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Differential Distribution of Vesicular Carriers During Differentiation and Synapse Formation

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Abstract Coated and noncoated vesicles participate in cellular protein transport. Both acetylcholine receptors (AChR) and acetylcholinesterase (AChE) are transported via coated vesicles, some of which accumulate beneath the neuromuscular synapse where AChRs cluster. To investigate the mechanisms by which these proteins are transported during postsynaptic remodeling, we purified coated vesicles from the bovine brain via column chromatography (Sephacryl S-1000) and raised monoclonal antibodies to epitopes of the vesicular membranes enriched in AChE. We assayed for AChE (coated vesicle enriched), hexosaminidase (lysosomal contaminants), NADH cytochrome C reductase (mitochondrial containing), and protein and demonstrated electron microscopically using negative staining that the vesicular fraction contained 95% pure coated vesicles. We then injected coated vesicle fractions and the fractions from which the coat was removed intraperitoneally into mice and obtained three monoclonal antibodies: C-33, C-172, and F-22. On immunoblots of purified vesicles and cultured skeletal muscle, mAb C-33 stained a 180 Kd band and mAb C-172 stained a 100 kd band. MAb F-22 stained 50 kd and 55 kd bands and was not characterized further. Immunofluorescent microscopy with C-33 and C-172 revealed punctate fluorescence whose distribution depends upon the stage of myotube development. Four days after plating, myotubes showed punctate fluorescence throughout the myotube, whereas those stained 8 days after plating showed a punctate perinuclear distribution. Myotubes innervated by ciliary neurons show punctate fluorescence limited to the nuclear periphery and most concentrated around nuclei which line up beneath neuronal processes. This differential vesicular distribution, observed during myotube differentiation and innervation, suggests that these vesicles participate in vesicular membrane traffic.

Key words: coated vesicles, monoclonal antibodies synapse formation, nerve-muscle cultures, immunofluorescent localization

In the developmental remodeling of the postsynaptic membrane, the target cell must transport numerous proteins to their proper destinations. Receptor proteins, such as the nicotinic acetylcholine receptors (AChRs), which are integral membrane proteins, reach their destination in the plasma membrane surface after undergoing post-translational modification and passing through many organelles. Secretory proteins do not become integral plasma membrane components but enter the extracellular space or become components of the basement membrane. Still other proteins never reach the plasma membrane and remain intracellular, often spending their lives in the one compartment. Ultimately the cell internalizes most membrane associated proteins, often transporting them intracellularly to a final destination in the lysosome. This protein traffic to and from the membrane requires membrane carriers which, in many systems, have been identified as coated vesicles. These unique structures, first described as pit-like depressions on the surface of the mosquito oocyte [Roth and Porter, 1964], subsequently have been seen in all animal and plant cells [Newcomb, 1980; Mersey et al., 1982]. Their distinguishing feature is a bristle-like protein coat, the triskilion [Ungewickell and Branton, 1981], which has a three-legged pinwheel structure [Kirchhauser et al., 1986]. The coat assem-

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bly contains three 180 Kd clathrin heavy chain and three 33–36 Kd light chain molecules [Keen, 1985]. The biochemistry and molecular biology of these proteins has been well characterized, and the cDNAs encoding its component polypeptides have been cloned and sequenced [reviewed by Pearse and Crowther, 1987].

In cultured skeletal muscle, as well as other eukaryotic cells, coated vesicles and coated pits exist in two distinct cellular patterns: a random distribution in the area of the nucleus and the golgi cisterna, and a more orderly arrangement along the cell surface [Bursztajn et al., 1987]. The size of these vesicles depends on their location within the cell. Coated pits and vesicles lying close to the plasma membrane are large, 100–250 nm in diameter, with a half-life of approximately 3 min [Steer and Klausner 1983; Klausner et al., 1985; Bursztajn et al., 1987]. Those in the Golgi area are considerably smaller (60–80 nm in diameter) and more nearly uniform in size [Keen, 1985; Bursztajn et al., 1987].

Cells also contain vesicles which lack a clathrin coat [Orci et al., 1986; Malhotra et al., 1989]. These nonclathrin coated vesicles (sometimes called noncoated vesicles) may play a role in exocytosis, transporting secretory and membrane proteins through the Golgi cisternae en route to the plasma membrane. Isolated nonclathrin coated vesicles are enriched in 160, 110, 98, and 61 KDa proteins [Serafini et al., 1991]. It is assumed that these proteins, referred to as α, β, γ, and σ, COPs, are the coat proteins and related vesicular proteins of nonclathrin coated vesicles [Duden et al., 1991]. Isolated clathrin coated vesicles have a similar set of major proteins consisting of clathrin heavy chain (180 KDa) and adaptor proteins of approximately 100 KDa, 50 KDa, and 17 KDa [reviewed by Keen, 1990; Pearse and Robinson, 1990]. Adaptors bind clathrin and appear to recognize a specific motif in the cytoplasmic tail of selected transmembrane receptors [Pearse, 1988; Trowbridge, 1991].

Coated vesicles participate in many biological functions, including endocytosis and membrane recycling at the neuromuscular junction [Huser and Reese, 1973], the steering of axon growth cones during developmental path finding [Bastiani and Goodman, 1984], intracellular sorting and secretion of protein [Friend and Farquhar, 1967; Steer and Klausner, 1983; Brodsky, 1988] and receptor-mediated endocytosis [Goldstein et al., 1979, 1985, 1988]. Coated pits and vesicles transport the majority of surface receptors; however, the subsequent intracellular sorting appears to depend upon the type of the receptor complex [Steer and Ashwell, 1990]. Asialoglycoprotein, LDL, and phosphomannosyl glycoprotein receptors all recycle, although their ligands undergo degradation in lysosomes. EGF receptors, like the muscle acetylcholine receptors, do not appear to recycle, and their final destination also appears to be the lysosome. Still other receptors, such as IgA, transcytose the cell and thus bypass the lysosomal proteases. Given the diversity of pathways known in other systems, we needed to develop specific tools to help us dissect vesicular membrane processing during postsynaptic development. Both coated and noncoated vesicles participate in protein transport, and removal of the clathrin coat enables the vesicle to deliver its contents to the endosome [Schmid et al., 1988; Griffiths et al., 1989]. We have therefore prepared monoclonal antibodies to both the membrane and the clathrin coat of coated vesicles. We have chosen the clathrin coated vesicle because its production has been well characterized compared to other intracellular compartments and it is one of the first compartments involved in membrane removal. Furthermore, the unique composition of the coated vesicles makes it relatively easy to isolate and identify. Moreover, during endocytosis coated vesicle lose their coat, and their membrane in some cases is reutilized. We have, therefore, prepared a panel of monoclonal antibodies against the clathrin coat and the membrane proteins to demonstrate the alterations these vesicles undergo during myotube differentiation and innervation.

MATERIALS AND METHODS

Isolation and Purification of Coated Vesicles

Using modified published procedures [Pearse, 1982; Bar-Zvi and Branton, 1986] we isolated coated vesicles from fresh bovine brains obtained from a local slaughterhouse and used within 1 h from the time of sacrifice. All procedures were carried out at 4°C. After removing the meninges we homogenized 500 gm of brain tissue in a Waring blender with three high speed 10 second bursts in two volumes of Buffer A [(0.1 M 2-N-morpholino) propanesulfonic acid (MES), 0.5 mM $MgCl_2$, 1 mM ethylene glycol bis (β-aminoethyl ether)-N,N ^{1}-tetra acetic acid (EGTA), 0.02% NaN_3, pH 6.5]. We obtained crude pellets of coated vesicles by centrifuging the homogenate for 30 min at 20,000*g*, collecting the supernatant and centrifuging it for 30

min at 34,000 rpm in a Beckman Type 35 rotor. We resuspended the pellets in 20 ml of buffer A, homogenized, mixed with an equal volume of a Ficoll-sucrose solution containing 12.5% (w/v) Ficoll-400 and 12.5% (w/v) sucrose in Buffer A, and centrifuged for 40 min at 19,000 rpm in a Beckman Type 35 rotor. Then we diluted the supernatant with three volumes of Buffer A, centrifuged for 1 h at 34,000 rpm, resuspended and homogenized the vesicle containing pellet in 3 ml of Buffer A, centrifuged for 5 min at 10,000*g* to remove aggregates, and loaded the mixture on a sephacryl S-1000 column (2.6 × 35 cm) pre-equilibrated with Buffer A. The 280 nm absorbance profile of the eluate showed two peaks. Because previous studies have shown that coated vesicles contain AchRs and AChE [Bursztajn and Fischbach, 1984; Porter-Jordan et al., 1986; Bursztajn et al., 1991], we collected 3 ml fractions, tested for the acetylcholinesterase [Ellman et al., 1961; Bursztajn et al., 1991], hexosaminidase [Hall et al., 1978], and NADH cytochrome C reductase [Mahler, 1955] activities, and pooled the fractions enriched in protein and AChE activity but low in hexosaminidase (a marker for lysosomes) and NADH cytochrome C reductase (a marker for mitochondria) activities. As shown in Figure 2, we obtained two protein peaks. We then pooled and rechromatographed the fractions in peak II which were enriched in protein and AChE activity. After obtaining a single peak from the second chromatography, we centrifuged it for 1 h at 34,000 rpm using a Beckman Type 35 rotor, and assessed the purity of the pellet of coated vesicles so obtained by SDS-polyacrylamide gel electrophoresis [Laemmli, 1970] and electron microscopy.

Enzymatic Assays

Acetylcholinesterase. As previously described [Berman et al., 1989; Bursztajn et al., 1991], we determined the acetylcholinesterase activity in a given fraction spectrophotometrically by adding the substrate, 0.075 M acetylthiocholine iodide, to a reaction mixture consisting of 0.1 M Tris-HCl, pH 8.9, 5 mM $CaCl_2$, 0.01 M 5,5'dithiobis-2-nitrobenzoic acid in 0.1 M Tris-HCl, pH 7.0, and 1.5 mg/ml $NaHCO_3$ (including tetra-isopropyl-pyrophosphoramide [iso-OMPA] 10^{-5} M, an inhibitor of nonspecific cholinesterase, to insure measurement of only AChE activity) and recording the absorbance at 412 nm (A_{412}).

Hexosaminidase. We performed the assay with 2.5 mM p-nitrophenyl-N-acetyl-β-D glucosaminide in 0.1 M sodium citrate buffer, pH 4.5, incubating for 30 min at 37°C and terminating the reaction by the addition of 0.75 ml of 0.2 M glycine buffer, pH 10.2. After centrifuging the mixture in a microfuge, we recorded the optical density of the supernatant (p-nitrophenol) at 400 nm. We obtained a calibration curve with several concentrations of freshly prepared p-nitrophenol in glycine buffer, pH 10.2.

NADH cytochrome C reductase assay. We assayed aliquots in 0.03 mM cytochrome C, 1.66 μM NaCN, 0.1 μM NADH and 10–50 μg of enzyme in 50 μM potassium phosphate buffer, pH 7.5, adding NADH to start the reaction and measuring product at 550 nm.

Preparation of Antibodies

We incubated coated vesicles in 0.75 M Tris-HCl buffer, pH 8.1, at 4°C for 1 h, sedimented them for 15 min in a Beckman airfuge at 130,000*g* and treated the pellet with 0.5% saponin in saline for 10 min. After washing the pellets three times with saline we applied them to a 7.5% SDS-polyacrylamide gel, excised the 180 K, 100 K, and 50 K bands identified by prestained molecular weight standards (Sigma), suspended them in 25 mM Hepes buffer, pH 7.5, and extracted overnight at 4°C. Using centrifugation at 1,000 rpm for 5 min, we separated the proteins from the gel, concentrated them with Amincon miniconcentrators, and produced a solution suitable for use as an immunogen.

We initially immunized female BALB/c mice (6–8 weeks old) subcutaneously with 50 μg of protein from each of the three protein bands emulsified in MPL + TDM (monophosphoryl lipid A + Trehalose dimycolate from RIBI Immunochemical Research) and then boosted the mice subcutaneously after 3, 7, and 11 weeks with proteins (50 μg) in emulsions of incomplete Freund's adjuvant. Two weeks after each boost elisa screening [Mayer and Walker, 1987] of antisera determined which mice gave the strongest reaction. Following the procedure of Debus et al., [1983], we fused their spleen cells with SP/2 myeloma cells, plated them in 96-well plates using HAT medium, and screened their supernatants by elisa. After twice subcloning colonies of interest using limiting dilution, we tested their supernatants by immunoblotting [for details of procedure see Ahle and Ungewickell, 1986]. For large scale production of mono-

clonal antibodies, we cultured hybridomas in large flasks and collected the supernatants or injected cells into mice for ascites production employing affinity chromatography on protein-A-sepharose according to manufacturer's instructions to purify the antibodies from the ascites fluid. We typed antibody subclasses with a kit obtained from Boehringer Mannheim Biochemicals. Typically, our 500 ml of supernatant yielded 12–25 mg of antibodies.

Electrophoretic Methods and Western Blots

We ran tris and saponin treated coated vesicles (180 K, 100 K, and 50 K) preparations on 7.5% SDS polyacrylamide gels (SDS-PAGE) [Laemmli, 1970] and either stained for protein or electrophoretically *trans*ferred proteins to nitrocellulose using a transfer apparatus (Bio-Rad). After incubation in PBS containing 0.05% Tween-20 (TPBS) and 5% powdered nonfat milk, we washed the immunoblots in TPBS and incubated them with the hybridoma supernatant (1:5 dilution) or purified antibodies obtained from ascites fluid (0.014 mg/ml). After further washings, we incubated the blots with anti biotinylated IgG, washed, and incubated for 1 h in vectastain ABC reagent (Vector Laboratories). Blots were washed in PBS and developed using 4-chloro-1-naphthol as a substrate.

Immunofluorescence Microscopy

As described previously [Bursztajn, 1984], we prepared cultured chick myotubes and ciliary neurons by removing pectoralis muscles from 11-day-old chick embryos, dissecting and plating them in collagenized 35 mm dishes (Falcon) containing coverslips. Three days after plating of muscle cells, we added ciliary neurons from 9-day-old embryos to the myotube cultures, maintained in Eagle's minimum essential medium (MEM) supplemented with horse-serum (10% vol/vol) and chick embryo extract (2% vol/vol). At various periods after plating, we fixed the cells for 30 min in 2% paraformaldehyde in a buffer containing 37.5 mM sodium phosphate, 0.75 M lysine, and 10 mM sodium periodate, permeabilized them with 0.1% Triton X-100 in PBS for 5 min, washed and incubated overnight at 4°C with C33 (180 K), C171 (100 K) or F22 (50 K) monoclonal antibodies. Then we washed the cells again and incubated them with biotinylated anti-mouse IgG followed by streptavidin Texas red as previously described [Bursztajn et al., 1989], and viewed them with a Zeiss epifluoroescence microscope and photographed with Kodak Tri-X film.

Electron Microscopy

To check the purity of coated vesicle fractions, we placed aliquots of fractions enriched in AChE activity on formvar coated grids and negatively stained with 2% uranyl acetate. We fixed cultured cells as described above and processed them for immunoelectron microscopy [Bursztajn et al., 1987; Park and Bursztajn, 1990] or fixed in 1% paraformaldehyde, 1% glutaraldehyde and processed for electron microscopic histochemistry [Bursztajn et al., 1991]. We postfixed the cells in 1% OsO_4, dehydrated in ethanol, and embedded in Epon. Thin sections were examined with a JEOL 100cx electron microscope.

RESULTS

Distribution and Purification of Vesicular Carriers

Coated vesicles participate in the transport of many proteins to and from the cell surface. In cultured muscle cells we find numerous coated vesicles (Fig. 1a) and certain populations of these vesicles contain both acetylcholinesterase (AChE) (Fig. 1b) and acetylcholine receptors (AChRs) (Fig. 1c). Our previous studies and those of others have shown that the vesicles initially transport these proteins from the Golgi complex, in which they initially reside, to the cell surface. At the end of this journey the AChRs reside in the lysosomes [Bursztajn and Fischbach, 1984; Porter-Jordan et al., 1986; Bursztajn et al., 1987; Park and Bursztajn, 1990]. Both coated and noncoated vesicles take part in the transport process. Because we found numerous coated pits during early stages of synapse formation [Bursztajn, 1984], we deemed it important to study the fate of these vesicles during muscle cell differentiation and at early stages of innervation.

Initial purification with a combination of Ficoll-sucrose gradients and chromatography on sephacryl S-1000 column produced two protein peaks (Fig. 2A), which we collected and assayed for lysosomes (hexosaminidase activity), mitochondria (NADH cytochrome C reductase activity), and coated vesicles (AChE activity). Enzymatic activity for AChE and electron microscopy indicated that fractions in peak number two (Fig. 2A) were enriched in coated vesicles. However, fractions in the second peak still contained some membrane contaminants, and therefore

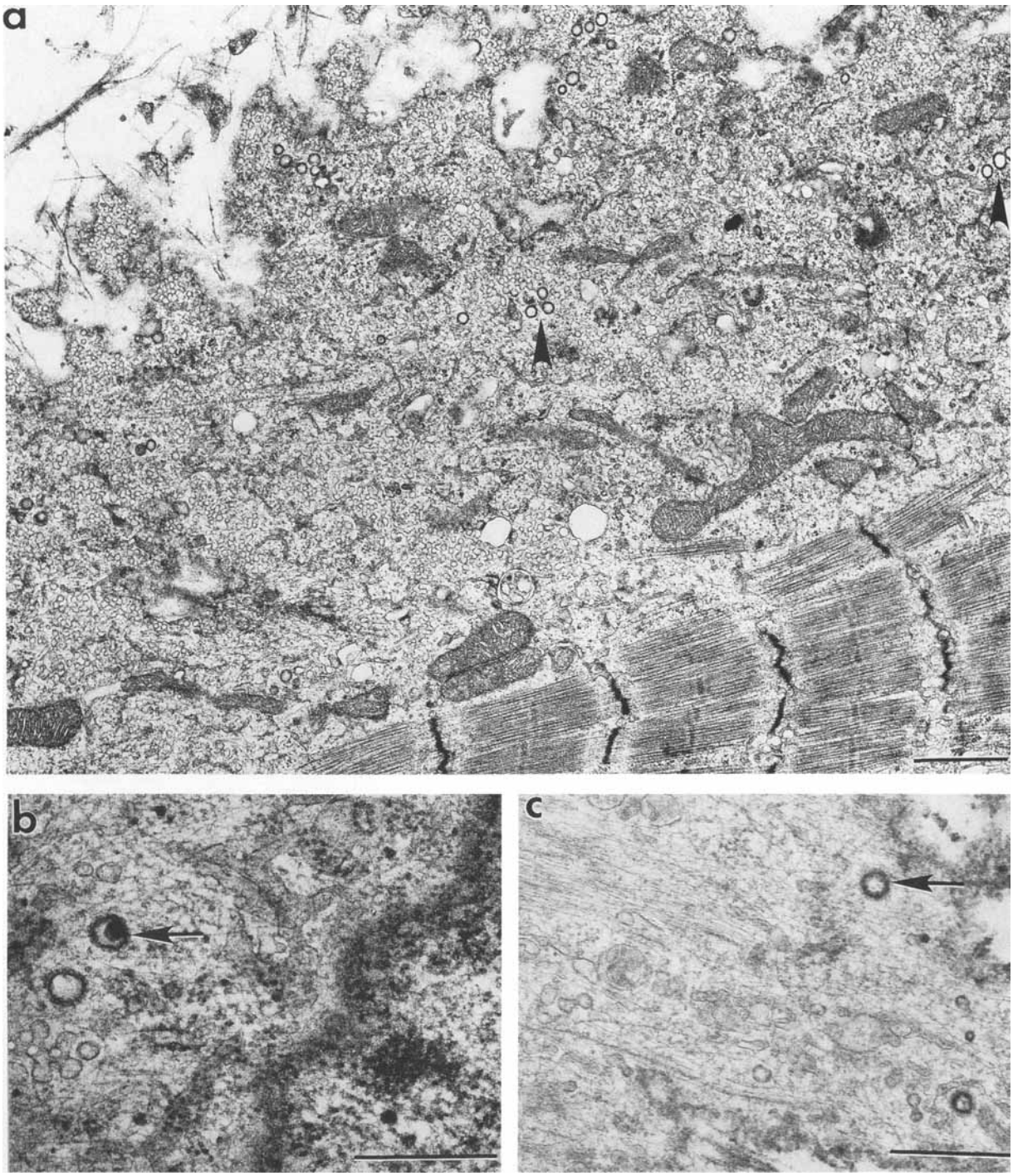

Fig. 1. Coated vesicles contain synaptic proteins. Cultured muscle cells processed for electron microscopy, 4 days after plating, show numerous coated vesicles scattered throughout the cytoplasm (**a:** arrowheads). Bar, 1 μm. Muscle cells stained for AChE show histochemical reaction product in coated vesicles (**b:** arrow). Muscle cells incubated with monoclonal antibody mAb 35, which binds to the immunogenic region of the AChR α-subunit, followed by streptavidin-HRP, show immunoreactivity in coated vesicles (**c:** arrow). Bar for b and c, 0.5 μm.

we pooled and rechromatographed them (fractions 33–48) on the sephacryl S-1000 column (Fig. 2B). The second elution profile generated one peak of protein, rich in AChE activity and devoid of lysosomal or mitochondrial contaminants according to both enzyme assays and electron microscopy. Electron micrographs of the fractions negatively stained with uranyl acetate revealed structures with polygonal coats characteristic of coated vesicles (Fig. 2c). In some cases

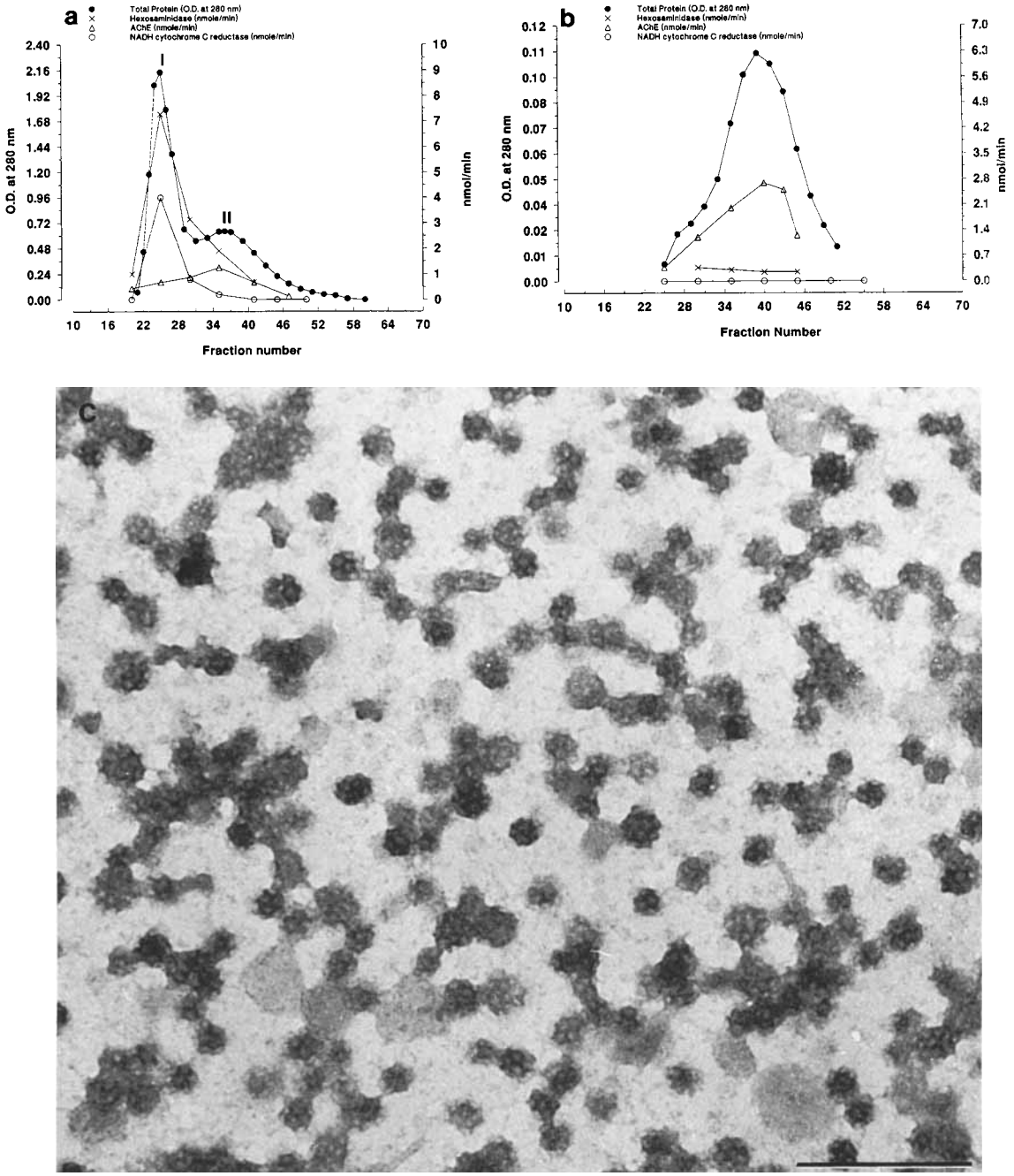

Fig. 2. Purification of coated vesicles from bovine brain. Coated vesicles were isolated by homogenization in a buffer containing 0.1 M 2-(N-morpholino) propane sulfonic acid, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.02% sodium azide, pH 6.5. **A:** After centrifugation and further homogenization, the homogenate was centrifuged in an equal volume of Ficoll-sucrose solution and passed over a sephacryl S-1000 column. Fractions were assayed for protein content, AChE activity, hexosaminidase, and NADH cytochrome C reductase. Two distinct peaks were observed. **B:** Fractions in the second peak were rechromatographed on the sephacryl S-1000 column and fractions enriched in AChE activity and protein content were collected and used as an antigen. **C:** Electron microscopy of fractions enriched for AChE activity. Negative staining with 2% uranyl acetate revealed a highly enriched population of coated vesicles. Bar, 0.5 μm.

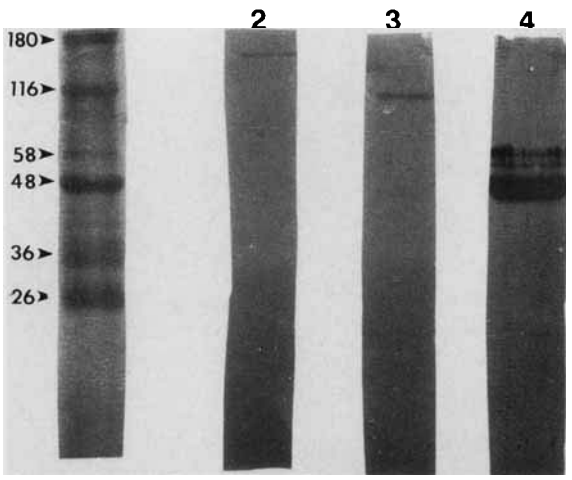

Fig. 3. Immunoblots of 180 K, 100 K, and 50 K proteins from coated vesicles. Affinity purified monoclonal antibodies C-33 (2), C-172 (3), and F-22 (4) were used to stain Western blots of proteins obtained from coated vesicles separated on denaturing SDS polyacrylamide gels. The mAb C-33 stains the 180 K band, mAb C-172 stains the 100 K band, and mAb F-22 stains a 50 K band and, in addition, a 55 K band. The blots were stained with VECTASTAIN ABC reagent, then washed and developed using 4-chloro-naphthol as substrate. Prestained standards consisting of triosephosphate isomerase 26.6 K, lactic dehydrogenase 36.5 K, fumarase 48.5 K, pyruvate kinase 58 K, fructose-6-phosphate 84 K, β-galactosidase 116 K, α_2 macroglobulin 180 K were used for comparison. The antibody F-22 labels a doublet which may be due to the ability of the antibody to recognize another polypeptide.

we could discern membranes beneath the coats, indicating that these clathrin cages surround a membrane. SDS-polyacrylamide gel electrophoresis showed four major bands. The dominant band was the 180 Kd clathrin heavy chain. Also present were proteins migrating at 100–110 Kd, 50–55 Kd, and 35–38 Kd. These molecular weights are consistent with the presence of adaptor complexes. We excised the 180 Kd, 100 Kd, and 50 Kd bands from the gel and used them to immunize animals. In some cases coated vesicles were incubated with 0.5 mM Tris, pH 8.8, to remove the clathrin coat [Porter-Jordan et al., 1986]. This procedure enhanced the production of antibodies to the noncoated vesicular membrane proteins.

Monoclonal Antibodies to Vesicular Polypeptides

We prepared microsomal membrane fractions from bovine brain and cultured skeletal myotubes and used them to screen the monoclonal antibodies using Elisa and Western blotting. The antibody (mAb) C-33 specifically recognized the 180 Kd protein (Fig. 3, lane 2). The mAb C-171 recognized the 100 Kd protein band (Fig. 3, lane 2) and mAb F-22 recognized 50 Kd and 55 Kd protein bands (Fig. 3, lane 4). The 55 Kd band may represent a polypeptide which is not a vesicular protein. The F-22 antibody was not characterized further in this study.

The presence of the 180 Kd and 100 Kd polypeptide in cultured chick myotubes was confirmed by Western blots. The postnuclear supernatants prepared from the myotubes were fractionated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blots using the antibodies prepared against clathrin and the 100 Kd vesicular proteins. Figure 4a shows that C-33 recognizes only a 180 Kd band, and C-172 recognizes only the 100 Kd band. This profile is very similar to that obtained from brain coated vesicles (Fig. 3). During the isolation of coated vesicles, tubulin may co-purify with the isolated proteins [Woodman and Warren, 1991]. To determine whether those antibodies recognize tubulin, we have purified tubulin from bovine brain and fractionated by SDS-PAGE (Fig. 4b). A doublet migrating at approximately 55 Kd can be seen (Fig. 4b). Immunoblots of proteins labeled with mAb C-33 or mAb C-172 revealed no immunoreactivity (Fig. 4b, lanes 2,3).

Localization of Vesicular Proteins Is Differentiation and Innervation Dependent

Four days after plating, immunofluorescent staining with mAb C-33 and mAb C-172 in differentiating myotubes and during early stages of innervation showed a punctate staining pattern characteristic of coated vesicles (Fig. 5a,c). We stained the nuclei with the DNA binding fluorescent dye bisbenzimide [Bruner and Bursztajn, 1986] and interchanged fluorescent filters to visualize both the nuclei and the vesicles [Fig. 5b,d]. Both antibodies showed an intense staining in the perinuclear area and finely punctate staining of the cytoplasm (Fig. 5a,c). The punctate staining encircling the nucleus suggests that some of these vesicles may be part of the Golgi cisternae. The antibody staining we have observed is specific because cells processed the same way as those shown in Figure 5a–d, except for omission of the primary antibody or incubation with irrelevant antibody, show no staining (Fig. 5e,f). Myotubes examined 8 days after plating and stained with mAb C-172 show punctate staining at opposite nuclear poles rather than a circular pattern (Fig. 6a). The presence of sarcomeres and the linear arrangement of nuclei indicate that these cells have fully differentiated

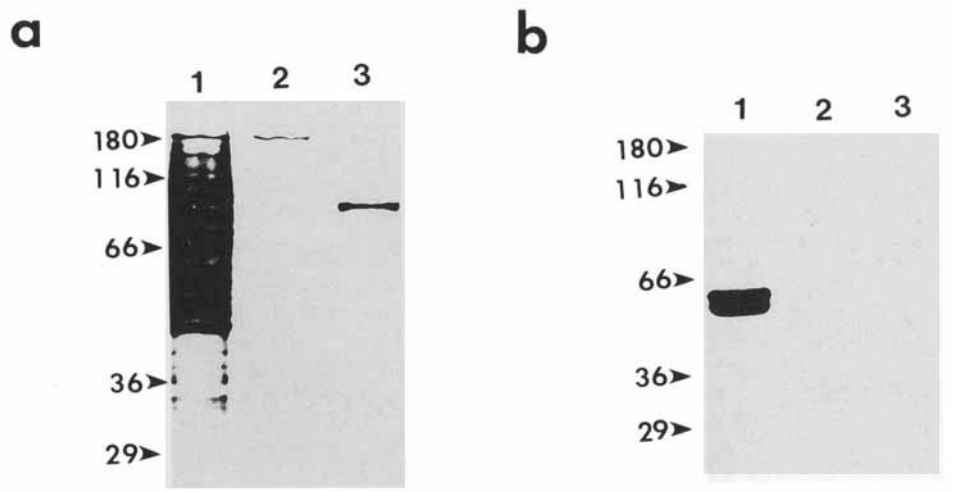

Fig. 4. Immunoblots of protein samples obtained from chick myotubes. **a:** Proteins were fractionated by SDS-PAGE, stained using *colloidal gold* (**lane 1**), immunoblotted using C-33 (**lane 2**), and C-172 (**lane 3**) monoclonal antibodies, and visualized using biotinylated second antibody and streptavidin-HRP, followed by peroxidase reaction. Arrows point to the following molecular weight standards: α_2 macroglobulin 180 K, β-galactosidase 116 K, bovine serum albumin 66 K, glyceraldehyde 3 phosphate dehydrogenase 36 K, and carbonic anhydrase 29 K. Note that monoclonal antibody C-33 recognizes only the 180 K clathrin heavy chains and C-172 recognizes only the 100 K vesicular protein. **b:** Monoclonal antibodies C-33 and C-172 do not recognize tubulin. We have purified tubulin from bovine brain and fractionated by SDS-PAGE (**lane 1**). Immunoblots of proteins labeled with mAb C-33 (**lane 2**) and mAb C-172 (**lane 3**) revealed no immunoreactivity.

(Fig. 6b,c). The mAb C-33 showed a similar staining pattern (figure not shown). When we co-cultured 8-day-old muscle cells with ciliary neurons, however, we found a redistribution of the 100 Kd vesicular protein. The punctate staining became perinuclear and the fluorescence appeared denser around the nuclei in these innervated cultures (Fig. 7) than in the noninnervated cultures. However, some nuclei have little or no punctate staining around them (Fig. 7a,b). The mAb C-172 heavily stained the myotube nuclei beneath the neuronal processes (Fig. 7b) as well as the ciliary neuronal soma. Such intense fluorescent staining of the neuronal soma made it impossible to resolve the punctate staining that was seen in the myotubes. We found few punctate speckles in the neuronal processes. Omitting the permeabilization reduced the number of fluorescent speckles with either mAb C-172 or C-33, suggesting a cytoplasmic location for many of the vesicular proteins.

DISCUSSION

For muscle cells to develop postsynaptic membrane specializations, appropriate structural molecules must target specific membrane domains. In many other systems, transport via coated and noncoated pits and vesicles figures prominently in sorting proteins and in transporting them to and from the cell membrane. Prior work has described at least two distinctive populations of coated vesicles which differ by both size and location. The larger coated pits and vesicles, at or near the plasma membrane, internalize certain membrane proteins via receptor-mediated endocytosis [reviewed by Goldstein et al., 1985], and in some cases, the membrane proteins recycle [Heuser and Reese, 1973; Patzak and Winkler, 1986; Valtora et al., 1990; Maycox et al., 1992]. The smaller coated vesicles lie near Golgi complexes and appear to help target proteins toward organelles such as lysosomes [Brown and Farquhar, 1984] and secretory granules [Orci et al., 1984].

In a limited number of cases, we know what tags proteins for transport to a specific organelle or plasma membrane domain. For example, a mannose-6 phosphate marker on lysosomal enzymes is required for their transport to the lysosomes [reviewed by Figura and Hasilik, 1986; Kornfeld and Mellman, 1989], and a C-terminal tetra-peptide KDEL is necessary for the retention of a group of soluble proteins in the lumen of the endoplasmic reticulum [Munro and Pelham, 1987]. The AChR receptor and the enzyme acetylcholinesterase are present in the Golgi complex [Fambrogh and Devreotes, 1978] and in coated vesicles where they are transported together to the cell surface [Bursztajn and Fischbach, 1984; Porter-Jordan et al., 1986], but we

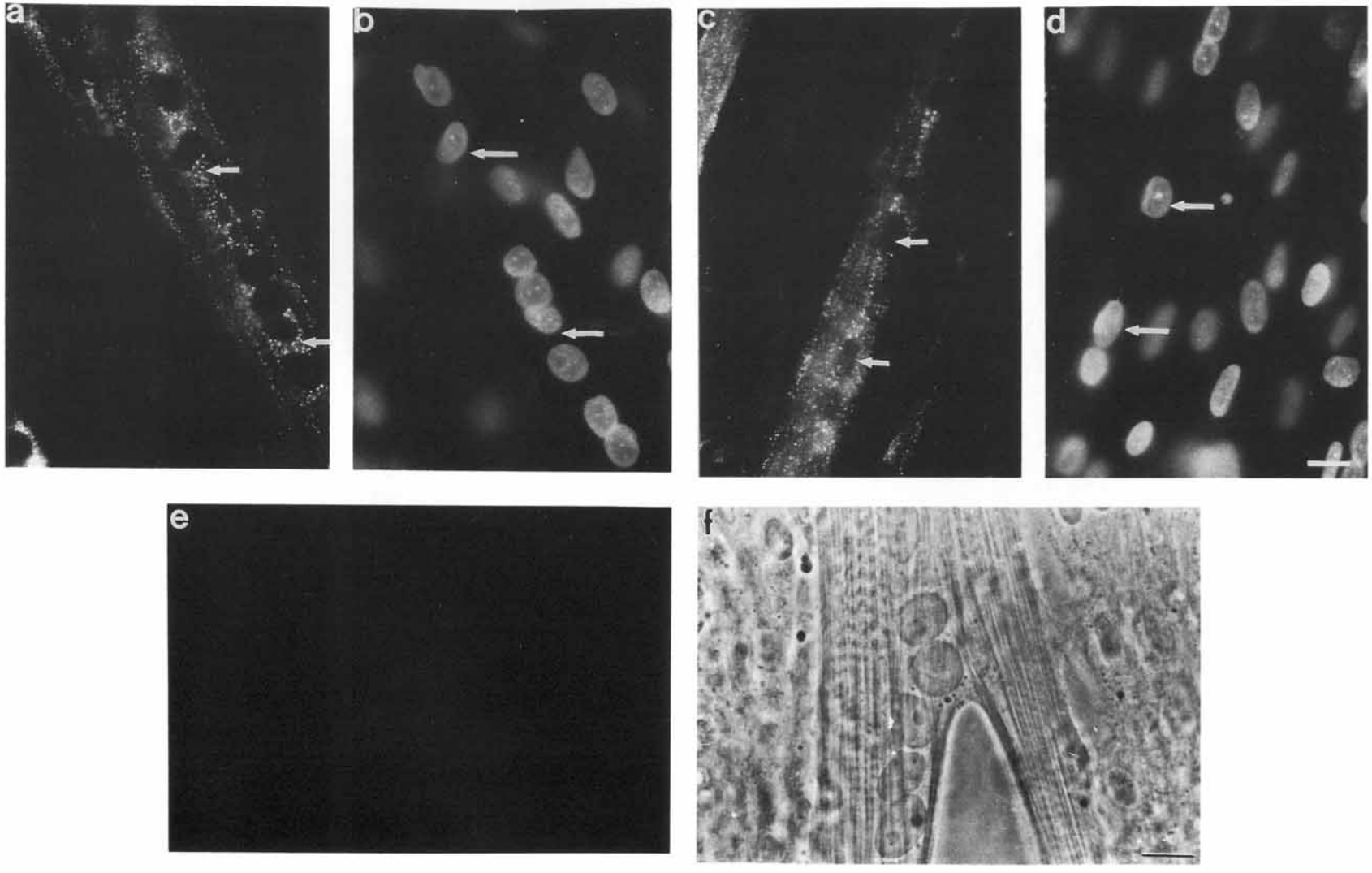

Fig. 5. Double label immunofluorescence of chick myotubes. Myotubes 4 days after plating were fixed and stained with monoclonal antibodies followed by anti-mouse IgG conjugated to Texas red and stained with bisbenzamide which binds to the nuclear DNA. **a:** Monoclonal C-33 gave punctate labeling throughout the cytoplasm with an apparent increase in the punctate structures around nuclei (arrows). Vesicles that lie in a different plane are out of focus. **b:** The same image as in a viewed with a fluorescent filter (BP365/LP420) which detects bisbenzamide staining of nuclei (arrows). **c:** Muscle cells from the same platings stained with mAb C-172. A similar punctate pattern as seen with mAb C-33 is observed. Arrows point to nuclei. **d:** The same image as in c viewed with a fluorescent filter that detects bisbenzamide staining of nuclei (arrows). **e:** Myotubes stained with an irrelevant monoclonal antibody (monoclonal antibody to neurofilament protein) show a lack of immunoreactivity. **f:** Phase contrast image of the same cells that were stained in e. Bar in a–f, 10 μm.

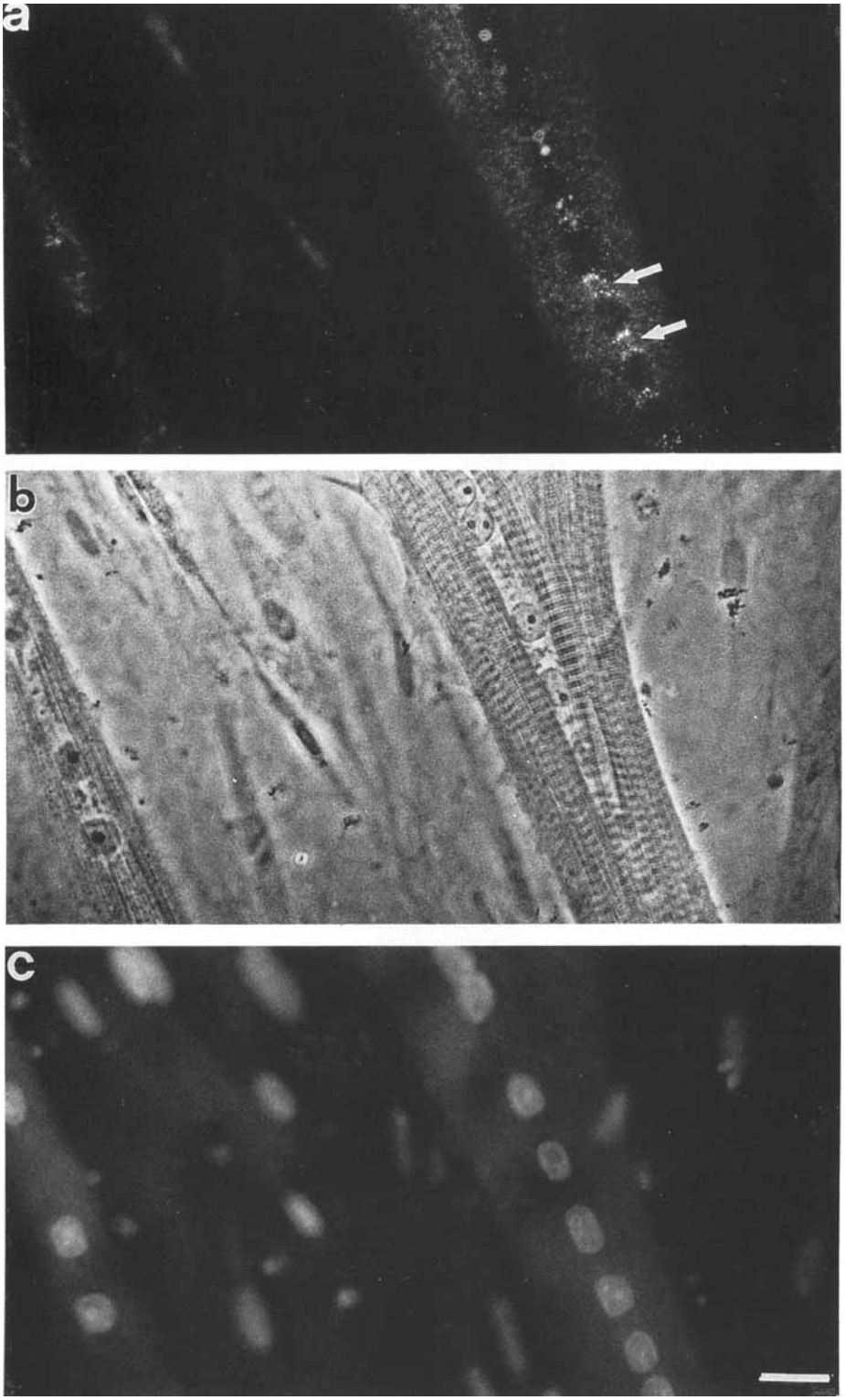

Fig. 6. Differences in staining patterns in differentiated myotubes. Muscle cells 8 days after plating stained with mAb C-172. **a:** Myotube stained with mAb C-172 and its fluorescence visualized with anti-mouse IgG–Texas red. Punctate structures are most predominantly concentrated at the opposite poles of the nuclei (arrows), giving a perinuclear orientation. Note that not all nuclei are surrounded by the punctate fluorescence. **b:** Phase contrast image of the same myotube as in a showing striations, which indicate the presence of sarcomeres. **c:** The same muscle cell showing nuclei stained with bisbenzamide. Bar in a–c, 10 μm.

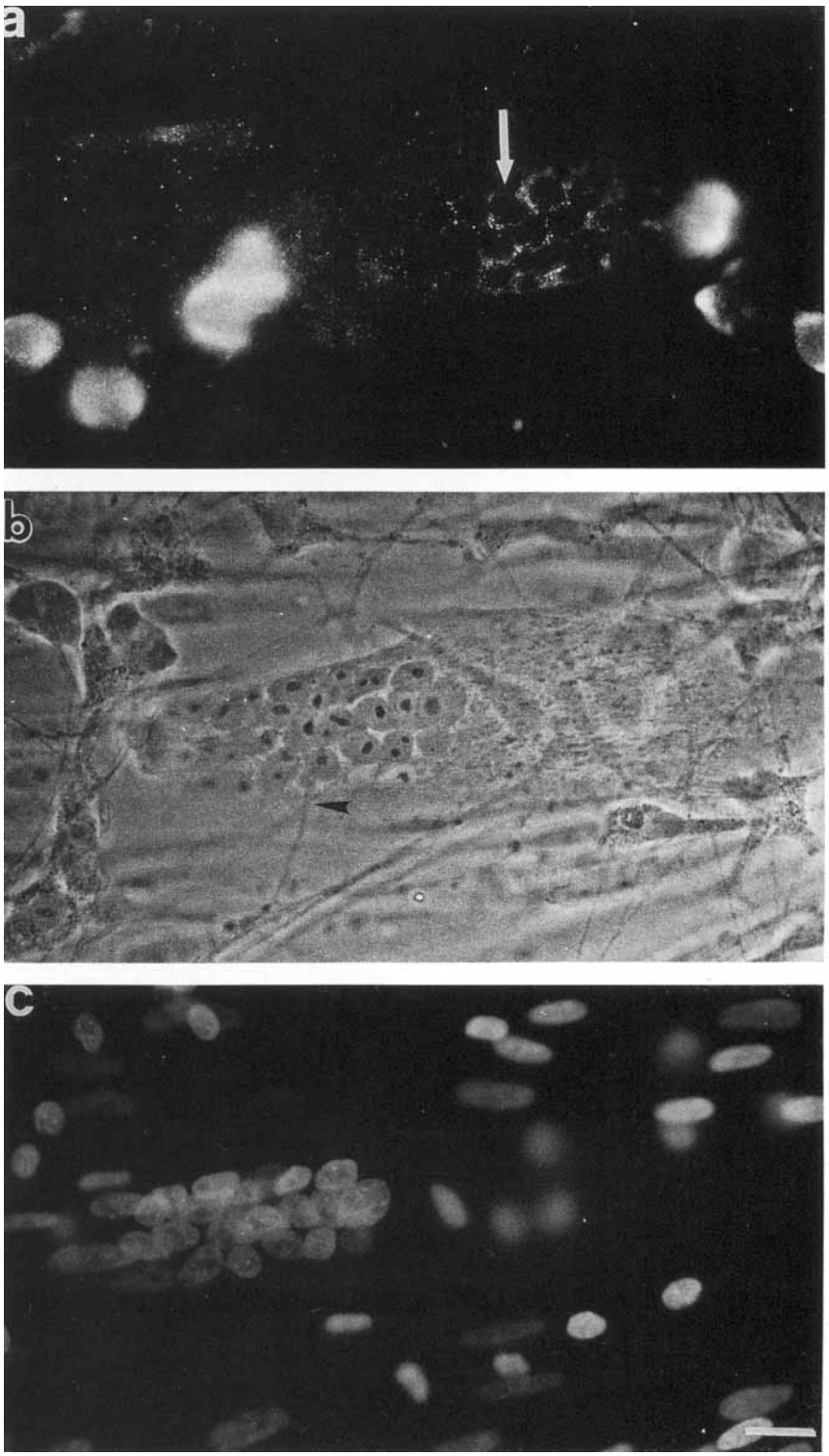

Fig. 7. Distribution of vesicular antigens in innervated myotubes. Muscle cells innervated by ciliary neurons 8 days after plating. **a:** Muscle cells stained with mAb C-172. Note the perinuclear punctate staining pattern (arrow). **b:** Phase contrast image of the same muscle cell as in a contacted by a few neuronal processes (arrowhead). **c:** The same muscle showing nuclei stained with bisbenzamide. Bar in a–c, 10 μm.

do not know what molecular markers direct them to these compartments. During endocytosis we find a high concentration of AChRs at coated pits which bud into the cytoplasm as coated vesicles. These quickly lose their coat and fuse with another compartment such as the endosome or lysosome [Bursztajn et al., 1987, 1991; Park and Bursztajn, 1990]. The coat protein is thought to be reutilized; however, the acetylcholine receptors are not known to recycle.

Can we discern specific patterns of vesicular involvement in the early stages of postsynaptic remodeling? To address this question we prepared monoclonal antibodies to isolated coated vesicles enriched for AChE enzymatic activity. We obtained three monoclonal antibodies, one of which recognizes the 180 Kd protein clathrin, and the other two recognize bands of 100 Kd and 50 Kd, the molecular weights of two vesicular adaptor proteins. The clathrin triskelions comprise the characteristic honeycomb lattice, and the 100 Kd adaptor proteins constitute the inner component of the coat. Presumably, the adaptors interact with the membrane of the vesicles containing the receptor molecules [Pearse and Crowther, 1987; Pearse and Robinson, 1990]. The adaptor proteins have been implicated in the sorting of vesicular cargo, as well as in supplying a signal or an "address label" directing the vesicle to a certain organelle or plasma membrane domain [Pearse and Robinson, 1990]. The Golgi complex coated pits, for example, contain different adaptor proteins than do coated pits of the plasma membrane [Chin et al., 1989].

The staining pattern we have obtained with Ab172, the 100 Kd protein, resembles the staining pattern obtained with the antibody to clathrin. This pattern of staining has been also reported with other adaptor proteins in a variety of cells. However, in muscle we observed a more restricted staining pattern [Robinson and Pearse, 1986; Robinson, 1987]. This may be due to the selective way we have isolated the coated vesicles in preparing our monoclonal antibodies. In our isolation procedures, we have collected fractions of coated vesicles enriched in the enzyme acetylcholinesterase (AChE). Our previous studies have shown that not all coated vesicles contained AChE enzyme activity [Bursztajn et al., 1991]. In the present study we have enriched for those vesicles capable of AChE transport and have made monoclonal antibodies to them.

In skeletal muscle, a distinct difference between endocytic and exocytic coated vesicles has been reported [Helmy et al., 1986; Porter-Jordan et al., 1986]. These investigators used an acetyl-cholinesterase-mediated density shift technique which allowed them to show that 75% of coated vesicles isolated from embryonic chick skeletal muscle were exocytic [Porter-Jordan et al., 1986]. Although we do not know what population of coated vesicles are endocytic or exocytic in our cultured muscle cells, it is of interest to note that the distribution of the 100 Kd vesicular proteins is more random and appears more highly concentrated in 4-day-old myotubes than 8-day-old myotubes. This observation is consistent with the idea that growing cells in the process of differentiation require more vesicular membrane which may be utilized for the addition of new membrane containing receptor molecules, or secretory proteins. The existing evidence, showing the involvement of coated vesicles in the transport of AChR and AChE [Bursztajn and Fischbach, 1984; Porter-Jordan et al., 1986; Bursztajn et al., 1987; Park and Bursztajn, 1990], indicates that during differentiation more vesicular membrane protein may be required for transport of crucial membrane markers to the proper destination or specific membrane domain. AChR clusters comprise one of the distinct membrane domains in cultured muscle cells. The coated vesicles appear to accumulate within the clusters [Pumplin and Bloch, 1990]. Whether there is an increase in exocytic vesicles at these membrane domains remains unknown.

Our present studies reveal that vesicular protein distribution depends on cell differentiation and innervation. In fully differentiated muscle cells (cells with sarcomeres), the vesicular proteins have a selective distribution with a frequent localization at opposite poles of the nucleus, whereas in undifferentiated cells the distribution is more diffuse. Innervation of muscle cells with ciliary neurons, which are known to form physiologically functional synapses, results in rearrangement of vesicular protein to form a circumferential distribution encircling the myotube nuclei. Although we do not know the nature of the protein(s) that confer the sorting signals, we think that specific interactions between the cytoplasmic domain and the contact with the neuron or factors released by the neuron may contribute to these topographic differences. Thus cellular differentiation as well as the initial contact of a neuron with muscle

cells may require rapid membrane addition and cytoskeletal assembly. This could result in changes in membrane fluidity which would facilitate addition of new membrane. Further experiments will allow us to determine whether the coated vesicles seen around the nuclei of muscle cells represent regions of newly synthesized membranous components containing molecules which can recognize signals from the cholinergic neuron and participate in postsynaptic membrane remodeling.

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