1986). Antibody binding was detected with peroxidase-conjugated secondary antibodies and a standard peroxidase color development reaction. The antibodies used in this study were specific for calreticulin or for calsequestrin (i.e. the anti-calreticulin antibody did not cross-react with calsequestrin, and the anti-calsequestrin antibody did not recognize calreticulin.

Immunofluorescence Microscopy - For intracellular localization of calreticulin and calsequestrin rabbit anti-smooth muscle cells was used. Polyclonal antibodies against calreticulin and calsequestrin were raised in rabbit and used at 1:30 and 1:30 dilutions, respectively. Antibodies conjugated secondary antibodies (1:30 dilution in PBS) were purchased from ICN Immunobiologicals (Mississauga, Ontario). For immunochemistry, rabbit anti-smooth muscle cells was used at 1:30 in PBS, and anti-calsequestrin was used at 1:30 dilution in PBS, respectively. All secondary antibody solutions contained 10% normal horse serum to reduce background staining. Primary antibodies were kept at 4°C overnight and the second antibody was applied at 1:50 dilution and incubated at room temperature for 2 h. The slides were then mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenylindol (DAPI) to stain nuclei.
Northern Blot Analysis - Total and poly(A)+ RNAs were isolated from various freshly removed rabbit tissues (Chomczynski and Sacchi, 1987) and 10 µg samples of poly(A)+ RNA or 20 µg of total RNA were separated by electrophoresis in denaturing formaldehyde - 0.8% agarose gel, stained with ethidium bromide and blotted onto nylon membranes (Maniatis et al., 1982). Hybridization was with 32P-labelled cDNA fragments was carried out as described earlier (Maniatis et al., 1982) in the presence of 10% dextran sulfate. The following restriction fragments were used for Northern blotting: Pst I - Bam HI restriction fragment of calreticulin (Fliegel et al., 1989a), and Hind III - Bam HI restriction fragment of cardiac calnexin (Scott et al., 1988), encoding the middle portion of coding region of calreticulin (Fliegel et al., 1989a) and the carboxy-terminal end of calnexin (Scott et al., 1988), respectively.

Figure 11. Hydroxylapatite chromatography of DEAE-Sephadex purified calreticulin. Illop, a DEAE-Sephadex fraction containing calreticulin was fractionated on a 1.5 x 30 cm hydroxylapatite column as described under "Experimental Procedures". The fraction size was 4 ml. Lane, SDS-PAGE of standard proteins and fractions 6 - 33 from the hydroxylapatite column. Molecular masses of standard proteins are indicated on the left side of the gel. The arrow indicates calreticulin.

Table 1. Distribution of marker enzymes in liver membrane fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mitochondria</th>
<th>Peroxisomes</th>
<th>Lysosome</th>
<th>Plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER I</td>
<td>29.6x10^6</td>
<td>62.6x10^6</td>
<td>8.7x10^6</td>
<td>0.0x10^6</td>
</tr>
<tr>
<td>ER II</td>
<td>137.6x10^6</td>
<td>52.6x10^6</td>
<td>38.4x10^6</td>
<td>0.0x10^6</td>
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<tr>
<td>Lysosome</td>
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<td>0.0x10^6</td>
<td>22.5x10^6</td>
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<tr>
<td>Peroxisome</td>
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<td>0.0x10^6</td>
<td>0.0x10^6</td>
<td>15.3x10^6</td>
</tr>
<tr>
<td>Plasma membrane</td>
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<td>0.0x10^6</td>
<td>0.0x10^6</td>
<td>3.2x10^6</td>
</tr>
</tbody>
</table>

Table 2. Distribution of marker enzymes in other membrane fractions.

<table>
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<tr>
<th>Fraction</th>
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<th>Peroxisomes</th>
<th>Lysosome</th>
<th>Plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER I</td>
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<td>Lysosome</td>
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<td>0.0x10^6</td>
<td>22.5x10^6</td>
</tr>
<tr>
<td>Peroxisome</td>
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<td>0.0x10^6</td>
<td>15.3x10^6</td>
</tr>
<tr>
<td>Plasma membrane</td>
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<td>0.0x10^6</td>
<td>3.2x10^6</td>
</tr>
</tbody>
</table>

NS = not determined