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Distribution of Calcium ATPase in the Sarcoplasmic Reticulum of Fast- and Slow-Twitch Muscles Determined with Monoclonal Antibodies

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Summary. Four monoclonal antibodies against the calcium ATPase in sarcoplasmic reticulum (SR) of rabbit fast-twitch skeletal muscle were characterized using SDS-PAGE, Western blots and immunofluorescence. The ultrastructural distribution of the antigens was determined using post-embedding immunolabeling. The antibodies recognized the calcium ATPase in the SR but not in transverse (T-) tubule or plasma membranes. The antibody, D12, had the same binding affinity for the calcium ATPase from fast-twitch (rabbit sternomastoid) and slow-twitch (rabbit soleus) fibers and the affinity fell by 30% after fixation for electron microscopy in both types of muscle fiber. Ultrastructural studies revealed that the density of D12 antibody binding to the terminal cisternae membrane of extensor digitorum longus (edl) and sternomastoid fibers was on average seven times greater than in the slow-twitch soleus and semimembranosus fibers. Since the affinity of the ATPase for the antibody was the same in SR from fast- and slow-twitch muscles, the concentration of calcium ATPase in the terminal cisternae membrane of fast-twitch fibers was seven times greater than in slow-twitch fibers. This conclusion was supported by the fact that the concentration of calcium ATPase in light SR membranes was six times greater in SR from fast-twitch fibers than in SR from slow-twitch fibers. The results provide strong evidence that the different calcium accumulation rates in mammalian fast- and slow-twitch muscles are due to different concentrations of calcium ATPase molecules in the SR membrane.

Key Words calcium ATPase · monoclonal antibodies · skeletal muscle · sarcoplasmic reticulum

Introduction

Skeletal muscle relaxes when calcium ions are removed from the sarcoplasm by the calcium ATPase in the SR membrane. The aim of this study was to characterize monoclonal antibodies to the calcium ATPase in rabbit fast-twitch muscle and to use the antibodies to study the distribution of the ATPase in mammalian muscles with different contraction speeds.

The distribution of calcium ATPase in mammalian muscles is important in understanding the different calcium accumulating capacities of isolated SR which are 4 to 11 times greater in fast-twitch fibers than in slower fibers (Sreter & Gergely, 1964; Heilmann et al., 1977). This may result from the specific activity of the calcium ATPase, the membrane density of the ATPase, or amounts of SR membrane in the fibers. Antibody studies suggest that the ATPase is not identical in all muscles (DeFoor et al., 1980; Damiani et al., 1981; Volpe et al., 1982), while polyclonal antibody and freeze-fracture studies suggest that there is 50% less ATPase in slow-twitch fibers (Beringer, 1976; Bray & Rayns, 1976; Heilmann, Müller & Pette, 1981; Jorgensen et al., 1982*b*). In contrast, ATPase crystal lattices (Dux & Martonosi, 1984) suggest that the different ATPase activities depend on the greater amount of ATPase containing membrane in faster fibers (Eisenberg & Kuda, 1976; Eisenberg, 1983). However, these differences in calcium ATPase density and SR content are too small to account for the different calcium-accumulating capacities in fast- and slow-twitch fibers. Either estimates of ATPase densities are in error, or the calcium ATPase is specific to fiber type.

In this study, we use monoclonal antibodies to determine the distribution of calcium ATPase in fibers from the fast-twitch, rat edl or rabbit sternomastoid muscles and slow-twitch rat and rabbit soleus muscles. The results show a 6- to 10-fold higher density of calcium ATPase in the fast-twitch muscles.

Materials and Methods

Muscle Preparation

Muscles were taken from rabbits (Canberra semi-lops), rats (Wistar) and toads (*Bufo marinus*) and dissected in Krebs solution (Dulhunty & Gage, 1985) into 0.5-mm fiber bundles for fluo-

rescence and electron microscopy. Sternomastoid muscles, used to prepare SR vesicles, are uniformly fast-twitch in rats (Dulhunty & Dlutowski, 1979) and in rabbits where (i) there are many indentations, 9.12 ± 0.23 (mean ± SEM); 628 junctions) per μm of terminal cisternae (Dulhunty & Valois, 1983) and (ii) twitches are fast: at 20°C, contraction times were 220 msec in soleus, 52 msec in edl and 49 msec in sternomastoid (*see* Table 3).

SR Vesicles for Antigen Isolation

Heavy SR was isolated from rabbit sternomastoid muscle (Saito et al., 1984). 'Triad rich' pellets (fractions 3 and 4) were washed twice in pyrophosphate buffer (Mitchell et al., 1983), homogenized in a Teflon pestle tissue grinder (Thomas) and repelleted (100,000 × *g* for 1 hr at 4°C) to remove residual filaments.

Monoclonal Antibodies

Kohler and Milstein (1975) and Banyard (1984) describe antibody and cell culture methods. Female mice [(Balb/c, CBA)F1] received three intramuscular injections of 'triad rich' fraction (the first emulsified in Freund's complete adjuvant and the others in Freund's incomplete adjuvant) and, three days before fusion, one intravenous injection of triad fraction in phosphate-buffered saline (PBS) containing (mM): Na_2HPO_4, 70; NaH_2PO_4, 26; NaCl, 138.

Antibody Assays

Dilutions and washes were in PBS. Microtitre wells (Removawell, Dynatech) were coated with microsomal fraction for 30 min, washed twice, blocked with 5% bovine serum albumin (Sigma) for 30 min, washed, exposed to the primary antibody for 1 hr at 4°C and washed three times before and once after a 1-hr exposure to the secondary antibody (100,000–200,000 cpm/50μl/well, iodinated with ^{125}I; Banyard, 1984), and radioactivity was measured. Antibodies that bound more strongly to fractions 3 and 4 than to microsomal fractions were studied further.

An enzyme-linked (ELISA) antibody conjugated with horseradish peroxidase was used to estimate relative numbers of D12 antibody binding sites on fast- and slow-twitch calcium ATPase and orthophenylene diamine was used as a substrate. Wells were coated with 10 dilutions of 5 μl of microsomal fraction from rabbit sternomastoid muscle or 15 μl of fraction 2 of sucrose gradients of microsomes from rabbit soleus muscle. The volumes were calculated from volumes required to give equivalent densities (measured with densitometry) of ATPase on SDS-PAGE gels. The wells were challenged with nonlimiting concentrations of D12 and second antibody and the optical density measured at 450 nm with a reference at 630 nm.

To estimate the relative binding affinity of D12 for the fast- and slow-twitch ATPase, one concentration of microsomal fraction or fraction 2 was challenged with an antibody dilution series (1×10^{-7} M to 5×10^{-10} M) and the amount of bound antibody measured with ELIZA. The sensitivity of calcium ATPase to denaturation during fixation for electron microscopy was evaluated by determining the amount of antibody bound to microsomal fraction or fraction 2 after incubation in the fixative solution (below) for 0 to 3 hr at 4°C.

Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immuno-Blotting

Standard methods (Laemmli, 1970; Tobin, Staehelin & Gordon, 1979) were used. All dilutions and washes were in Tris-buffered saline (TBS: Tris/HCl (pH 7.4), 25 mM; NaCl, 140 mM, Na azide, 0.02%). 20% powdered skim milk blocked nonspecific binding. The blots were washed twice with 0.05% tween-20 and then in TBS after exposure to each antibody (Bers & Garfin, 1985). The second antibody was either alkaline phosphatase- or horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (Silenus Laboratories). In some cases reactions were developed with 4-chloro-1-naphthol (Sigma) or 3,3′-diaminobenzidine (Sigma).

Immunofluorescence Studies

Fresh or fixed (in 0.1% glutaraldehyde/Krebs) muscle fibers were frozen at rest length, sectioned and mounted on gelatine-coated slides, incubated for 15 hr in culture supernatants, washed in PBS, incubated for 1 hr with fluorescein-conjugated rabbit anti-mouse antibody (Dako), washed and mounted in 50% glycerol in PBS. The slides were viewed under a Leitz Orthoplan Microscope with fluorescence and camera attachments.

Immunoelectron Microscopy

Muscles fixed (0.1% glutaraldehyde/2% paraformaldehyde in Krebs for 1 hr) at rest length were embedded in L.R. Gold Resin (London Resin Company) using recommended procedures. Dilutions and rinses of sectioned material were in 0.5% Tween-20 in PBS. Grey or silver sections were mounted on formvar-coated grids, blocked for 15 hr with 50% fetal calf serum in Dulbecco's Modified Eagle Medium (Gibco Laboratories), rinsed, exposed to the first antibody (filtered and diluted 1 : 1) for 1 hr, washed, exposed to the second antibody (5 nm gold-labeled goat anti-mouse antibody: Janssen Pharmaceuticals) for 1 hr, washed in distilled water and stained with uranyl acetate.

Distribution of Calcium ATPase in the SR Membrane

The distribution of calcium ATPase was measured on micrographs of immunolabeled thin sections. The D12 antibody had a similar affinity for calcium ATPase from fast- and slow-twitch muscles and antigenicity was depressed by the same amount after fixation (*see* Results). Therefore the distribution of gold-conjugated antibody bound to D12 was used to measure the distribution of calcium ATPase in the SR membranes. The total area of SR differs in rat edl and soleus fibers (Davey & Wong, 1980) so that the gold particle density in a micrograph could not be used to assess the densities of calcium ATPase in the SR membrane. However, the gold particle density in the terminal cisternae provided a quantitative measure of the relative densities of calcium ATPase in the SR membranes of edl and soleus fibers. The methods used to count gold particles are outlined in Fig. 1. The T-tubules were swollen after the light fixation and were clearly defined (*TT*, Fig. 1*A*). The tramline appearance of triad junctions (*TJ*, Fig. 1*A*) marked the tubular border of the terminal cisternae. The profile of the terminal cisternae could sometimes be seen,

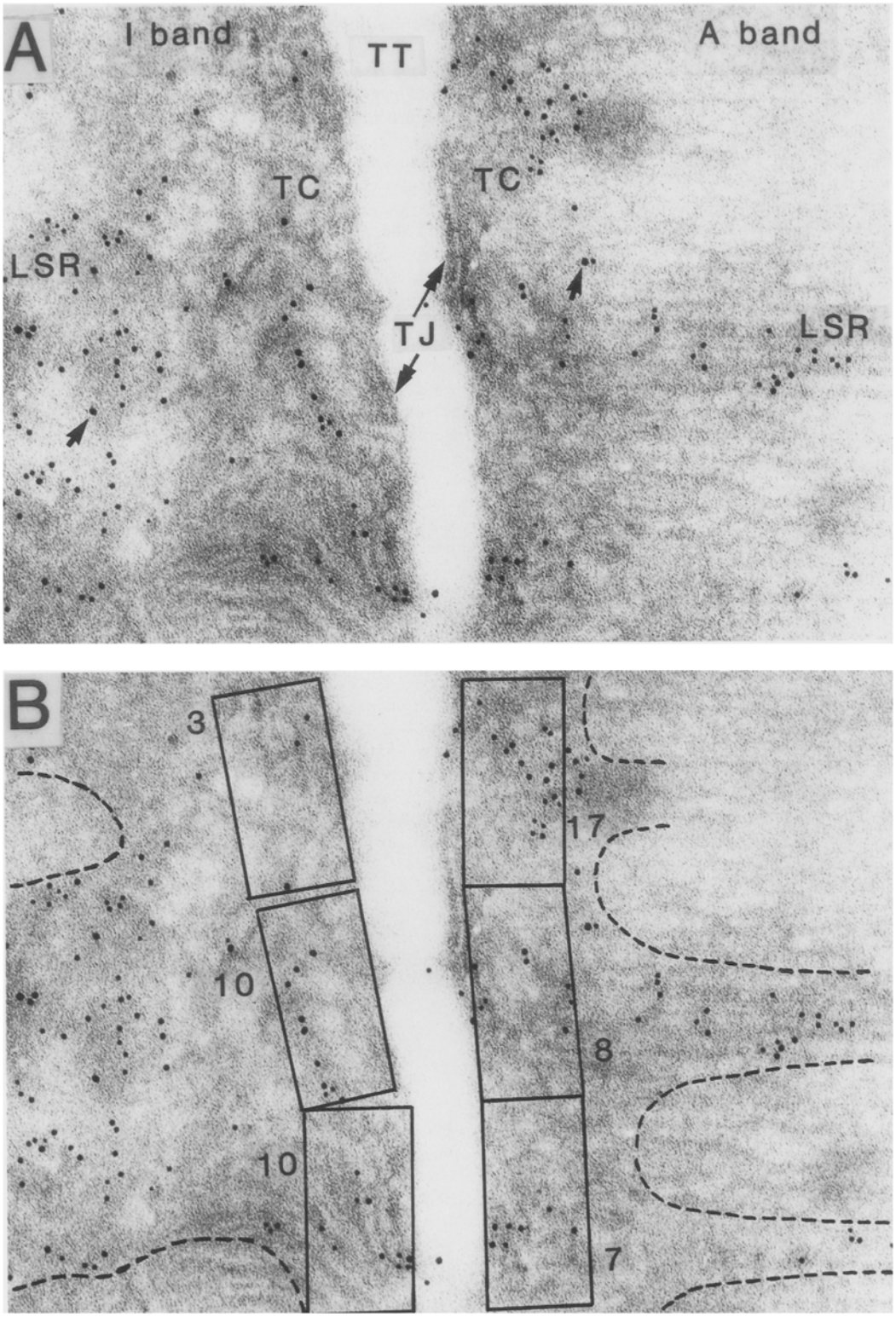

Fig. 1. Methods used to measure the density of gold particles in the terminal cisternae membrane. *A* shows a typical micrograph labeled first with the D12 antibody and then with the gold-conjugated second antibody. The small black dots (single headed arrows) are 10-nm gold particles. *TT* marks a transverse tubule, the double-headed arrows extending from *TJ* point to two clearly sectioned triad junctions, *LSR* marks longitudinal SR and *TC* mark terminal cisternae. Note that, as previously described (Eisenberg, 1983), there is significantly more longitudinal SR and gold labeling in the I band than in the A band. The approximate borders of SR and terminal cisternae have been outlined with a broken line in *B* and 100 × 200 nm rectangles drawn within the terminal cisternae. The number of gold particles counted within each rectangle are shown. The magnification is ×150,000

but the membrane was usually not clearly defined. It was assumed that areas extending 100 nm in a longitudinal direction from the triad junction, covering areas seen to be occupied by terminal cisternae, contained terminal cisternae membrane sectioned in a plane that was coincident with the plane of the section. This assumption was justified by the fact that thin section (Eisenberg, 1983) and freeze-fracture (Dulhunty & Valois, 1983) images show the terminal cisternae extending longitudinally for at least 150 to 200 nm from the triad junction, before branching into longitudinal SR. Rectangular areas, 100 × 200 nm, were drawn over the terminal cisternae with one long edge abutting the junctional gap (Fig. 1*B*) and the numbers of gold particles within the areas counted.

The densities of gold particles in the 100 × 200 nm rectangles are proportional to the relative densities of calcium ATPase if the rectangles cover the same area of membrane in edl and soleus fibers, i.e., if the membrane geometry is the same in the two types of fiber. The errors in this assumption are minimal in rat edl and soleus fibers since the structure of the terminal cisternae is similar in the two types of muscle (Dulhunty & Valois, 1983; Dulhunty, Gage & Lamb, 1986). Shallow indentations are more numerous in edl but do not much increase the surface area and only a small part of their structure is located within 100 nm of the triad junction (Dulhunty & Valois, 1983).

The measurements of gold particle density provide a quantitative measure of relative densities of calcium ATPase in the membrane but not the absolute density of calcium ATPase because (a) the true area of membrane within the rectangles is unknown, (b) some antigenicity is lost during fixation (*see* Results) and (*c*) the antibodies react only with antigenic sites close to the surface of the thin section. The density of particles in the terminal cisternae is proportional to the density of calcium ATPase in the SR membrane.

In early studies, 10 to 15 photographs were taken of different areas in each fiber at a magnification of 25,000. The micrographs were enlarged three times and 10 to 30 rectangular areas such as those shown in Fig. 1*B* counted on each micrograph. The average densities of gold particles proved to be remarkably constant in each micrograph from one fiber, and plots of running averages showed that a constant average was achieved after counting 4 to 5 micrographs. Thereafter, six micrographs were analyzed in each fiber. The selection of areas to photograph was based on the quality of fixation and sectioning of the tissue and the orientation of the triad junctions to the plane of section. Longitudinally sectioned junctions such as that illustrated in Fig. 1 were selected.

It could be argued that the results were subject to error because of differential effects of fixation on different types of fiber, arising from differences in fiber size or in muscle geometry. Such errors were avoided because (a) rat edl and soleus fibers have similar diameters (Dulhunty et al., 1986) (b) all micrographs

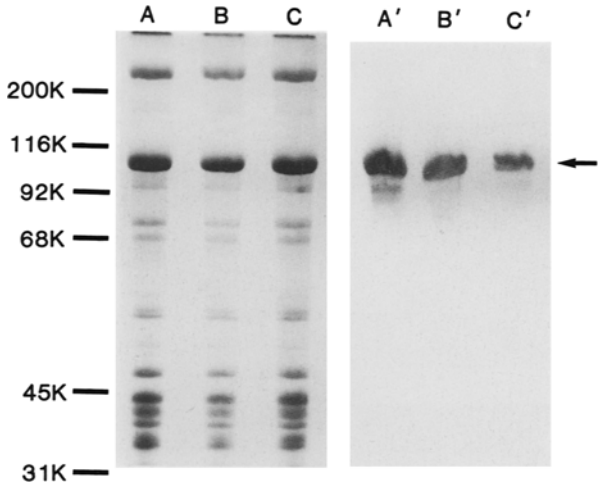

Fig. 2. Specificity of the monoclonal antibody D12 for the calcium ATPase. Lanes *A*, *B*, and *C* show Coomassie Blue staining of SDS-PAGE electrophoresis of microsomal fractions from rabbit sternomastoid muscle: 20 μl of microsomal fraction were used in *A*, 2 μl in B and 10 μl in *C*. Lanes *A′*, *B′* and *C′* show Western blots of the muscle proteins in *A*, *B*, and *C*, labeled with D12 and counterlabeled with horseradish peroxidase-conjugated sheep antimouse immunoglobulin. The arrow indicates the position of the calcium ATPase. The mass of molecular mass standards are shown (×10³) on the left

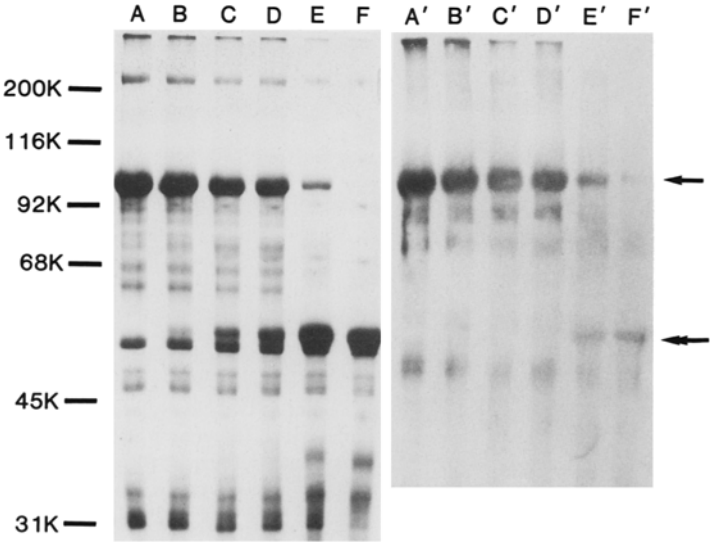

Fig. 3. Effect of tryptic digestion on proteins from microsomal fractions of rabbit sternomastoid muscle, showing the transfer of immunoreactivity from the calcium ATPase (100,000 daltons) in the control gels, to a 55,000-dalton peptide which appears after tryptic digestion. Lanes *A* to *F* show muscle proteins separated from 40 μl of microsomal fraction with SDS-PAGE electrophoresis and stained with Coomassie Blue. *A*, control, no trypsin. Times in trypsin (min): *B*, 0; *C*, 1; *D*, 2; *E*, 10; *F*, 30. Lanes *A′* to *F′* show Western blots of lanes *A* to *F*, stained with the D12 antibody and counterlabeled with horseradish peroxidase-conjugated sheep antimouse immunoglobulin. The single arrow shows the calcium ATPase and the double arrow marks the 55,000-dalton peptide, which appears after tryptic digestion. The mass of molecular mass standards are shown (×10³) on the left

were taken within 5 μm of the fiber surface where preservation was best, and (c) all muscles were dissected into 5-mm bundles of fibers before fixation.

Results

Characterization of Monoclonal Antibodies

Four monoclonal antibodies (A9, B11, D12 and F2) to the calcium ATPase in the SR were identified using the criteria outlined below. D12 showed the strongest immunoreactivity and will be described in most detail.

SDS-PAGE and Western Blotting

The antibodies recognized a single antigen at 100,000 daltons (Fig. 2), i.e, the molecular mass of SR calcium ATPase (Meissner & Fleischer, 1974; Jorgensen et al., 1982; Saito et al., 1984; MacLennan et al., 1985). Peptides of 45,000 and 55,000 daltons appear after tryptic digestion of the calcium ATPase (Stewart, MacLennan & Shamoo, 1976) and the antigenic site for the D12 antibody was transferred to the 55,000-dalton band (Fig. 3).

The SR calcium ATPase was relatively insensitive to endogenous proteolytic degradation: treatment of microsomal material for 16 hr at 37°C was required for significant breakdown, and this was accompanied by loss of immunoreactivity, suggesting that endogenous proteolysis destroyed the D12 antigenic site.

Immunofluorescence

Longitudinal sections of sternomastoid fibers, labeled with the antibodies (Fig. 4*B*), had striations of the same frequency as the sarcomeres bands seen with hematoxylin-eosin staining (Fig. 4*C*). The fibers showed little endogenous fluorescence and nonspecific activity was undetectable (Fig. 4*A*).

Fluorescence was concentrated in transverse bands with longitudinal extensions into low activity areas separating the high activity areas (Fig. 4*B*). Colabeling with D12 and an alpha-actinin antibody (Fig. 4*D*) showed that the D12 antigen was mostly in the I band, which contains the greatest density of SR (Eisenberg & Kuda, 1976; Eisenberg, 1983) and the highest concentration of calcium ATPase. Therefore the fluorescence pattern was consistent with the antibody binding to the calcium ATPase in the SR membrane. The four antibodies showed similar patterns of fluorescence in fresh and lightly fixed (0.1% glutaraldehyde) muscle.

Electron-Microscopic Studies

Nonspecific antibody binding to thin-sectioned muscle was low: a few gold particles are visible in the control micrograph in Fig. 5*A* which was chosen because it provided an unusual example of nonspecific activity. In contrast, there was a high density of particles, particularly in the I band, of sections exposed to the D12 antibody (Fig. 5*B* and *C*). Mem-

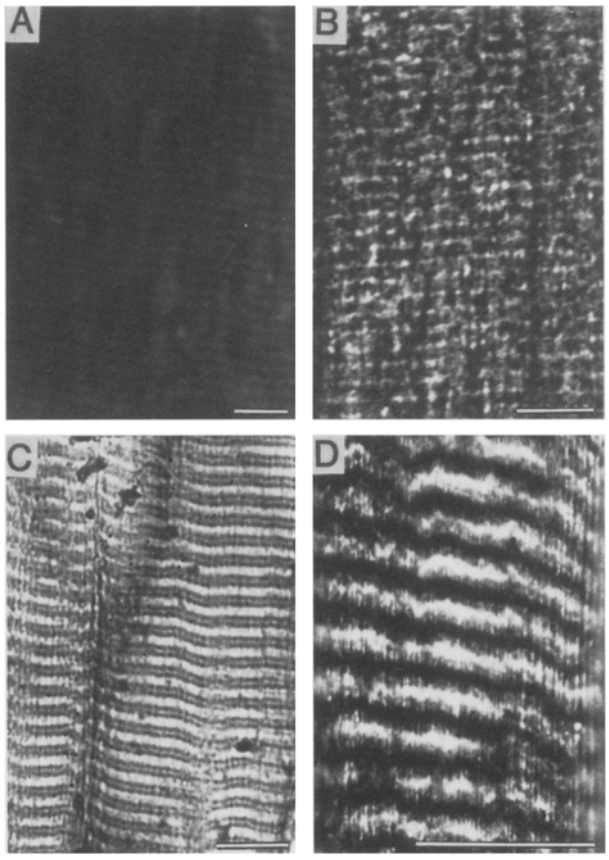

Fig. 4. Immunofluorescence localization of the D12 antigen in rabbit sternomastoid muscle. *A*, *B*, and *C* are light micrographs of frozen longitudinal sections of fixed (0.1% glutaraldehyde) muscle fibers, labeled with primary antibody and counterlabeled with fluorescein-conjugated rabbit anti-mouse antibody. *A* is a control section incubated with a nonmuscle primary antibody, showing very low background activity. *B* is a section incubated with D12 as the primary antibody, showing a striated fluorescence staining pattern. *C* is a light micrograph of a frozen longitudinal section of fixed muscle fibers stained with hematoxylin and eosin to show the sarcomere striations. *D* is a section of unfixed muscle fibers, which was primarily incubated with D12 and an alpha actinin antibody. The section shows the coincidence of the two antibodies in the I band of the muscle fibers. The calibration bars are 20 μm

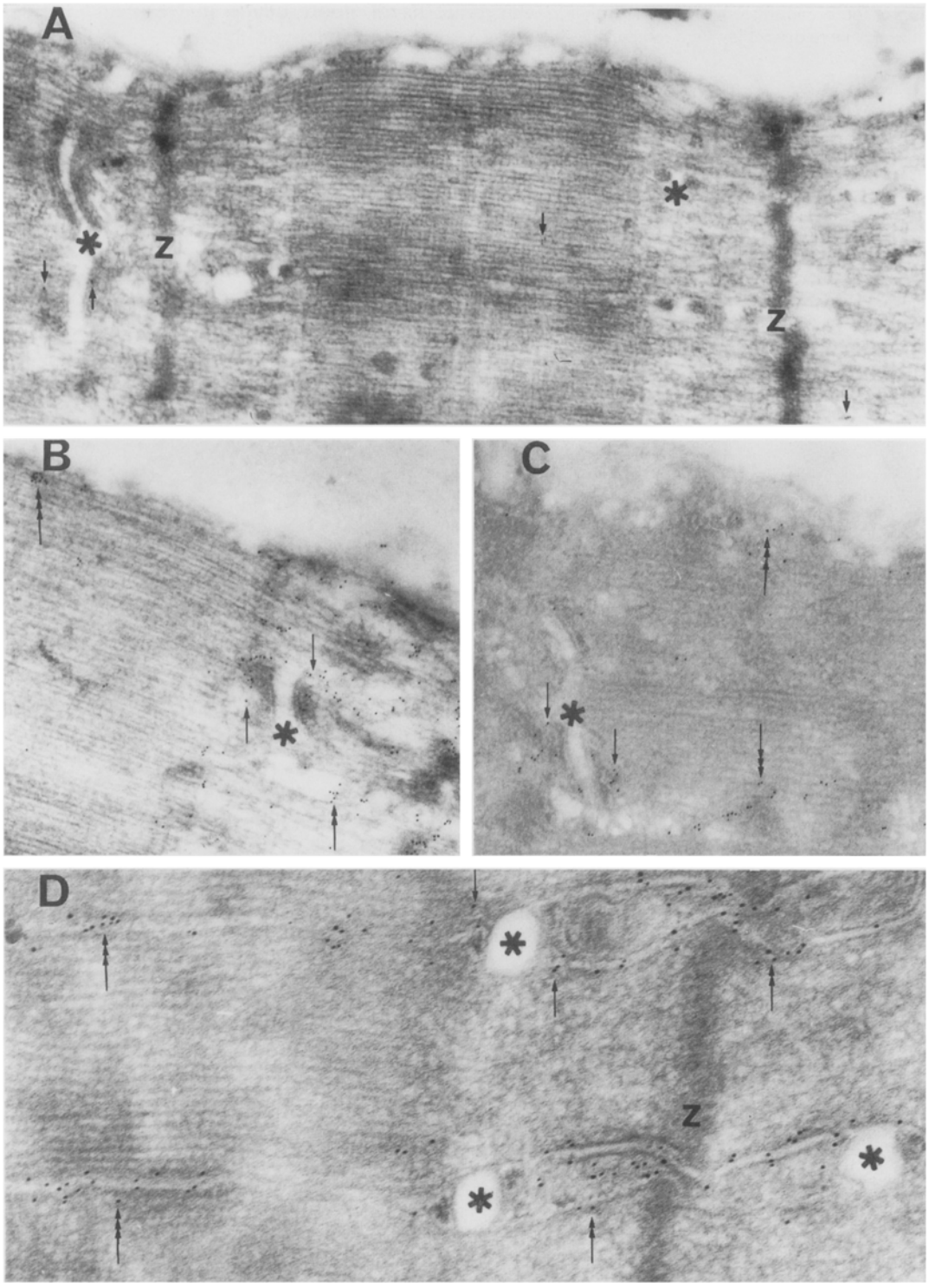

Fig. 5. Electron microscopic localization of the D12 antigen in rabbit sternomastoid muscle. Longitudinal thin sections of resin-embedded muscle fibers were incubated with primary antibody and counterlabeled with 5 nm gold-conjugated goat antimouse antibody. *A* is a control micrograph incubated with a nonmuscle primary antibody. The micrograph was chosen because it shows rare examples of nonspecific labeling (short arrows). *B*, *C* and *D* show examples sections incubated first with the D12 antibody. The sections show 5 nm gold particles associated with the membranes of the terminal cisternae and longitudinal SR, but not with T-tubule or surface membranes. The single-headed long arrows point to gold particles in the terminal cisternae, the two and three headed arrows point to gold particles in the longitudinal SR of the I band and A band, respectively. *Z* indicates the Z-line and the *'s mark T-tubules. The magnifications are ×40,700 in *A*, 79,200 in *B*, ×57,000 in *C* and ×95,000 in *D*

Table 1. Immunoreactivity of four antibodies to muscles from different sources (column 1), assessed with immunofluorescence in unfixed material, and with immunofluorescence and electron microscopy in fixed material[a]

	Unfixed fibers				Fixed fibers			
	A9	B11	D12	F2	A9	B11	D12	F2
Rabbit SM[b]	+	++++	++++	+++	+	+++	+++	++
Rat edl	+	++++	++++	+++	+	+++	+++	+
Rabbit soleus							+	
Rat soleus	+	+	+	+	−	+	+	−
Toad sartorius	+	++	++	+	−	+	+	−

[a] The results obtained with fluorescence and electron microscopy were essentially the same and have been lumped together. The relative intensities of fluorescence and densities of gold particles were assessed qualitatively and are indicated by the number of +'s in the columns. − indicates no visible fluorescence or no gold labeling.
[b] SM, sternomastoid muscle.

branes of the T-tubules and SR are visible in the lightly fixed muscle, and gold particles were associated with the terminal cisternae in the I band and longitudinal SR in the I and A bands (Fig. 5*D*). Calcium pump protein is the main component of the terminal cisternae and SR membranes (MacLennan et al., 1971) and is distributed uniformly over the membrane, except in the junctional area (Franzini-Armstrong, 1975; Jorgensen et al., 1982*b*). The other major SR protein, calsequestrin, is confined to the terminal cisternae (Jorgensen, Kalnins & MacLennan, 1979). Therefore the distribution of gold particles in the electron micrographs again suggests that the antibody is binding to the calcium ATPase.

Specificity of Antibodies to the Calcium ATPase in the SR

Gold particles were confined to the terminal cisternae and longitudinal SR: very few particles were seen in T-tubules, or associated with T-tubule membranes (Fig. 5). The surface membrane was devoid of particles (Fig. 5*B* and *C*). Therefore the antigenic sites on the SR calcium pump were not present on the calcium ATPase in the T-tubule membrane (Malouf & Meissner, 1979), indicating that there are structural differences between the calcium ATPase in the SR and the calcium ATPase in the T-tubule membranes.

Immunological Properties and Distribution of the Calcium ATPase in the SR Membrane of Muscles with Different Contractile Properties

The relative activities of the four antibodies to the calcium ATPase were assessed and the antibodies used to obtain an initial qualitative comparison of the distribution of the ATPase in rat, rabbit and toad muscles using fluorescence and electron microscopy.

Density of Antibody Binding

The density of A9, B11, D12 and F2 antibody activity was compared in rabbit sternomastoid, rat edl, rat and rabbit soleus and toad sartorius muscles, in fixed (fluorescence and electron microscopy) and unfixed (fluorescence microscopy only) tissue. Within the mammalian fast-twitch muscles, B11 and D12 were more active than the A9 and F2 antibodies. Rat and rabbit soleus and toad sartorius muscles had lower activities than other muscles (Table 1). The fluorescence in rat edl (Fig. 6*A*) was brighter and more uniform than in soleus (Fig. 6*B*), where two immunologically distinct populations of fibers were indicated by the different fluorescence intensities.

Fixation did not affect the relative distribution of the B11 or D12 antibodies, although there was higher activity in unfixed material. Fixation masked the antigenic sites for the weaker A9 and F2 antibodies in soleus and toad sartorius fibers (Table 1). Therefore the apparent specificity of A9 and F2 for the fast-twitch calcium ATPase in fixed muscles (Table 1) was an artifact of fixation.

Affinity of D12 Antibody for Fast- and Slow-Twitch Calcium ATPase

The results in Table 1 suggest that, if the affinity of the antibody for the calcium ATPase is the same in fast- and slow-twitch fibers, there is considerably

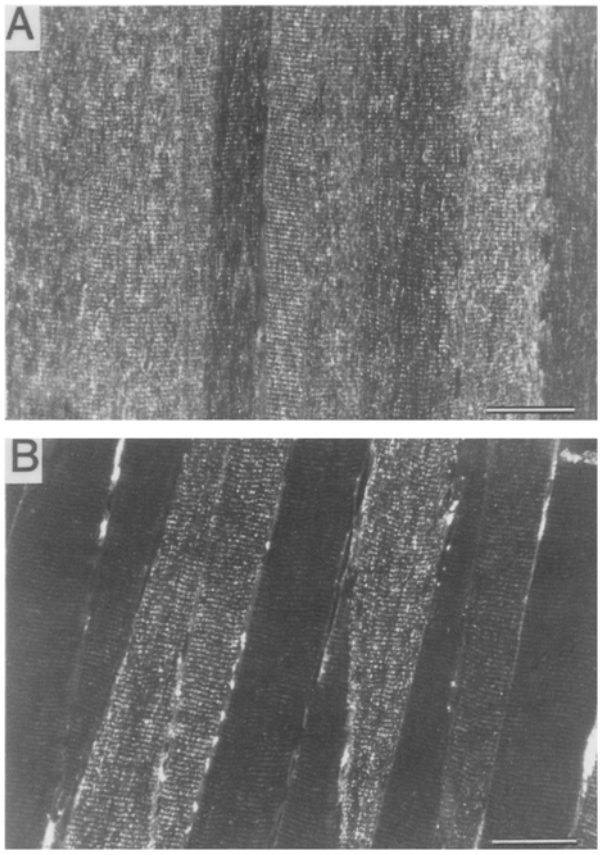

Fig. 6. Immunofluorescence localization of the D12 antigen in rat edl (*A*) and soleus (*B*) fibers. *A* and *B* are light micrographs of frozen longitudinal sections of fixed (0.1% glutaraldehyde) muscle fibers, labeled with D12 and counterlabeled with a fluorescein-conjugated rabbit anti-mouse antibody. *A* is a typical section of an edl muscle showing relatively uniform fluorescent staining of all fibers in the section. *B* is a typical section of a soleus muscle showing one population of fibers with bright fluorescent staining and a second population with less fluorescent staining. The calibration bar is 50 μm

more calcium ATPase in the faster fibers. The relative affinity of the D12 antibody for the calcium ATPase in fast- and slow-twitch muscles was measured so that D12 could be used to accurately determine the relative distributions of calcium ATPase in the two types of muscle.

The D12 antibody bound calcium ATPase from rabbit sternomastoid and soleus muscles over the same range of antigen concentrations (Fig. 7*A*), suggesting that there were equal numbers of binding sites on the ATPase molecules. An estimate of the *relative* binding affinity (Fig. 7*B*) showed similar affinities of D12 for fast and slow calcium ATPase. There was a parallel 30% reduction in antigenicity of the calcium ATPase from both muscles after fixation (Fig. 7*C*).

Table 2. The average density of gold particles in 1 μm^2 of terminal cisternae of rat edl and soleus fibers, measured as described in Materials and Methods[a]

	Mean (No/μm^2)	±SEM
Rat edl	316.5	±1.0
Rabbit sternomastoid	340.8	±2.0
Average fast	323.4	±1.4
Rat soleus	49.6	±0.6
Rabbit soleus	35.0	±4.0
Average slow	44.1	±2.0

[a] In rat, the results were obtained from 20 edl and 20 soleus fibers from 3 animals and at least 120 cisternae were counted in each fiber. In rabbit, results were obtained in 8 sternomastoid fibers from 2 animals and 12 soleus fibers from one animal with at least 90 observations in each fiber. The results are shown as mean ±1 SEM.

The similar antibody binding affinities and effects of fixation meant that the density of antibody binding in the terminal cisternase of fast- and slow-twitch muscles could be used to ascertain the relative concentrations of calcium ATPase in the membranes.

Results obtained during the course of the affinity studies suggested that, as indicated by the fluorescence and electron microscopic observations (Table 1), there was considerably less calcium ATPase in the slow-twitch muscles than in the faster muscles. Microsomal fractions of sternomastoid muscle could be used as a source of calcium ATPase from fast-twitch fibers, but it was necessary to use the sucrose gradient fraction 2 (Saito et al., 1984) of rat soleus muscles to obtain equivalent concentrations of slow-twitch calcium ATPase. When the fast-twitch microsomal fraction was run on SDS-PAGE at 3.3 times the concentration of the slow-twitch fraction 2, bands of calcium ATPase of equal intensity (measured by densitometry) were obtained.

Distribution of Calcium ATPase in the SR Membrane

Thin sections of rabbit sternomastoid (Fig. 5) and rat edl fibers (Fig. 8*A* and *B*) showed higher antibody activity, and therefore a higher calcium ATPase content than soleus fibers (Fig. 8*C* and *D*). When gold particles were counted over a standard area of terminal cisternae membrane (*see* Materials and Methods) the average density of particles in rat edl was six times higher than the density in the slower rat soleus fibers (Table 2). There was an

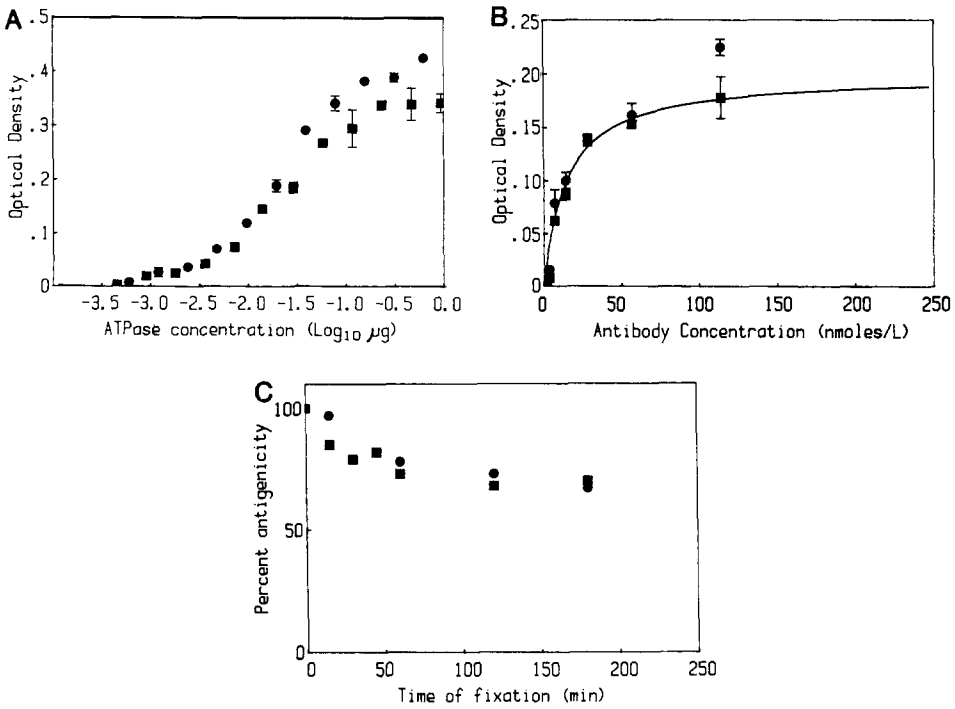

Fig. 7. Estimates of D12 antibody binding to calcium ATPase from fast-twitch rabbit sternomastoid muscle (circles) and the slow-twitch rabbit soleus (squares) using ELISA assays. The quantitative methods used for the experiments in this figure are given in Materials and Methods. *A* shows the relative antigenicity of the calcium ATPase. Increasing concentrations (horizontal axis) of SR vesicles from sternomastoid and soleus fibers, containing equivalent concentrations of calcium ATPase, were coated onto dishes and probed with a constant concentration of the D12 antibody. *B* shows the relative binding affinity of the antibody for the calcium ATPase was determined by measuring the degree of binding of varing concentrations of the D12 antibody (horizontal axis) to a constant concentration of SR vesicles. The results in *A* and *B* are shown as mean ± 1 SEM where this is greater than the dimensions of the symbol. *C* shows the relative stability of the antibody binding to fixation in 0.1% glutaraldehyde/2% formaldehyde. SR vesicles containing similar calculated amounts of calcium ATPase were fixed for various times (0 to 180 min) before addition of the D12 antibody

even greater difference between the fast- and slow-twitch fibers in rabbit where the terminal cisternae in sternomastoid fibers contained 10 times more gold than in soleus (Table 2). Due to the limited number of observations in the rabbit muscle the data has been lumped together with that from rat muscle in Table 2. The ratio of the average gold densities in slow- and fast-twitch fibers was 1 to 7, suggesting that the ratio of calcium ATPase in the SR membranes was 1 to 7.

The fast- and slow-twitch muscles contained immunologically discrete populations of fibers: there is no overlap between the densities of gold particles in the two muscles (Fig. 9*A* and *B*). As with fluorescence intensity (Fig. 6*B*), particle densities revealed one population of fibers in rat edl (Fig. 9*A*) but at least two populations of fibers in rat soleus (Fig. 9*C*). Rabbit soleus contained only one population of fibers which overlapped the lowest density population in rat soleus (Fig. 9*B*). The densities of gold in rat and rabbit sternomastoid fibers were similar.

Relative Concentrations of Calcium ATPase Isolated from Fast- and Slow-Twitch Sarcoplasmic Reticulum

The relative calcium ATPase densities in fast- and slow-twitch fibers, suggested by the ultrastructural studies, should be similar to the relative concentration of calcium ATPase in isolated SR from the two types of muscle. The calcium ATPase content in SR from rabbit, sternomastoid and lower back muscles (psoas and latissimus dorsi, which have relaxation times similar to edl, Table 3) were compared with SR from the slow-twitch rabbit soleus and semimembranosus muscles. Since it is likely that calcium ATPase content is directly related to relaxation rates, the contraction and relaxation times for the muscles used in this study are given in Table 3 and data for edl is included for comparison. The relative concentrations of calcium ATPase were determined using densitometry on SDS-PAGE gels. Corrections were made for the content of protein of nonmembrane origen in the fractions (i.e., myosin

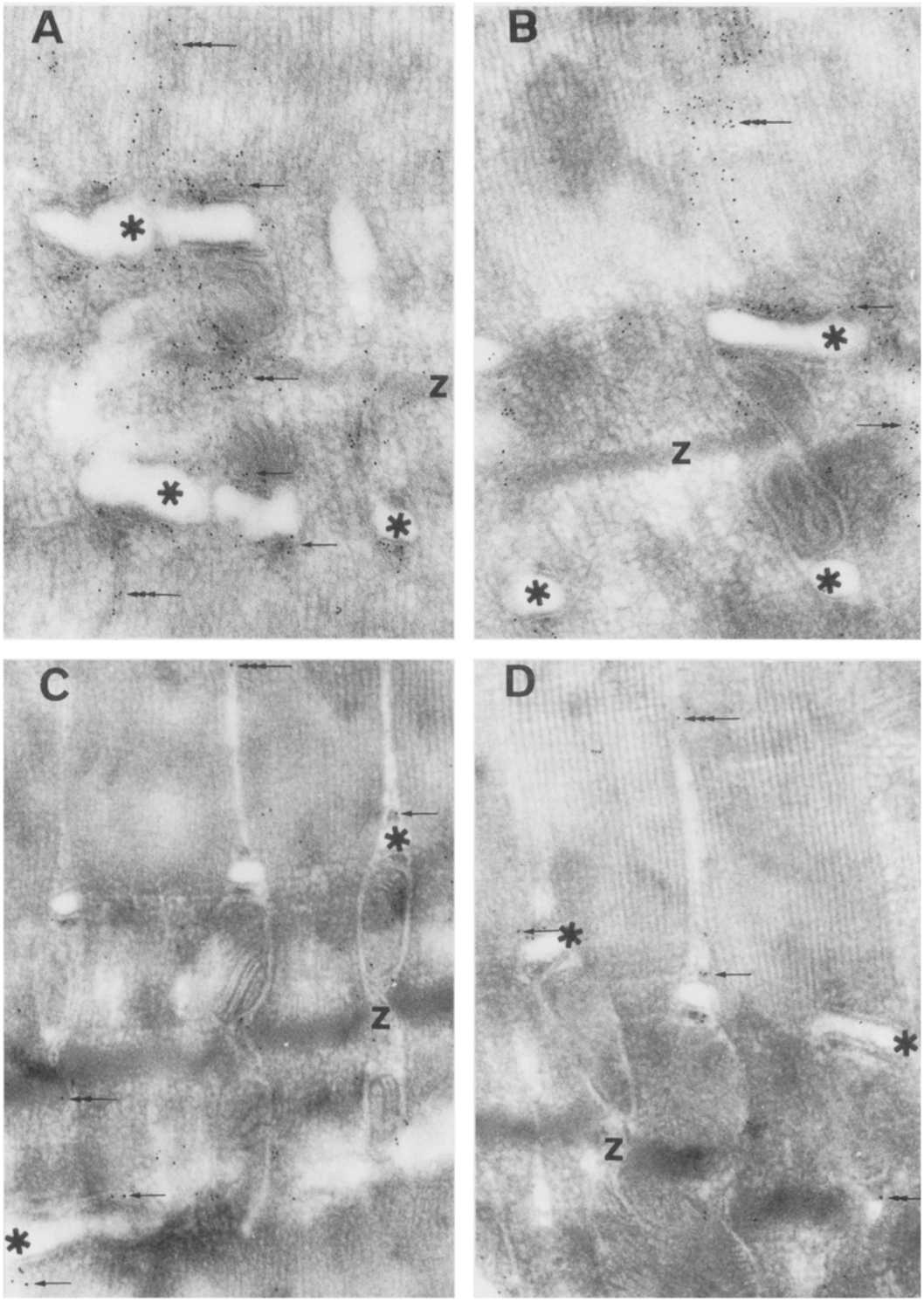

Fig. 8. Electron microscopic localization of the D12 antigen in rat edl (*A* and *B*) and soleus (*C* and *D*) muscles. Longitudinal thin sections of resin-embedded muscle fibers were incubated with D12 antibody and counterlabeled with 5 nm gold-conjugated goat anti-mouse antibody. *A* and *B* show typical examples of edl fibers with many 5-nm gold particles associated with the terminal cisternae and longitudinal SR membranes. *C* and *D* show typical examples of soleus fibers with 5-nm gold particles sparcely distributed over the terminal cisternae and longitudinal SR membranes. The symbols are defined in the legend to Fig. 5. The magnifications are ×70,300 in *A* and ×72,200 in *B*

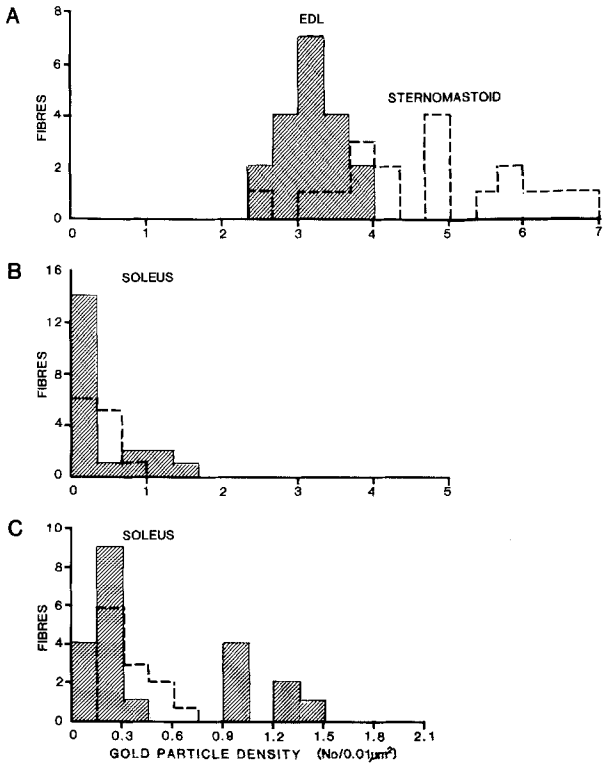

Fig. 9. Histograms of the distribution of gold particle densities counted in electron micrographs of the terminal cisternae in edl and soleus fibers. The histograms show the same data used to obtain the average gold particle densities given in Table 2. The numbers of fibers are shown on the ordinate, and ranges of gold particle densities (No/0.01 μm^2) are shown on the abscissa. *A* shows data obtained from edl fibers, and *B* shows data obtained from soleus fibers, using the same scale as *A* to allow comparison of the two sets of data. *C* shows the soleus data on an expanded particle density scale to display the two populations of fibers in rat soleus muscles

and calsequestrin). The ratio of calcium ATPase in light SR (sucrose gradient fraction 2) from soleus and sternomastoid fibers was 1 to 7. In the slow-twitch (soleus and semimembranosus) and fast-twitch (psoas and latissimus dorsi) preparations the ratio of calcium ATPase in light SR was 1 to 5. The mean ratio of calcium ATPase in light SR from slow- and fast-twitch fibers was 1 to 6. This value is remarkably similar to the ratio of 1 to 7 obtained with the very different immunoelectron microscopy technique. The results confirm the large difference between the calcium ATPase contents of the SR membranes in fast and slow-twitch fibers.

The Distribution of Calcium ATPase in Fibers from the Toad Sartorious Muscle

The D12 antibody to mammalian calcium ATPase molecules showed good cross-reactivity with amphibian calcium ATPase indicating homology between the molecules in different species. The striated immunofluorescence in amphibian fibers (Fig. 10*A*) was similar to that seen in mammals (Fig. 4), while the differential fluorescence staining in neighboring fibers was in contrast to the uniform staining in fast-twitch mammalian muscles (Figs. 4 and 6).

The density of gold particles in immunolabeled thin sections was roughly equivalent to that seen in the least-active rat soleus fibers. The highest density of gold particles was in the I band, associated with terminal cisternae membranes (Fig. 10*B* and *C*), and a few particles were seen in the A band, associated with the membranes of the longitudinal SR.

Table 3. Contraction and 50% relaxation times of rabbit muscles used to isolate light SR for assessment of calcium ATPase content[a]

Muscle		Contraction time (msec)	50% relaxation time (msec)
Sternomastoid	(n = 7)	48.7 ± 1.8	44.0 ± 1.0
edl	(n = 7)	52.3 ± 1.3	55.7 ± 3.5
psoas	(n = 2)	52.0 ± 3.1	80.0 ± 0.0
Latissimus dorsi	(n = 2)	72.0 ± 0.0	62.0 ± 1.0
Semimembranosus	(n = 2)	232.0 ± 5.6	440.0 ± 28.0
Soleus	(n = 8)	253.3 ± 9.8	720.0 ± 28.0

[a] The results are given as mean ±1 SEM, and the number of observations is given in parentheses.

Discussion

The monoclonal antibody, D12, recognized the calcium ATPase in the SR of fibers from fast- and slow-twitch muscles and had a similar relative affinity for the ATPase from the two types of muscles. The relative affinities were not affected by fixation. A *quantitative* measure of the density of antibody bound to terminal cisternase membrane showed that there were seven times more antibody in fast-twitch fibers than in slow-twitch fibers. Antibody distribution was assessed over a similar area of membrane in all muscles and was independent of the content of SR in the fibers and effects of fiber diameter on fixation. Therefore, the density of calcium ATPase in the SR membrane was seven times greater in the fast-twitch fibers. This result was confirmed by the relative concentration of calcium ATPase in light SR membranes, which was measured and found to be six times greater in fast-twitch fibers. The difference in calcium ATPase content, determined with two very different techniques, accounts for the different calcium-accumu-

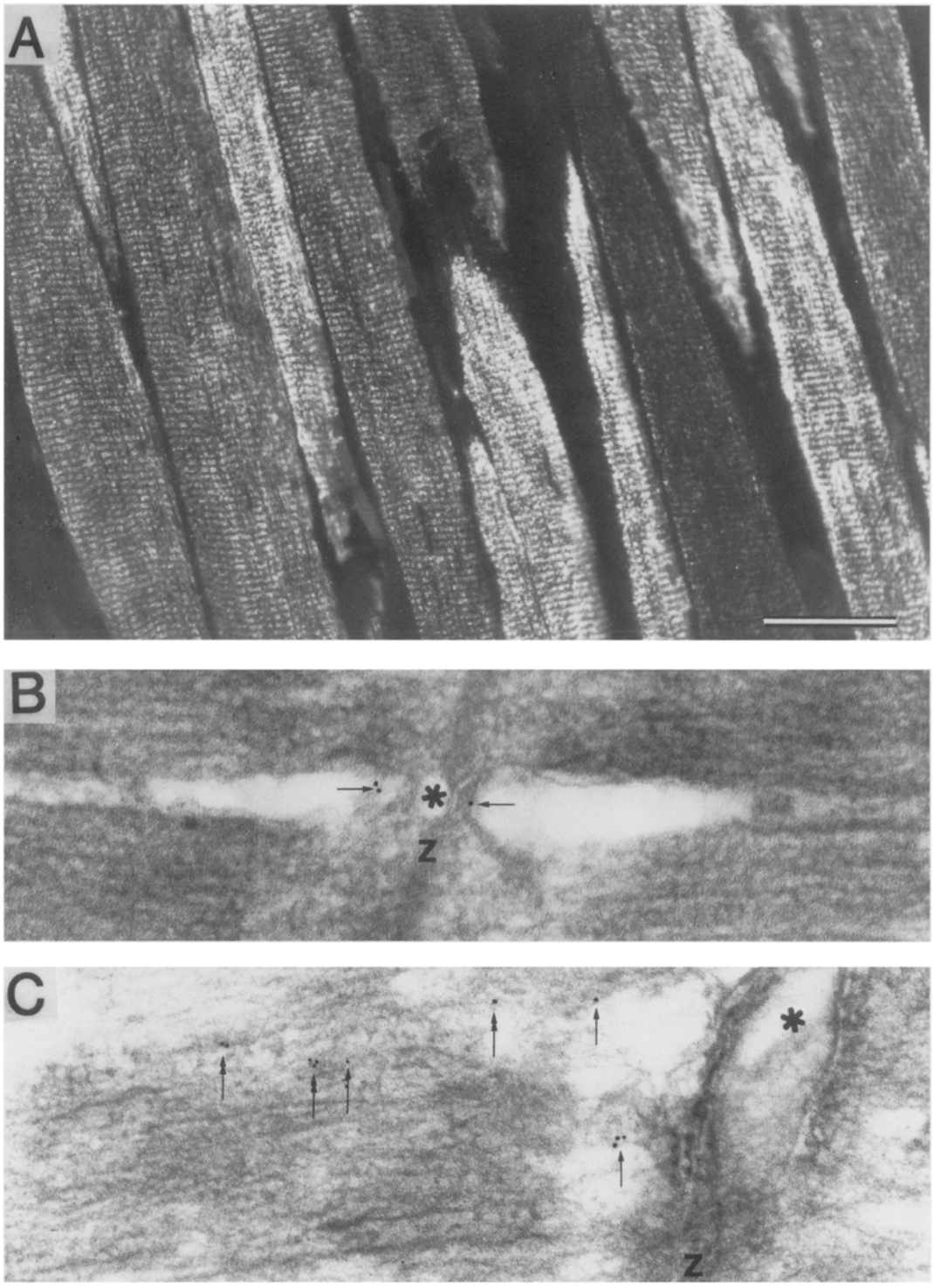

Fig. 10. Localization of the D12 antigen in toad sartorius fibers using fluorescence and electron microscopy. *A* shows fluorescent staining of a longitudinal section of a fixed (0.1% glutaraldehyde) toad sartorius muscle, incubated with D12 and counterlabeled with fluorescein-conjugated rabbit anti-mouse antibody. The light micrograph clearly demonstrates the variety of fluorescent staining intensity seen in fibers from the toad sartorius muscle. The calibration bar is 50 μm. *B* and *C* show examples of longitudinal thin sections of toad sartorius fibers, incubated with D12 and counterlabeled with 5 nm gold-conjugated goat anti-mouse antibody. The electron micrographs show a low density of gold particles associated with the terminal cisternae (single arrows) and longitudinal SR (double arrows). The *s are placed in T-tubules. The magnifications are ×87,500 in *B* and ×112,500 in *C*

lating capacities of fast- and slow-twitch muscles and the five- to sixfold difference in the rate of tension-independent heat measured in rat edl and soleus fibers (Wendt & Gibbs, 1973).

The results do not support polyclonal antibody (Jorgensen et al., 1979, 1982*b*) and freeze-fracture (Beringer, 1976; Bray & Rayns, 1976) studies that suggest there is only a twofold difference between the SR calcium ATPase densities in fast- and slow-twitch fibers. Both techniques could have significantly underestimated the differences between the ATPase densities. First, polyclonal antibodies to ATPase from mixed muscles could have contained an excess of antibodies to slow ATPase (Jorgensen & Campbell, 1986) and antibody activity was related to fiber-type rather than isometric relaxation times. Indicators of fiber-type, myosin ATPase activity and Z-line thickness, are not always indicative of contraction or relaxation times (Close, 1972; Dulhunty & Dlutowski, 1979). The different conclusions drawn from this study and from Jorgenson et al. (1982*b*) could be explained if the calcium ATPase density was more closely related to relaxation times than to fiber-type. Secondly, the difference in the numbers of 7–9 nm particles in freeze fracture of fast- and slow-twitch SR would be less than the difference between calcium ATPase densities if the other SR proteins in soleus (Pette & Heilmann, 1979) form 7–9 nm particles.

The results suggest that the calcium ATPase contents are indeed closely correlated with relaxation rates. The greatest difference in calcium ATPase contents (1 : 7) was found between soleus and sternomastoid fibers, which also have the greatest difference in relaxation times (720 msec compared with 44 msec). A smaller difference (1 : 5) was found between the combined latissimus dorsi and psoas preparations (mean relaxation time of 71 msec) and the combined soleus and semimembranous preparations (mean relaxation time of 580 msec).

The lower concentration of calcium ATPase in the SR membrane of soleus fibers suggests that, in contrast to faster rabbit white muscles (MacLennan et al., 1971), the SR membrane is not mostly calcium ATPase. In fact it has been clearly shown that slow-twitch muscles contain several other proteins in addition to the calcium ATPase (Pette & Heilmann, 1979; Tada & Katz, 1982). The different protein composition of SR membrane in fast- and slow-twitch fibers may have been responsible for the differential effects of fixation on the affinities of the different antibodies. The fact that the monoclonal antibodies cross-reacted with all muscles examined confirms other evidence that there are strong structural similarities between the calcium ATPase in a diversity of muscles (Dux & Martonosi, 1984). It is not clear whether the specificity of some antibodies for a fiber-type (Volpe et al., 1982; Jorgensen & Campbell, 1986) is the result of inherent differences between ATPase molecules, or whether the differences are imposed by different membrane environments around the molecules.

The density of gold particles in fast-twitch fibers (300 to 400 per μm^2, Table 2) was considerably lower than the density of 7–9 nm particles seen in freeze-fracture replicas of the terminal cisternae membrane (about 4000 per μm^2, *see*, e.g., Heilmann et al., 1981). This discrepancy might be expected since the antibodies bind most easily to antigenic sites on the surface of the section. Clearly, only a fraction of the membrane seen in a section is coincident with the surface of the section and available to the antibodies. In addition, the antigenicity lost after fixation would further reduce the density of antibody binding in fixed material.

A calcium ATPase in the T-tubule and plasma membranes of skeletal muscle (Malouf & Meissner, 1979; Hidalgo, Gonzalez & Lagos, 1983) has the same molecular mass as the calcium ATPase from the SR, but different functional properties (Hidalgo et al., 1983). The antibodies used in this study did nto recognize an antigen on the surface or T-tubule membranes, confirming previous findings that there are immunological, and presumably structural, differences between the two calcium ATPase molecules (Jorgensen et al., 1979, 1982*b*).

The toad sartorius muscle contained several populations of fibers with different densities of antibody binding. If antibody activity is related to contraction times, as it is in mammalian edl and soleus muscles, the diversity of activity in toad sartorius suggests that the muscle contains fibers with a variety of contraction times. This may be a common feature of amphibian muscles: the iliofibularis in *Xenopus laevis* contains at least five fiber-types with a wide range of contractile and histochemical properties (Lannergren & Smith, 1966; Lannergren, 1979) and distributions of myosin isoenzymes (Lannergren & Hoh, 1984).

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