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A Monoclonal Antibody to the Ca^{2+}-ATPase of Cardiac Sarcoplasmic Reticulum Cross-Reacts With Slow Type I but Not With Fast Type II Canine Skeletal Muscle Fibers: An Immunocytochemical and Immunochemical Study

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Ca^{2+}-ATPase of the sarcoplasmic reticulum was localized in cryostat sections from three different adult canine skeletal muscles (gracilis, extensor carpi radialis, and superficial digitalis flexor) by immunofluorescence labeling with monoclonal antibodies to the Ca^{2+}-ATPase. Type I (slow) myofibers were strongly labeled for the Ca^{2+}-ATPase with a monoclonal antibody (II D8) to the Ca^{2+}-ATPase of canine cardiac sarcoplasmic reticulum; the type II (fast) myofibers were labeled at the level of the background with monoclonal antibody II D8. By contrast, type II (fast) myofibers were strongly labeled for Ca^{2+}-ATPase of rabbit skeletal sarcoplasmic reticulum. The subcellular distribution of the immunolabeling in type I (slow) myofibers with monoclonal antibody II D8 corresponded to that of the sarcoplasmic reticulum as previously determined by electron microscopy. The structural similarity between the canine cardiac Ca^{2+}-ATPase present in the sarcoplasmic reticulum of the canine slow skeletal muscle fibers was demonstrated by immunoblotting. Monoclonal antibody (II D8) to the cardiac Ca^{2+}-ATPase binds to only one protein band present in the extract from either cardiac or type I (slow) skeletal muscle tissue. By contrast, monoclonal antibody (II H11) to the skeletal type II (fast) Ca^{2+}-ATPase binds only one protein band in the extract from type II (fast) skeletal muscle tissue. These immunopositive proteins coelectrophoresed with the Ca^{2+}-ATPase of the canine cardiac sarcoplasmic reticulum and showed an apparent M_r of 115,000. It is concluded that the Ca^{2+}-ATPase of cardiac and type I (slow) skeletal sarcoplasmic reticulum have at least one epitope in common, which is not present on the Ca^{2+}-ATPase of sarcoplasmic reticulum in type II (fast) skeletal myofibers. It is possible that this site is related to the assumed necessity of the Ca^{2+}-ATPase of the sarcoplasmic reticulum in cardiac and type I (slow) skeletal myofibers to interact with phosphorylated phospholamban and thereby enhance the accumulation of Ca^{2+} in the lumen of the sarcoplasmic reticulum following β–adrenergic stimulation.

Key words: Ca^{2+}-ATPase of sarcoplasmic reticulum, immunofluorescence, myofibers types I (slow) and II (fast), II D8 monoclonal antibody, II H11 monoclonal antibody

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INTRODUCTION

Recent immunofluorescence, immunochemical, and biochemical studies [Jorgensen and Jones, 1986] have shown that phospholamban, first described as a cardiac sarcoplasmic reticulum protein [Kirchberger et al., 1974; Tada and Katz, 1982; Tada and Inui, 1983] is localized in the sarcoplasmic reticulum of type I (slow) but not type n (fast) skeletal muscle fibers. Since phospholamban upon phosphorylation is believed to interact with the Ca^{2+}-ATPase of the cardiac sarcoplasmic reticulum and thereby modulate the Ca^{2+} fluxes across the sarcoplasmic reticulum [Tada and Katz, 1982; Tada and Inui, 1983], one might expect that the Ca^{2+}-ATPases of the sarcoplasmic reticulum in cardiac and type I (slow) skeletal muscle fibers have certain structural characteristics in common, which are absent from the Ca^{2+}-ATPase of type II (fast) myofibers. If this is indeed the case, one would predict that one or more of a panel of monoclonal antibodies to the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum would specifically immunolabel the Ca^{2+}-ATPase in type I (slow) but not type II (fast) myofibers of skeletal muscle tissues.

To assess this possibility, monoclonal antibody (II D8) to the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum [Pepper et al., 1986] was used for immunocytochemical and immunochemical studies of canine skeletal muscle. The results demonstrate that a protein antigenically and structurally related to the Ca^{2+}-ATPase of the canine cardiac sarcoplasmic reticulum is present in type I (slow) but is apparently absent from type II (fast) skeletal muscle fibers. Similar studies with a monoclonal antibody (II H11) to the Ca^{2+}-ATPase of rabbit skeletal sarcoplasmic reticulum [Pepper et al., 1986] showed that a protein antigenically and structurally related to the Ca^{2+}-ATPase of rabbit skeletal sarcoplasmic reticulum but distinct from the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum is present in type II (fast) but is apparently absent from type I (slow) skeletal muscle fibers.

MATERIALS AND METHODS

Preparation of Ca^{2+}-ATPase of the Sarcoplasmic Reticulum

The Ca^{2+}-ATPase of the sarcoplasmic reticulum from rabbit fast (psoas) skeletal muscle and the Ca^{2+}-ATPase of the sarcoplasmic reticulum from canine ventricular muscle were purified as previously described [Nakamura et al., 1983]. Both Ca^{2+}-ATPases electrophoresed as a single band on SDS-polyacrylamide gels and showed an apparent M_r of 115,000. The specific activities of the Ca^{2+}-ATPases purified from rabbit skeletal and dog ventricular muscle were determined to be 1,005 μmol ADP/mg/hr and 425 μmol ADP/mg/hr, respectively, using the assay previously described by Nakamuraetal. [1983].

Preparation of Sarcoplasmic Reticulum Vesicles

Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were purified as described by MacLennan [1970], with the modification of Campbell and MacLennan [1981], in the presence of protease inhibitors. Sarcoplasmic reticulum vesicles from canine ventricular muscle were purified as described by Jones and Cala [1981] in the presence of protease inhibitors.

Preparation of Muscle Extracts

Bundles of canine skeletal muscle fibers were dissected from gracilis (34% ± 10% type I fibers), superficial digitalis flexor (89% ± 3% type I fibers), and extensor carpi radialis (14% ± 6% type I fibers) muscle and quickly frozen in liquid nitrogen. Similarly, canine ventricular muscle tissue was dissected and quickly frozen in liquid nitrogen. Frozen muscle tissue was homogenized in 4 volumes 0.25 M sucrose, 20 mM Tris maleate, pH 7.0, per gram of tissue with a Tekmar polytron for three 30 sec bursts in the presence of protease inhibitors: pepstatin A (1 μM), andpain (1 μM), leupeptin (1.4 μM), chymostatin (0.7 μg/ml), benzamidine (0.8 mM), aprotinin (125 U/ml), iodoacetamide (1 mM), and PMSF (1.0 mM). The extract was centrifuged for 20 min at 3,000g (R_{max}), and me supernatant (muscle extract) was filtered through six layers of cheesecloth. All muscle extracts were frozen in liquid nitrogen and stored at -70°C. Protein was determined by the method of Lowry et al [1951] as modified by Peterson [1977].

Preparation of Monoclonal Antibodies (mAb) to Cardiac Ca^{2+}-ATPase of the Sarcoplasmic Reticulum

One-month-old-female BALB/c mice were immunized by intraperitoneal injections of 500 μg canine cardiac Sarcoplasmic reticulum vesicles in Freund's complete adjuvant. Three additional injections of 500 μg sarcoplasmic reticulum vesicles in Freund's incomplete adjuvant were given at 1 month intervals. On each of the fourth, third, and second days preceding hybridoma fusion, the selected mouse was immunized with 500 μg cardiac Sarcoplasmic reticulum. Hybridomas were obtained according to the procedure described by Kennett, McKeam, and Bechtal [1980] by fusing isolated spleen cells from an immunized mouse with NS-1 myeloma cells. After 10-14 days of incubation, hybridoma supernatants demonstrated to be positive for canine cardiac Sarcoplasmic reticulum by solid-phase immunodot assay as described by Hawkes, Niday, and Gordon [1982] were selected. Positive cell cultures were subsequently assayed for antibodies able to recognize epitopes on cardiac Ca^{2+}-

ATPase of the sarcoplasmic reticulum by SDS-PAGE immunoblotting [Towbin et al., 1979]. Positive cell cultures were subcloned by limiting dilution.

mAb to Skeletal Ca^{2+}-ATPase of the Sarcoplasmic Reticulum

mAb to skeletal Ca^{2+}-ATPase was prepared as described above for mAb to cardiac Ca^{2+}-ATPase except that sarcoplasmic reticulum vesicles purified from rabbit skeletal muscle were used instead of sarcoplasmic reticulum vesicles of canine ventricular muscle.

Purification of mAb

mAb antibodies were purified from the supematants of antibody-producing hybridoma cultures by precipitation with 50% saturated ammonium sulfate. The pelleted antibodies were resolubilized in phosphate buffered saline (50 mM NaH_2PO_4, 0.9% NaCl, pH 7.4), and the ammonium sulfate was removed by chromatography on a PD-10 column (Pharmacia Fine Chemicals).

Immunoblotting

Ca^{2+} ATPase was identified on nitrocellulose blots of various muscle extracts and purified sarcoplasmic reticulum proteins by an indirect immunoperoxidase staining. Samples were first separated by Laemmli [1970] SDS-PAGE using 5% to 16% gradient slab gels and then transferred to nitrocellulose paper (0.45 μm pore size) electrophoretically [Towbin et al., 1979] for 1.5 hr at 1.0A using 25 mM Tris, 195 mM glycine, 20% methanol as the transfer buffer. Nitrocellulose blots were blocked with BLOTTO-Bovine Lacto Transfer Technique Optimizer (50 mM NaH_2PO_4, 0.9% NaCl, pH 7.4, 5% nonfat dry milk) for 60 min at room temperture [Johnson et al., 1984]. The blots were transferred to a seal-a-meal bag with 0.5-2.5 μg/ml mAb in BLOTTO for a 60 min incubation with gentle agitation. The blots were briefly rinsed with water and washed two times for 10 min each in BLOTTO to remove free and nonspecifically bound antibody. The blots were then incubated with goat antimouse IgG-peroxidase-linked secondary antibody (Cooper Biomedical) at a 1:1,000 dilution in BLOTTO for 60 min with gentle agitation. The blots were again briefly rinsed with water and washed two times for 10 min each with BLOTTO. Color development was accomplished using the peroxidase substrate 4-chloro-l-naphthol. A mixture of 100 ml 20 mM Tris, 200 mM NaCl, pH 7.5, and 20 ml 4-chloro-l-naphthol (3 mg/ml in ice-cold methanol) was added to the blots followed by the addition of 0.06 ml 30% H_2O_2 (4°C). The reaction was terminated by thoroughly rinsing the blots with water and allowing them to air dry.

Dissection, Fixation, and Sectioning of Skeletal and Cardiac Muscle Tissue

Fixed and unfixed bundles of canine skeletal muscle fibers of three adult mongrels were used. Bundles of canine skeletal muscle fibers were dissected from gracilis (34% ± 10% type I fibers), superficial digitalis flexor (89% ± 3% type I fibers), and extensor carpi radialis (14% ± 6% type I fibers) muscle. Subsequently, the dissected tissues were quickly frozen in liquid nitrogen-cooled isopentane [Jorgensen and Jones, 1986] and 6-8 μm transverse cryostat sections were cut and stored at —20°C for up to 4 weeks and used for either immunofluorescence labeling or cytochemical staining as described below. Bundles of myofibers to be fixed (2-3 in diameter) were dissected from the gracilis muscle and immediately tied to applicator sticks at 100-120% of their rest length. Subsequently, the myofibers were fixed in 2% paraformaldehyde for 2 hr and then in a mixture of 2% paraformaldehyde and 0.3% glutaraldehyde for 3 hr as previously described [Jorgensen et al., 1979]. Storage, infusion with sucrose, and cryosectioning (6-8 μm) were performed as previously described [Jorgensen et al., 1979].

Immunofluorescence Labeling

The indirect immunofluorescence labeling of fixed and unfixed cryostat sections from adult canine skeletal muscle tissue was carried out as previously described [Jorgensen and Jones, 1986]. Sections were first labeled with either mAb II D8 to Ca^{2+}-ATPase of canine cardiac sarcoplasmic reticulum (0.5 mg/ml), or mAb II Hll to Ca^{2+}-ATPase of rabbit skeletal sarcoplasmic reticulum (15 μg/ml). The fluorescence-conjugated gamma globulin fraction of goat antimouse gammaglobulin (1/20 dilution, Cooper Biomedical) was used as the secondary reagent. The sections were examined in a Zeiss photomicroscope provided with an epifluorescence attachment and a phase contrast condenser.

Cytochemical Staining

Transverse sections of unfixed skeletal muscles were stained for myosin ATPase after alkaline preincubations (pH 10.4) as described by Guth and Samaha [1969]. This method specifically labels the myosin ATPase of only fast skeletal muscle fibers.

RESULTS

Characterization of mAb to Ca^{2+}-ATPase of Cardiac and Skeletal Sarcoplasmic Reticulum

mAb 11 D8 was obtained from a panel of hybridoma clones whose supernatants recognized epitopes on car-

diac sarcoplasmic reticulum vesicles (both nonjunctional and junctional cardiac sarcoplasmic reticulum). mAb 11 D8 was chosen for its ability to bind an epitope of the Ca^{2+}-ATPase of the sarcoplasmic reticulum from canine cardiac muscle and an inability to recognize epitopes of the Ca^{2+}-ATPase of sarcoplasmic reticulum of rabbit skeletal muscle in SDS-PAGE immunoblots (Fig. 1; right). Similarly, mAb II Hll was obtained from a panel of hybridoma clones whose supematants recognized epitopes on skeletal sarcoplasmic reticulum vesicles. mAb II H11 was selected on the basis of its ability to bind an epitope of the Ca^{2+}-ATPase of the sarcoplasmic reticulum from rabbit skeletal muscle and an inability to bind to epitopes of the Ca^{2+}-ATPase of the sarcoplasmic reticulum from canine ventricular muscle (Fig. 1, left).

The specificity of H D8 for the Ca^{2+}-ATPase of the sarcoplasmic reticulum in canine ventricular muscle was demonstrated by its ability to bind only one polypeptide band present in SDS-PAGE immunoblots of extracts from this tissue (Fig. 1, lane CE). Similarly, the specificity of II H11 for the Ca^{2+}-ATPase of the sarcoplasmic reticulum in canine skeletal muscle (carpi radialis; 14% ± 6% type I fibers) was demonstrated by its ability to bind only one polypeptide band present in SDS-PAGE immunoblots of extracts from this tissue (Fig. 1, lane SE). The immunoreactive peptides coelectrophoresed with the Ca^{2+}-ATPase of the canine cardiac sarcoplasmic reticulum and showed an apparent M_r of 115,000.

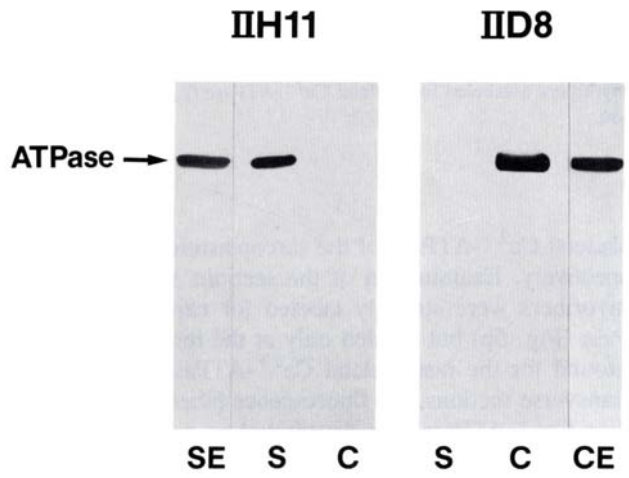

Fig. 1. Immunoblot staining of muscle extracts from canine ventricular muscle (**lane CE**), muscle extracts from canine skeletal muscle (carpi radialis; **lane SE**), purified rabbit skeletal (**lanes S**) and purified canine cardiac (**lanes C**) ATPase with mAb II H11 (antiskeletal ATPase) and II D8 (andcardiac ATPase). Canine muscle extracts (100 ug), purified rabbit skeletal ATPase (1 μg), and purified canine cardiac ATPase (2.5 μg) were separated by SDS-PAGE (5-16% gradient gels) and transferred electrophoretically to nitrocellulose paper as described in Materials and Methods. Nitrocellulose blots were blocked with PBS-BLOTTO (50 mM Na_2 HPO_4, 0.9% NaCl, pH 7.4, 5% nonfat dry milk), incubated with monoclonal antibodies, washed to remove excess unbound antibody, and incubated with goat anti-mouse IgG peroxidase-linked antibody. Following washing, color development was accomplished using 4-chloro-l-naphthol as a substrate. Nitrocellulose blots were stained with either 0.5 μg/ml II H11, skeletal-specific anti-Ca^{2+}-ATPase mAb or 2.5 μg/ml II D8, cardiac-specific anti-Ca^{2+}-ATPase mAb. ATPase designates the 115,000 Da Ca^{2+}-ATPase.

Localization of a Cardiac Ca^{2+}-ATPase-Like Protein in Type I (Slow) and Type II (Fast) Canine Skeletal Muscle Fibers

Transverse cryostat sections from adult canine gracilis muscle containing a mixture of type I (34% ± 10%) and type II myofibers [Armstrong et al., 1982] were labeled with mAb II D8 to canine cardiac Ca^{2+}-ATPase of the sarcoplasmic reticulum by the indirect immunofluorescence labeling technique. Examination of the sections showed that some myofibers were strongly labeled, whereas others were labeled at the level of the background (Fig. 2a).

The relationship between the distribution of the myofibers strongly labeled for the cardiac Ca^{2+}-ATPase and the type I (slow) and type n (fast) fibers was determined by labeling serial transverse sections of unfixed gracilis muscle tissue with mAb II D8 to Ca^{2+}-ATPase of canine cardiac sarcoplasmic reticulum (Fig. 2a) or mAb II H11 to Ca^{2+}-ATPase of rabbit skeletal sarcoplasmic reticulum (Fig. 2c) or stained cytochemicaUy for the alkali stable myosin ATPase (Fig. 2b). It has previously been shown that strong labeling with antibodies to the Ca^{2+}-ATPase of rat skeletal sarcoplasmic reticulum [Jorgensen et al., 1979] and cytochemical labeling for alkali-stable myosin ATPase are specific markers for type II (fast) myofibers [Gum and Samaha, 1969]. The results showed that all type I (slow) myofibers, unlabeled for the alkali-stable myosin ATPase (white fibers. Fig 2b), were strongly labeled with mAb II D8 to the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum (Fig. 2a) but were unlabeled with mAb II H11 to the Ca^{2+}-ATPase of skeletal sarcoplasmic reticulum (Fig. 2c). By contrast all type II (fast) myofibers, darkly labeled for the alkali-stable myosin ATPase (black fibers, Fig. 2b), were strongly labeled with mAb II H11 to the Ca^{2+}-ATPase of skeletal sarcoplasmic reticulum (Fig. 2c) but were unlabeled with mAb II D8 to the Ca^{2+}-ATPase of the cardiac sarcoplasmic reticulum (Fig. 2a). Thus it appears that a protein antigenically related to the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum is present in type I (slow) but apparently is absent from type n (fast) canine skeletal fibers. In addition, a third kind of myofiber, named type X in the present paper, was identified in canine gracilis muscle, where it constitutes approximately 5% of the total myofibers (Fig. 3, stars). In contrast to type I (slow) and type II (fast) myofibers, type X myofibers are

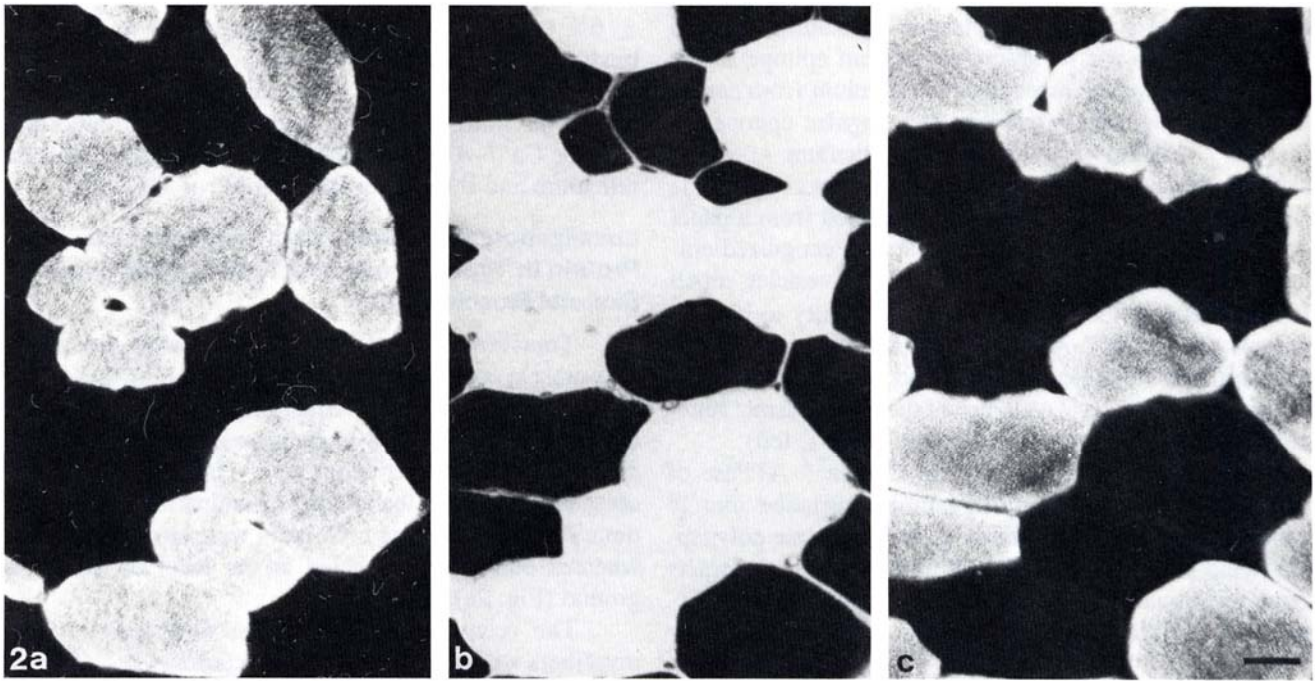

Fig. 2. Transverse serial cryosections of unfixed gracilis muscle stained **a:** by the indirect immunofluorescence technique with mAb to Ca^{2+}-ATPase of canine cardiac sarcoplasmic reticulum (II D8), **b:** cytocheinically for alkali-stable myosin ATPase which is a specific marker for type II (fast) myofibers and **c:** with mAb to Ca^{2+}-ATPase of rabbit skeletal sarcoplasmic reticulum (II H11) by the indirect immunofluorescence staining technique. There is a positive correlation between the myofibers positively stained for cardiac Ca^{2+}-ATPase (a, white) and alkali-stable myosin ATPase (b, white) and myofibers unlabeled for skeletal Ca^{2+}-ATPase (c, black). Bar = 20 μm.

strongly labeled with mAb to both the cardiac (II D8; stars, Fig. 3a) and the fast skeletal Ca^{2+}-ATPase of the sarcoplasmic reticulum (II Hll; stars. Fig. 3c). Whereas type I (slow) and type II (fast) myofibers appeared light and dark, respectively, following the cytochemical labeling for alkali-stable myosin ATPase (Fig. 3b), type X myofibers appeared grey (stars, Fig. 3b).

Similar immunofluorescence studies of serial transverse sections from superficial digitalis flexor muscle (89% ± 3% type I myofibers) and from extensor carpi radialis muscle (14% ± 6% type I myofibers) were also carried out. As in gracilis muscle, it was observed in both superficial digitalis flexor (Fig. 4a) and extensor carpi radialis muscle (Fig. 4b) that myofibers strongly labeled for the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum were unlabeled for the alkaline-stable myosin ATPase (Figs. 4c, d).

Localization of the Cardiac Ca^{2+}-ATPase and the Skeletal Ca^{2+}-ATPase in Canine Myocardial Fibers

Transverse cryostat sections from adult canine atrial muscle tissue (Fig. 5) were labeled with mAb II D8 (Fig. 5a,b) and II H11 (Fig. 5c) to the canine cardiac and skeletal Ca^{2+}-ATPase of the sarcoplasmic reticulum, respectively. Examination of the sections showed that all myofibers were strongly labeled for cardiac Ca^{2+}-ATPase (Fig. 5b) but labeled only at the level of the background for the fast skeletal Ca^{2+}-ATPase (Fig. 5c). In transverse sections, the fluorescence labeling for the cardiac Ca^{2+}-ATPase was distributed as an irregular network (Fig. 5a, arrows) corresponding in general to the distribution of the sarcoplasmic reticulum in myocardial cells.

Subcellular Distribution of the Ca^{2+}-ATPase in Type I (Slow) Skeletal Muscle Fibers

Previous ultrastructural studies have determined the qualitative and quantitative distribution of the sarcoplasmic reticulum in relation to the A and I bands in mammalian skeletal muscle [Eisenberg, 1983]. Thus it is feasible to assess whether the immunofluorescence staining patterns observed in transverse and longitudinal cryostat sections of type I myofibers after labeling with antibodies to the cardiac Ca^{2+}-ATPase correspond to the general distribution of the sarcoplasmic reticulum in skeletal muscle fibers (Fig. 6) [Jorgensen et al., 1979]. In longitudinal cryosections of type I myofibers in fixed

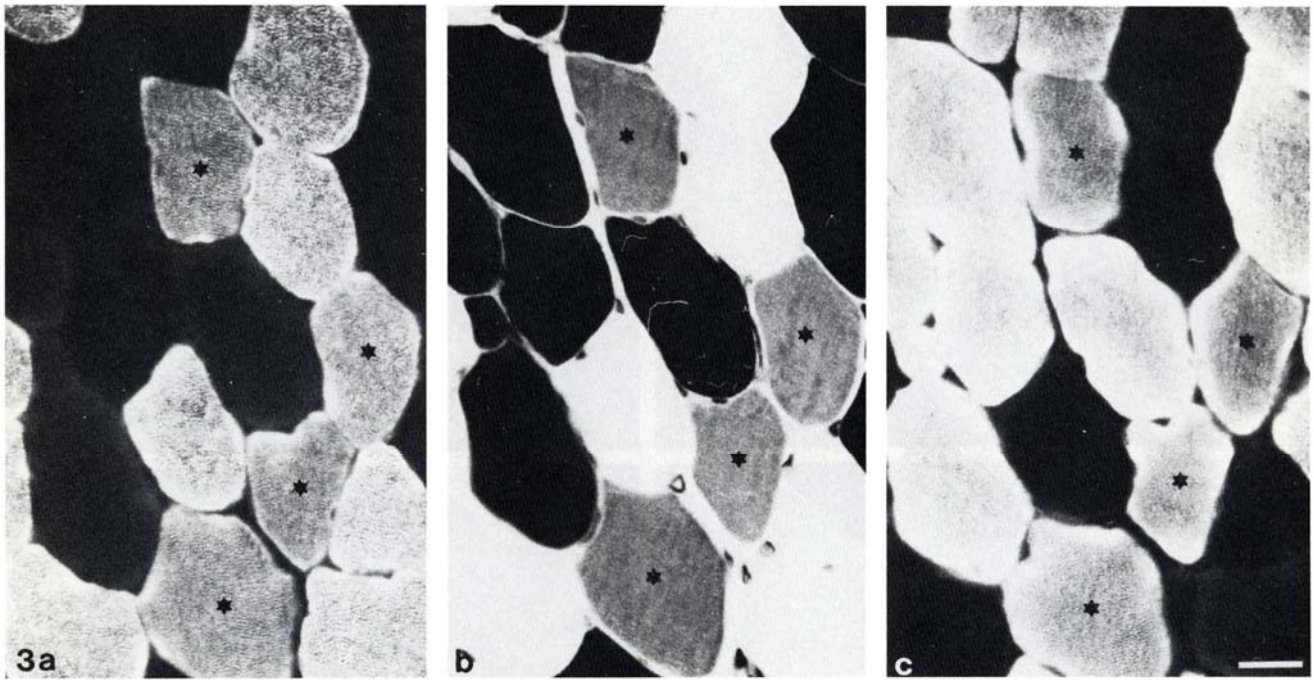

Fig. 3. Transverse serial cryostat sections of gracilis muscles labeled **a:** by the indirect immunofluorescence staining techniques with mAb to cardiac Ca^{2+}-ATPase (II D8), **b:** cytochemically for the alkali-stable myosin ATPase, and **c:** mAb to skeletal Ca^{2+}-ATPase (II H11) by the indirect immunofluorescence staining technique. In addition to type I (b, white) and type II myofibers (b, black), which are positively labeled for cardiac Ca^{2+}-ATPase (a) and skeletal Ca^{2+}-ATPase (c), respectively, a third kind of myofiber, type X (stars) is present. In contrast to types I and II myofibers, type X myofibers are strongly labeled with antibodies to both the cardiac (a) and the skeletal Ca^{2+}-ATPase (c) of the sarcoplasmic reticulum. Note that type X myofibers appeared grey following cytochemical labeling for alkali-stable myosin ATPase (stars, b). Bar = 20 μm.

gracilis muscle, strong immunofluorescence staining was present in the I band region following labeling with antibodies to cardiac Ca^{2+}-ATPase. Immunofluorescence labeling of the A band region was generally confined to the central part (Fig. 6d). The fluorescent labeling in the I band region was frequently resolved into fluorescent rods the length of which equaled the length of the I band (Fig. 6d). The rods were arranged parallel to one another and to the longitudinal axis of the fiber. The distance between the centers of two neighboring rods in an I band varied from 0.2 to 1.0 μm. In transverse sections of type I myofibers in gracilis muscle, a polygonal staining pattern was seen following immunolabeling with antibodies to cardiac Ca^{2+}-ATPase (Fig. 6e). The number of sides in each polygon varied from four to six, and the distance between the centers of neighboring polygons ranged from 1.1 to 1.5 μm.

Thus it appears that the subcellular distribution of the cardiac Ca^{2+}-ATPase-like protein as determined by immunofluorescence labeling is very similar to that of the sarcoplasmic reticulum in mammalian type I fibers as determined by electron microscopy [Eisenberg, 1983]. These results support the conclusion that the protein in slow skeletal fibers antigenically related to the cardiac-Ca^{2+}-ATPase is fairly uniformly distributed throughout the sarcoplasmic reticulum of type I (slow) fibers.

Identification and Characterization of Ca^{2+}-ATPase in Extracts From Canine Cardiac and Skeletal Muscle Tissues

To characterize the protein(s) present in slow but not in fast skeletal fibers that is antigenically related to the Ca^{2+}-ATPase of canine cardiac sarcoplasmic reticulum, the protein in extracts of canine slow (superficial digitalis flexor; 89 ± 3% type I fibers), fast (extensor carpi radialis; 14% ± 6% type I fibers), and cardiac muscle tissues were separated by SDS-PAGE and subsequently immunoblotted with mAb II D8 to the canine cardiac Ca^{2+}-ATPase (Fig. 7, left). The results showed that the 11 D8 specifically bound to a single band with an apparent Mr value of 115,000 in all three extracts (Fig. 7, left). However, qualitatively, the amount of cardiac Ca^{2+}-ATPase-like protein per milligram of extracts from superficial digitalis muscle tissue (89 ± 3% type I myofibers) was much higher than that of extensor carpi radialis muscle tissue (14 ± 6% type I myofibers). It is likely that the low amount of cardiac Ca^{2+}-ATPase-like protein in the extensor carpi radialis muscle tissue originates

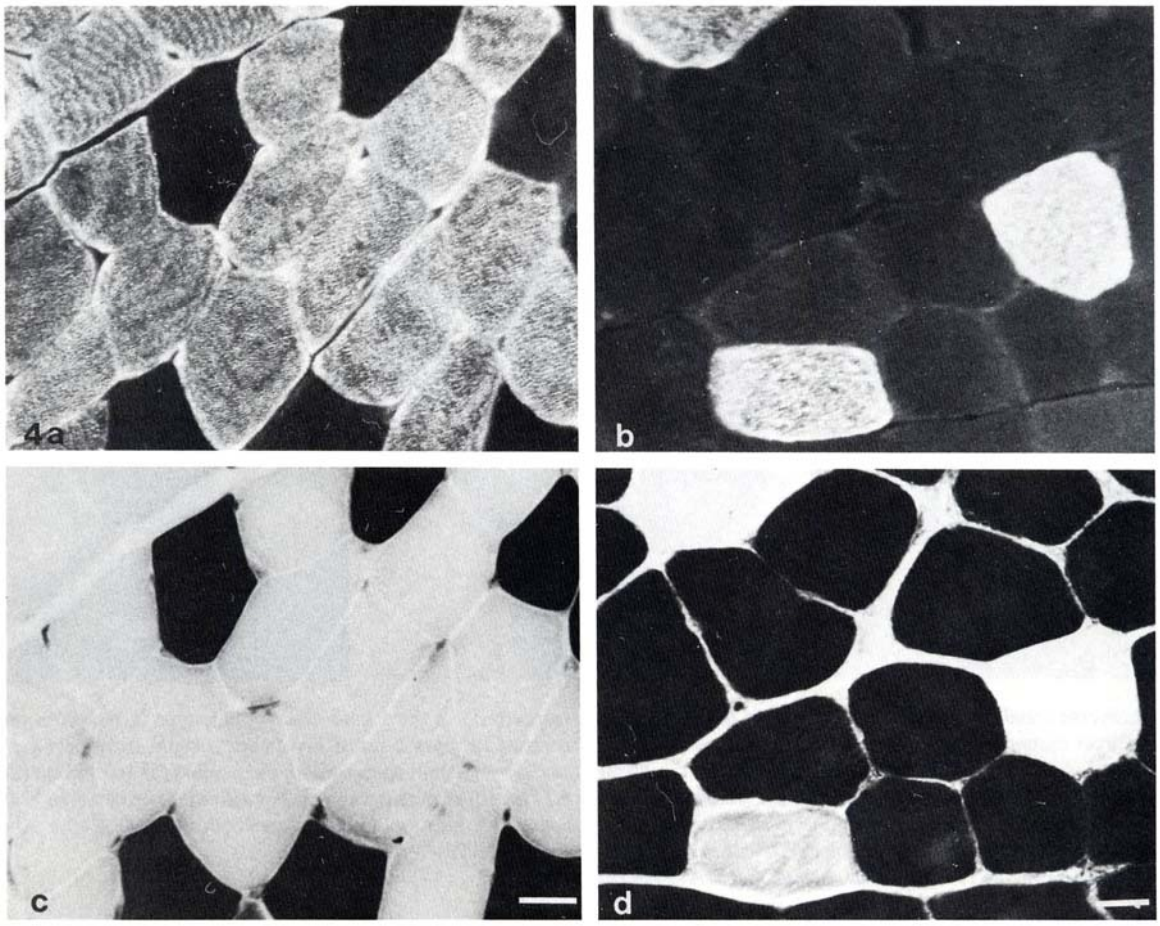

Fig. 4. Transverse cryostat sections of unfixed muscle tissue from superficial digitalis flexor (**a** and **c**) and extensor carpi radialis (**b** and **d**) stained with mAB 11 D8 to cardiac Ca^{2+}-ATPase (a and b) and cytochemically for alkali-stable myosin ATPase (c and d), a marker for type II (fast) myofibers. There is a positive correlation between myofibers positively stained for cardiac Ca^{2+}-ATPase (a and b, white) and myofibers unlabeled for alkali-stable myosin ATPase (c and d, white). Bar = 20 μm.

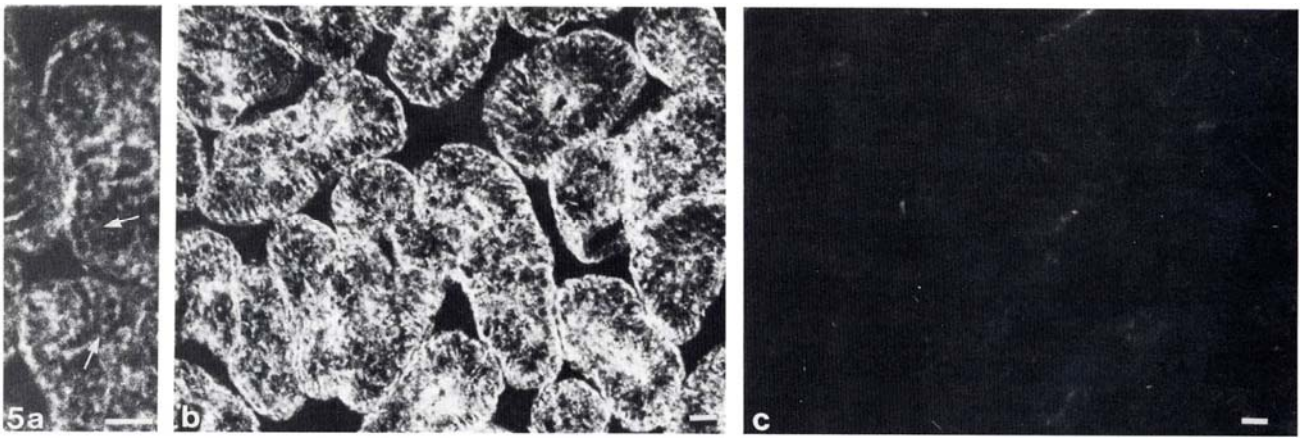

Fig. 5. Transverse cryosections of unfixed canine atrial muscle tissue stained with mAb 11 D8 to cardiac Ca^{2+}-ATPase (**a** and **b**) and II H11 to skeletal Ca^{2+}-ATPase (**c**). All myocardial cells are strongly labeled for cardiac Ca^{2+}-ATPase but unlabeled for skeletal Ca^{2+}-ATPase of the sarcoplasmic reticulum. Occasionally, an irregular networklike staining pattern was observed (a, arrows). Bar = 20 μm.

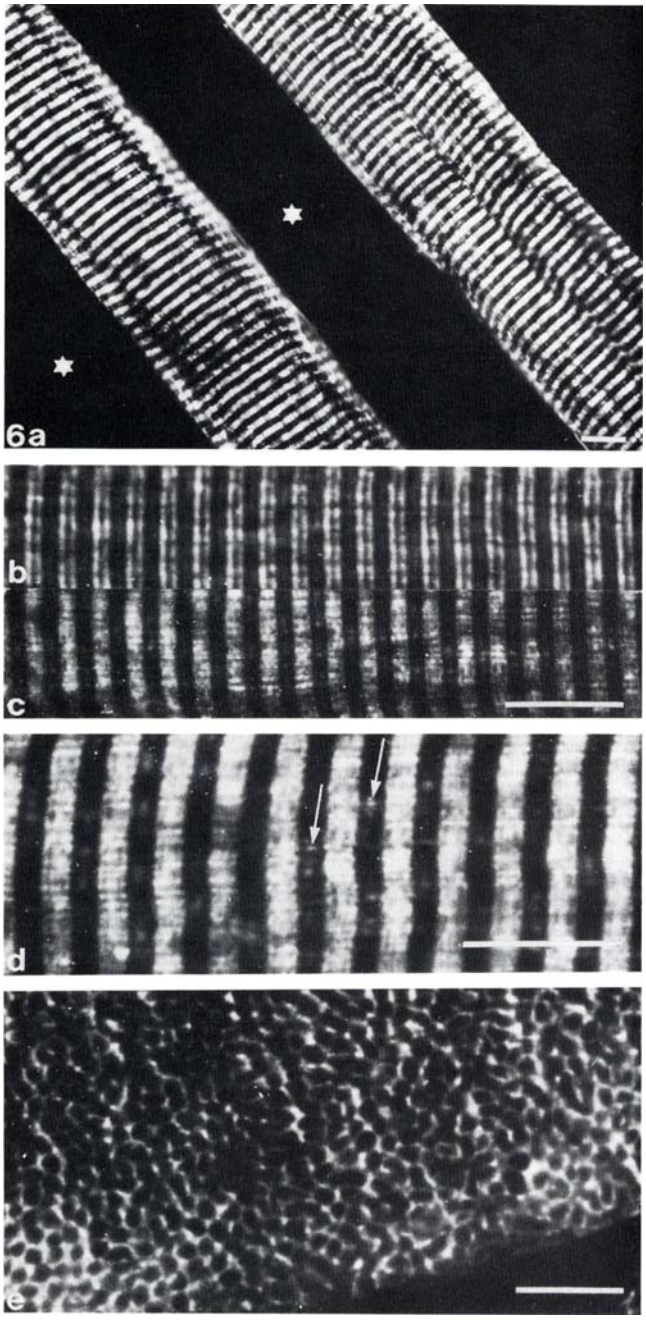

Fig. 6. Adult canine skeletal (gracilis) tissue fixed, cryostat-sectioned longitudinally (**a-d**) and transversely (**e**), and labeled with mAb to Ca^{2+}-ATPase (a,c,d,e). Note that, whereas some myofibers are strongly stained (a) with antibodies to cardiac Ca^{2+}-ATPase, others are very weakly labeled (a, stars). The fluorescence staining pattern in c was compared with the position of the A and the I bands in the same field as seen by phase-contrast microscopy in b. Regular fluorescent staining in the I band region consists of short rods running parallel to the long axis of the myofiber. The length of these strands equals the width of the I band (b). Regular fluorescent staining in the A band region consists of small, bright foci in the central region of the A band (d, arrows). In transverse sections of type I myofibers, a polygonal staining pattern was observed (e). Bar = 10 μm.

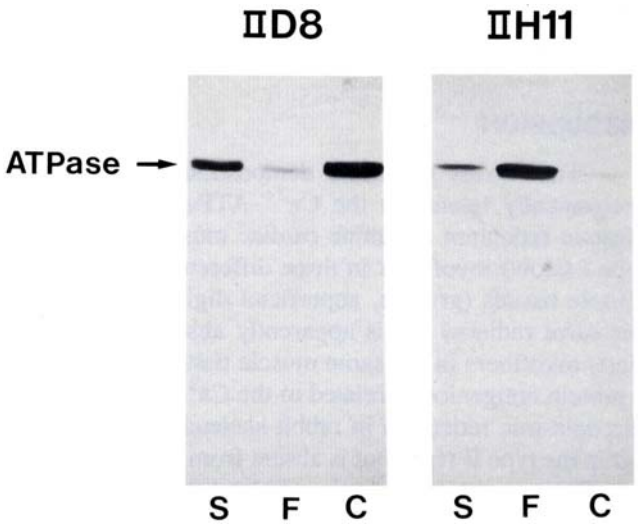

Fig. 7. Immunoblot staining of fast, slow, and cardiac muscle extracts with mAb II D8 (anticardiac Ca^{2+}-ATPase) and mAb II H11 (antiskeletal Ca^{2+}-ATPase). Canine muscle extracts (100 μg) were separated by SDS-PAGE (5-16% gradient gels) and transferred electrophoretically to nitrocellulose paper and immunoblotted as described in Figure 1 and in Materials and Methods. Nitrocellulose blots were stained with either 2.5 μg/ml II D8, cardiac-specific anti-Ca^{2+}-ATPase mAb (**left**); or 0.5 μg/ml II H11, skeletal-specific anti-Ca^{2+}-ATPase mAb (**right**). **Lanes S,F,** and **C** designate slow skeletal muscle extract, fast skeletal muscle extract, and cardiac muscle extract, respectively. ATPase designates the 115,000 Da Ca^{2+}-ATPase.

from type I (slow) myofibers, which constitute 14 ± 6% of the total myofibers of this muscle tissue.

To characterize the protein(s) present in fast but not in slow canine skeletal fibers that is antigenically related to the Ca^{2+}-ATPase of rabbit skeletal sareoplasmic reticulum, proteins in extracts of the same canine slow, fast, and cardiac muscle tissues were separated by SDS-PAGE and subsequently immunoblotted with mAb II H11 to rabbit skeletal Ca^{2+}-ATPase (Fig. 7). The results showed that this antibody specifically bound to a single band with an apparent Mr of 115,000 in the extracts from the fast and slow canine skeletal muscle tissue. However, this antibody did not bind any of the proteins present in the extract from canine cardiac muscle tissue. Qualitatively, the amount of the rabbit skeletal Ca^{2+}-ATPase-like protein per milligram of extract in extensor carpi radialis muscle tissue (14 ±6% type I myofibers) was much higher than that of superficial digitalis muscle tissue (89 ± 3% type I myofibers). It is likely that the low amount of rabbit skeletal Ca^{2+}-ATPase-like protein in the superficial digitalis muscle tissue originates from type II (fast) myofibers, which constitute approximately 10% of the total myofibers in this muscle tissue. The amount of rabbit skeletal Ca^{2+}-ATPase-like protein per milligram

of extract in canine cardiac muscle was barely detectable by the immunoblotting technique used (Fig. 7, right).

DISCUSSION

The results presented demonstrate that a protein antigenically related to the Ca^{2+}-ATPase of the sarcoplasmic reticulum in canine cardiac muscle is present in type I (slow) myofibers in three different canine skeletal muscle tissues (gracilis, superficial digitalis, and extensor carpi radialis) but is apparently absent from type II (fast) myofibers in the same muscle tissues. By contrast, a protein antigenically related to the Ca^{2+}-ATPase of the sarcoplasmic reticulum in rabbit skeletal muscle is present in the type II (fast) but isabsent from the type I (slow) myofibers in the same canine skeletal muscle tissue and from canine myocardial fibers.

The subcellular distribution of the cardiac Ca^{2+}-ATPase-like protein in type I myofibers as determined by indirect immunofluorescence labeling with mAb II D8 to cardiac Ca^{2+}-ATPase corresponds to that of the sarcoplasmic reticulum in mammalian type I myofibers as determined by stereological analysis at the ultrastructural level of resolution [Eisenberg, 1983]. This result suggests that the distribution of the cardiac Ca^{2+}-ATPase-like protein in type I myofibers is similar to that of phospholamban [Jorgensen, 1986] and that both proteins are uniformly distributed throughout the nonjuctional sarcoplasmic reticulum in type I myofibers. Thus it is possible that the cardiac Ca^{2+}-ATPase-like protein and phospholamban, as proposed for cardiac sarcoplasmic reticulum [Kirchberger et al., 1974; Tada and Katz, 1982], can interact and modulate the Ca^{2+} fluxes across the sarcoplasmic reticulum in type I (slow) myofibers.

Structurally, the apparent M_r in SDS gels of the cardiac Ca^{2+}-ATPase-like protein present in the sarcoplasmic reticulum of type I (slow) skeletal myofibers was found to be 115,000, indistinguishable from that of the Ca^{2+}-ATPase of the cardiac sarcoplasmic reticulum [Nakamura et al., 1983; Pepper et al., 1986]. Additional evidence for the structural similarity between the Ca^{2+}-ATPases of the sarcoplasmic reticulum in cardiac and slow skeletal muscle was obtained by comparing immunoblots of tryptic peptide maps from extracts of canine cardiac and slow skeletal muscles [Pepper et al., 1986].

Combining these results with the immunofluorescence and immunochemical studies presented here shows that the Ca^{2+}-ATPase of the sarcoplasmic reticulum from adult cardiac and type I (slow) skeletal myofibers have at least one epitope in common that is not present in the Ca^{2+}-ATPase of the type II (fast) skeletal sarcoplasmic reticulum. They also demonstrate that the Ca^{2+}-ATPase of type II (fast) skeletal sarcoplasmic reticulum has at least one epitope not present in the Ca^{2+}-ATPase of type I (slow) and cardiac sarcoplasmic reticulum, thus providing strong evidence that distinct isoforms of the Ca^{2+}-ATPase of the sarcoplasmic reticulum are present in adult fast and slow skeletal fibers. This interpretation is consistent with recent results obtained by cloning and sequencing two distinct cDNAs encoding for two different Ca^{2+}-ATPases present in neonatal rabbit skeletal muscle. These two distinct cDNAs were tentatively identified as the neonatal fast and slow isoforms of the Ca^{2+}-ATPase [Brandl et al., 1986]. Since a probe from the 3′-nontranslated region of the slow muscle cDNA hybridized with large transcripts of mRNAs from both slow skeletal and cardiac muscle, it was also proposed that the cardiac and slow isoforms of the Ca^{2+}-ATPases are encoded by the same gene.

There is now an extensive body of evidence demonstrating the similarity of some but not all of the contractile proteins in cardiac and slow skeletal muscle [Perry, 1985; Whalen, 1985]. It has been shown, for example, that the beta forms of ventricular myosin and slow skeletal muscle myosin are products of the same gene [Lompre et al., 1984]. The present study and the study of Brandl et al. [1986] demonstrate the similarity of the Ca^{2+}-ATPases of the sarcoplasmic reticulum in cardiac and slow skeletal muscle.

A majority of previous biochemical studies have suggested that the difference in the Ca^{2+}-ATPase and Ca^{2+} uptake activity of the isolated sarcoplasmic reticulum vesicles was due to quantitative rather than qualitative differences [Jorgensen, 1986; Pette and Vrbova, 1985]. The presence of distinct isoforms of the Ca^{2+}-ATPase in slow and fast skeletal muscle tissue has previously been suggested on the basis of immunochemical studies [Damiani et al., 1981]; however, the immunocytochemical and immunochemical studies presented here are me first to report on the characterization of two monoclonal antibodies of which one, II D8, specifically binds the Ca^{2+}-ATPase of the sarcoplasmic reticulum in either cardiac or type I (slow) skeletal fibers, whereas the other, II H11, specifically binds the Ca^{2+}-ATPase of type 11 (fast) fibers.

The nature of the type X fibers in canine gracilis muscle and their possible relationship to types I and n myofibers are presently unknown. It is possible that they correspond to the small number of fibers in rat and rabbit skeletal muscle tissues previously shown by immunocytochemical studies to be strongly labeled with antibodies specific to either the slow or the fast isoform of the heavy chain of myosin from skeletal muscle [Gauthier and Lowey, 1977; Lutz et al., 1979].

It is interesting that slow and cardiac muscle fibers in addition to the similarities of their Ca^{2+}-transport

systems of the sarcoplasmic reticulum both contain the same isoform of the regulatory protein troponin C [Perry, 1985], which upon binding of Ca^{2+} elicits muscle contraction. Thus it is conceivable that the gene expression of the major Ca^{2+} binding proteins relevant to the contraction relaxation cycle is similarly regulated in cardiac and type I (slow) muscle fibers.

Ultrastructural [Eisenberg and Salmons, 1981] and biochemical [Pete and Vrbova, 1985] studies of muscle fibers during the transformation of a fast muscle into a slow one by long-term, low-frequency stimulation showed that induced structural and molecular changes of the sarcoplasmic reticulum occur concurrent with the changes in the contractile properties of the fibers but well before the appearance of the slow isoform of myosin and other contractile proteins. Thus Pette and Vrbova [1985] suggested that the transformed sarcoplasmic reticulum rather than the contractile apparatus might play an important role in the early changes of the contractile properties of these fibers.

The availability of antibodies specific for the slow and the fast isoforms of the Ca^{2+}-ATPase will now enable us to use immunocytochemical labeling as a tool to determine at the cellular level the time course of the transition from the fast to the slow isoform of the Ca^{2+}-ATPase in response to long-term, low-frequency stimulation. Comparison of the results of these studies with similar ones of the time course of the transition from the fast to the slow isoform composition of myosin in the very same fibers should enable us to determine whether the slow form of the sarcoplasmic reticulum is indeed assembled and thus is likely to change the Ca^{2+} concentration and Ca^{2+} fluxes before the slow isoform of myosin and the other contractile proteins start to appear.

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