Association of Clathrin with Microsomes Isolated from Canine Myocardium

David R. Caprette, Mark L. Entman, and W. Barry Van Winkle
From the Section of Cardiovascular Sciences, Department of Medicine, Baylor College of Medicine, Houston, Texas

SUMMARY. We and others have observed specialized regions of sarcoplasmic reticulum membranes that resemble coated vesicles, in the I-band region of myocardial cells. These structures have been named "corbular" sarcoplasmic reticulum, and are distinct in appearance from Golgi-associated coated vesicles, in that they are larger and contain a flocculent material that has been identified as calsequestrin. Whereas it has been suggested that these structures have a role in cardiac calcium metabolism, their function(s) and the molecular identity of the characteristic "bristle" coat remain unknown. Microsomes enriched in sarcoplasmic reticulum were prepared from canine ventricular muscle by Polytron homogenization in pH 6.5 buffer, followed by differential centrifugation. Protein was released by incubation in 50 mM Tris/HC1, pH 8, followed by centrifugation. We found these extracts to be enriched in a protein that was identical to brain clathrin in mobility on a Sepharose 4B gel filtration column, final position of the native protein following nondenaturing electrophoresis, relative mobility in denaturing (sodium dodecyl sulfate) electrophoresis on 6% and 7.5% gels, and antigenicity to anti-clathrin IgG. These findings confirmed the presence of clathrin triskelions in the cardiac microsome extract. On this basis, we suggest that clathrin may be a component of the electron dense "coat" of corbular sarcoplasmic reticulum. (*Circ Res 58: 120–126, 1986*)

COATED vesicles (CV) are ubiquitous organelles found in varying amounts in most eukaryotic cells (Pearse, 1980). Despite their involvement in diverse cellular functions, e.g., receptor mediated endocytosis (Anderson et al., 1978; Goldstein et al., 1979), membrane recycling (Heuser and Reese, 1973; Pearse and Bretschmer, 1981) and secretion (Kartenbeck, 1980), CV possess a common feature, the characteristic bristle coat seen in electron micrographs. This coat has been shown to be a closed lattice made up of protein subunits called triskelions (Ungewickell and Branton, 1981), several associated proteins of 100,000–110,000 daltons (Unanue et al., 1982; Zaremba and Keen, 1983), and a triplet of 50,000–55,000 daltons that have been identified as α- and β-tubulin and an "assembly protein" (Kelly et al., 1983; Pfeffer et al., 1983). The triskelion is a unique three-armed macromolecule, identifiable by rotary shadowing in the electron microscope. It consists of three identical 175,000 dalton "heavy" polypeptide chains called clathrin (Pearse, 1975) and three heterogeneous "light" chains of 32,000–35,000 daltons, termed clathrin-associated proteins, or CAP (Lisanti et al., 1982).

We and others (Forbes and Sperelakis, 1980) have noted the presence of CV-like structures in cardiac cells, associated with sarcoplasmic reticulum (SR) membranes in the I-band region of mammalian ventricular and atrial muscle (Fig. 1) and in the perinuclear area in association with Golgi membranes. CV have been observed "free" in the cytoplasm of neonatal skeletal muscle cells (Bursztajn and Libby, 1981), but have not been seen in association with SR membrane or transverse tubules in adult skeletal muscle. The CV-like structures associated with cardiac SR have also been called "corbular SR" (Sommer and Waugh, 1976), and are distinct in appearance compared with Golgi-associated CV. They contain flocculent material in the lumen that has been identified as calsequestrin (Jorgensen and Campbell, 1984). A role for these structures in cardiac calcium metabolism has been suggested, based on their membrane continuity with the terminal portions of the SR membrane (Forbes and Sperelakis, 1980). However, the presence of clathrin in the bristle coat of these SR-associated, calsequestrin-containing vesicles has not been confirmed.

As part of our studies to determine the role of corbular SR in myocardial cell function, we report the identification of clathrin triskelions in extracts of an SR-enriched microsomal fraction of canine myocardium. On the basis of these results, we suggest that it is highly likely that clathrin is a component of the electron-dense coat of corbular SR.

**Methods**

**Brain Clathrin Preparation**

Crude CV were prepared from trimmed cortices by the method of Pearse (1975), and were further purified by sucrose gradient centrifugation. Brains were removed from Nembutal-anesthetized dogs, placed in ice-cold isotonic saline solution, and stripped of their meninges. Subse-
FIGURE 1. Panel a: thin section electron micrograph of canine left ventricular papillary muscle. Coated vesicles (arrows) associated with membrane of the sarcoplasmic reticulum (SR) occur only in the region of the Z-band and I-band (bar = 0.5 μm). Panel b: thin section of canine papillary illustrating association of two coated vesicles (arrows) with the transverse (T) tubule, and similarity of their flocculent contents (bar = 0.5 μm).

Subsequent steps were carried out at 0–4°C. The trimmed cortices were placed in 1 ml/g buffer A, consisting of 100 mM 2[N-morpholino]ethanesulfonic acid (MES), 1 mM EGTA, 0.5 mM MgCl₂, and 5 mM NaN₃, pH 6.5, and homogenized 3 × 10 seconds at high speed in a Waring Blender. The homogenate was centrifuged 30 minutes at 20,000 g, and the supernatant was filtered and recentrifuged, then centrifuged for 60 minutes at 85,000 g. The pellets (crude microsomes) were washed by resuspension in 50 ml buffer A, with several strokes of a Dounce homogenizer with a loose-fitting pestle, followed by centrifugation for 30 minutes at 85,000 g. CV were further purified by sucrose gradient centrifugation, as described by Keen et al. (1979). The coat protein was isolated from either coated vesicles or crude microsomes by suspension in 50 mM Tris/HCl, pH 8, by Dounce homogenization, followed by stirring for 30 minutes. Vesicular material was separated from soluble protein by centrifugation for 60 minutes at 165,000 g.

Myocardial Clathrin Preparation

The following is a modification of our preparation for SR-enriched fractions from canine myocardium (Van Win-
thyroglobulin (669,000 daltons), apoferritin (443,000 daltons), β-amylase (200,000 daltons), and alcohol dehydrogenase (150,000 daltons), obtained from Sigma. Void volume was determined with blue dextran 2000, and $K_v = (V_e - V_0)/(V_t - V_0)$ was determined for each protein standard, where $V_e =$ elution volume, $V_0 =$ void volume, and $V_t =$ total volume. Protein in each extract was desalted on a column of Sephadex G-25 equilibrated with 20 mM NH$_4$HCO$_3$ and lyophilized. Samples were prepared for chromatography by resuspension in buffer B with 40 mM dithiothreitol (DTT), and were degassed before loading 2 to 4 ml of sample per run. Elution was performed at 12-14 ml/hr with buffer B, and 2-ml fractions were collected. Fractions were identified by absorbance at 280 nm. Protein was recovered by desalting and lyophilization, as described above, and pellets were resuspended in distilled water.

Electrophoresis

Electrophoresis was performed on slab gels prepared from a stock solution of 30% acrylamide/0.8% bis-acrylamide. Protein bands were visualized by staining with Coomassie-Weber stain, followed by destaining with 7.5% methanol, 10% acetic acid. Relative amounts of clathrin triskelions or heavy chain polypeptide in different fractions were estimated by scanning Coomassie-stained bands in an LKB laser densitometer. Molecular weights of polypeptides were estimated from standard curves prepared from protein standards (Sigma).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on fractions of brain and heart microsomal preparations, after protein estimation by the biuret method. Samples were diluted to 2-4 mg/ml with distilled water, then mixed 1:1 with buffer C, consisting of 125 mM Tris base, 80 mM DTT, 20% glycerol, 10% SDS, and 0.002% bromophenol blue dye (BPP). After 5 minutes at room temperature, the solubilized samples were centrifuged 5 minutes in a Beckman microfuge (10,000 g) and loaded in amounts of 50-150 µg biuret protein into sample wells of 6% SDS gels with 3% stacking gel, while preserving 280 nm. Protein was recovered by desalting and lyophilization, as described above, and pellets were resuspended in distilled water.

Electron Microscopy

Samples containing crude microsomes or coated vesicles were examined by negative stain electron microscopy employing 1% uranyl acetate (pH 4.5) on Formvar-coated, carbon-stabilized grids. Papillary muscles from canine left ventricle were processed for electron microscopy by the method described by Bloom et al. (1980). Reactivity of clathrin-enriched fractions with anti-clathrin IgG was assessed by the “Western” blotting technique with radiolabeled protein A (Towbin et al., 1979; Burnette, 1981).

Results

Electron Microscopy

Coated vesicles occurred in precise locations in canine ventricular muscle cells in agreement with previous descriptions. Most often, they occurred as specializations in the I-band region of each sarcomere, with their membranes continuous with those of saccoplasmic reticulum (Fig. 1a). We also observed them attached to the transverse (T) tubule membrane (Fig. 1b), suggesting that this species of coated vesicle represents a mechanism of transfer between the SR lumen and the extracellular space.

Stereological analyses of electron micrographs of canine myocardium indicated that the coated vesicles associated with SR or T-tubule membranes occupy 0.3% of the volume of cardiac ventricular cytoplasm. In addition to the flocculent matrix, SR-coated vesicles were distinguished from Golgi-associated coated vesicles by their size, the former being 75.1 ± 2.5 nm (mean ± sd) in diameter, compared to 51.3 ± 1.6 nm for the latter. Both possessed a 20-nm-thick bristle coat, as observed previously (Forbes and Sperelakis, 1980).

Preparative

Coated vesicles isolated from canine brain exhibited the same morphology as those isolated from other tissues. Yields of brain and heart microsomes, following the wash step, were 2.13 ± 0.2 and 1.23 ± 0.2 mg biuret protein/g wet weight starting tissue, respectively. SDS-PAGE of brain microsomes revealed a major high molecular weight band shown by others to be clathrin heavy chain (Pearse, 1976; Garbern and Wu, 1981). The molecular weight of canine brain clathrin heavy chain, estimated from a
standard curve of molecular weight vs. relative mobility \( R_f \) was 165,000 daltons. A faint band (band 1) with the same \( R_f \) as brain clathrin was revealed by electrophoresis of crude cardiac microsomes (Fig. 2). Extraction of the membranes with 50 mM Tris/HCl, pH 8, resulted in enhancement of this band in the soluble fraction. Yields of the crude microsome extracts were \( 77 \pm 19 \) and \( 88 \pm 28 \) \( \mu g/g \) starting tissue for brain and heart, respectively. Band 1 represented 15% of heart microsome extract protein.

Calibration of the Sepharose 4B column yielded \( K_v \) values of 0.44, 0.47, 0.60, and 0.81 for thyroglobulin, apoferritin, catalase, and aldolase, respectively. Chromatography of brain extract yielded three major protein peaks. Fractions containing the second peak (\( K_v = 0.29 \)) were pooled. Analysis of this protein peak by SDS-PAGE revealed a single major polypeptide band with a molecular weight of 165,000 daltons (lane E, Fig. 2; lane A, Fig. 5). The elution patterns for both extracts are shown in Figure 3. The most prominent protein peaks from heart microsome extract eluted either in the void fraction or with a \( K_v \) of 0.63. A minor peak with a \( K_v \) identical to that of brain triskelions was identified, and fractions were pooled as indicated in Figure 3. Subsequent gradient SDS-PAGE showed this fraction to be enriched with band 1 protein.

Nondenaturing PAGE of CV extract revealed a single major band ('native' clathrin) migrating to a position of 4.1% acrylamide monomer concentration (%T) on the gradient gel (Fig. 4). Extracts from heart microsomes also revealed this band (band 2), but other proteins of lower apparent molecular weight were also evident. The final %T was the same for band 2 and brain clathrin. As shown in Figure 4, treatment with DTT did not affect the final %T for either band 2 or brain clathrin.

Nondenaturing electrophoresis was carried out on DTT-treated heart and brain extracts with 3–15% linear gradient gels, and the protein bands were transferred to nitrocellulose paper (Fig. 4). Following immunoblotting of identical transfers with anticalthrin antibody, brain triskelions and band 2 of heart extract were labeled. Irrelevant protein bands and control transfers were not labeled.

To ensure the presence and predominance of clathrin in band 2 compared with triskelion protein from brain microsomes, a form of two-dimensional...
FIGURE 4. Analysis of coated vesicle and heart microsome extracts by nondenaturing PAGE on linear gradient gels of 3–15% polyacrylamide with a continuous buffer system. T: brain triskelion band. 2: band 2 from heart microsome extract. Lanes A and C: 100 µg coated vesicle extract. Lanes B and D: heart microsome extract, 100 and 270 µg, respectively. Dithiothreitol (40 mM) was included in the sample buffer in lanes C and D. Lanes E and F: X-ray film exposed 16 hours to a "Western" transfer of samples identical to those shown in lanes C and D. Transfers were exposed to anti-clathrin IgG and radioiodinated protein A. The bands correspond to the positions of brain clathrin triskelions (E) and band 2 of heart extract (F).

electrophoresis was performed. Gel pieces containing band 2 and the clathrin band from brain microsome extract were removed from preparative nondenaturing gels, denatured, and SDS-PAGE was carried out (Fig. 5). Electrophoresis on 6% polyacrylamide gels resolved a single band with R_f of 0.37, corresponding to 165,000 daltons.

Discussion

The principal question addressed by this study is whether or not the protein clathrin is associated, in significant amounts, with cardiac SR. Cardiac microsomes, prepared by Polytron homogenization of ventricular tissue followed by differential centrifugation, are enriched in SR (Van Winkle et al., 1978). Whereas other membranes vesicles are present in the microsomal fraction, the only membranes likely to contribute clathrin, as determined by morphological examination of intact tissue, are corbular SR and the Golgi-associated coated vesicles. The Golgi-associated structures are smaller in diameter than corbular SR, and are much fewer in number, located primarily in the perinuclear region of the myocardial cell. We therefore sought to determine if the protein clathrin could be isolated from suspensions of cardiac microsomes.

Clathrin lattices are dissociated into subunits by incubation in buffers of low ionic strength and alkaline pH, i.e., >7.5, and by buffers containing monoamines such as Tris base (Schook and Puszkin, 1983). To preserve the structure of clathrin that may have been associated with SR membranes, we prepared the microsomes in buffer containing MES (pKa = 5.96) at pH 6.5. Clathrin lattices were subsequently dissociated into subunits by incubation in Tris buffer, pH 8. To identify cardiac clathrin, we compared analyses of extracts of cardiac microsomes with extracts known to contain clathrin. These extracts were prepared from coated vesicles and crude microsomes from canine brain, and clathrin was purified further by gel filtration on a column of Sepharose 4B.

Coated vesicles isolated from canine brain by the method of Pearse (1975) exhibited the same morphology in negatively stained electron micrographs as that reported for coated vesicles isolated from porcine or bovine brains (Pearse, 1975, 1976). Examination of crude microsome preparations by both electron microscopy and SDS-PAGE revealed canine brain to be an especially rich source of coated vesicles and clathrin (Fig. 2). Structures resembling brain-coated vesicles were not seen in negatively stained preparations of cardiac microsomes, although obscure vesicles of the size and shape of coated vesicles occasionally were seen. We offer three possible explanations for this observation. First, corbular SR vesicles would represent a very small minority of the population of vesicles in a crude microsome suspension. Second, the degree of contrast obtained in negative staining is based on the principle that proteinaceous structures repel the
heavy metal stain, whereas spaces surrounding the proteins stain darkly. The high protein:l lipid ratio of SR vesicles relative to brain vesicles may obscure the postulated clathrin lattices in negatively stained preparations. Third, a characteristic of corbular SR and other forms of junctional SR is the presence of high molecular weight "feet" proteins (Dolber and Sommer, 1984; Seiler et al., 1984). In addition to the repulsion of stain by SR feet proteins, an interaction of clathrin with these extrinsic proteins may affect the configuration of the lattice structure itself. These structures may have to be distinguished through the use of immunocytochemical labeling using antibodies to clathrin and feet proteins, or, perhaps, through the use of transmission electron microscopic goniometry (specimen tilting).

The difference between our estimate of 165,000 daltons for the molecular weight of brain clathrin heavy chain and the estimate by others (Pearse, 1975; Pfeffer et al., 1983) of 175,000–180,000 daltons may reflect our choice of protein standards and the gel electrophoresis system. We did not attempt to estimate the molecular weight of "native" clathrin triskelions by gel filtration, since the three-armed structure of the triskelion gives an effective molecular radius greater than that expected for a globular protein of the same molecular weight. Thus, the elution volume for triskelions (approximate molecular weight, 640,000 daltons) was less than that of thyroglobulin (669,000 daltons).

SDS-PAGE of cardiac microsomes prepared at pH 6.5 revealed a faint band with the same Rf as brain clathrin heavy chain. This suggests the presence of clathrin in cardiac SR. Enhancement of this band in the soluble fraction by extraction at pH 8 of cardiac microsomes suggests that the alkaline pH released the clathrin coat from corbular SR. Protein bands in this molecular weight range must be greater than 2,000 daltons apart in order to be resolved, assuming a minimum resolution distance of 0.5 mm between bands. To test the hypothesis that the band from the cardiac source was a different protein with an identical apparent molecular weight, we analyzed high pH extracts by electrophoresis underondenaturing conditions. Analysis of coated vesicle or brain microsome extract revealed a single high molecular weight protein band, representing "native" clathrin triskelions. A band in heart extract migrated to the same percent acrylamide concentration (%T) on the gradient gel. Since the subunit structure of the triskelion has been shown to be independent of disulfide bonding (Gargern and Wu, 1981), electrophoresis was also performed under reducing conditions. This reduced the number of protein bands in the cardiac microsome extract, but the mobility of band 2 was unaffected, and remained the same as that of brain clathrin triskelions.

An additional piece of evidence (Fig. 4) for molecular identity between band 2 of heart extract and brain triskelions is their cross-reactivity with affinity-purified rabbit IgG antibody to bovine brain clathrin. The immunological similarity among clathrins isolated from canine brain and heart tissue and from bovine brain reflects the absence of significant variation in structure of clathrin heavy chain among tissues of diverse species.

A form of two-dimensional PAGE, employing nondenaturing followed by SDS-PAGE on 6% polyacrylamide slab gels, confirmed that both the brain triskelion band and the corresponding band in heart extract consisted primarily of subunits with the same Rf, corresponding to 165,000 daltons. We chose a 6% resolving gel in order to achieve good resolution of bands at the molecular weight of clathrin heavy chain, i.e., with Rf between 0.3 and 0.7. We consider the identical mobilities of band 2 protein and brain clathrin in both nondenaturing and SDS-electrophoresis systems to be strong evidence that band 2 represents native clathrin triskelions.

In transmission electron micrographs, corbular SR vesicles resemble clathrin-coated vesicles that have been identified in micrographs of diverse tissues. Most roles postulated for coated vesicles in other tissues have been dynamic, in which assembly of clathrin lattices leads to vesicle formation, followed by transport of substances into or out of the cell, or between intracellular structures. An association of clathrin with the electron-dense coat of corbular SR might suggest that such "coated SR" is involved in a dynamic transport process. The association of corbular SR vesicles with the terminal portions of the SR membrane, and the presence of calcequinin in the electron-dense matrix, suggest a role for these structures in the regulation of calcium levels in the myocardial cell. One possible function could be the transport of excess calcium from the lumen of the SR membrane to the transverse (T)-tubule for delivery to the T-tubule lumen. A proposed alternative role for clathrin is the formation of stable clathrin-coated "pits," in which a specialized region of the membrane is segregated for a purpose such as the approximation of similar receptor complexes. The clathrin lattice, if associated with corbular SR, might serve to segregate and stabilize portions of SR containing of high concentration of calcequinin, and/or to bind the "feet" proteins to the membrane surface. The exact function of corbular SR, and the complete biochemical makeup of the electron-dense coat, remain unknown.

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Address for reprints: David R. Caprette, Section of Cardiovascular Sciences, Department of Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030.

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References


Forbes MS, Sperelakis N (1980) Structures located at the levels of the Z bands in mouse ventricular myocardial cells. Tissue Cell 12: 467–489


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