In situ localization of cholesterol in skeletal muscle by use of a monoclonal antibody

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1Division of Space Life Sciences, Universities Space Research Association, and 5Life Sciences Research Laboratories, National Aeronautics and Space Administration/Johnson Space Center, Houston, Texas 77058; 2Research Space Management Group, Massachusetts General Hospital, Boston, Massachusetts 02114; 3Division of Exercise Physiology, University of Alabama at Birmingham, Birmingham, Alabama 35294; and 4Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia 30912

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Clarke, Mark S. F., Charles R. Vanderburg, Marcas M. Bamman, Robert W. Caldwell, and Daniel L. Feeback. In situ localization of cholesterol in skeletal muscle by use of a monoclonal antibody. J Appl Physiol 89: 731–741, 2000.—A common perception is that cholesterol, the major structural lipid found in mammalian membranes, is localized nearly exclusively to the plasma membrane of living cells and that it is found in much smaller quantities in internal membranes. This perception is based almost exclusively on cell fractionation studies, in which density gradient centrifugation is used for purification of discrete subcellular membrane fractions. Here we describe a monoclonal antibody, MAb 2C5-6, previously reported to detect purified cholesterol in synthetic membranes (Swartz GM Jr, Gentry MK, Amende MM, Blanchette-Mackie EJ, and Alving CR. Proc Natl Acad Sci USA 85: 1902–1906, 1988), that is capable of detecting cholesterol in situ in the membranes of skeletal muscle sections. Localization of cholesterol, the dihydropyridine receptor of the T tubule, and the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum (SERCA2) by means of double and triple immunostaining protocols clearly demonstrates that cholesterol is primarily localized to the sarcoplasmic reticulum membranes of skeletal muscle rather than the sarcolemmal or T tubule membranes. The availability of this reagent and its ability to spatially localize cholesterol in situ may provide a greater understanding of the relationship between membrane cholesterol content and transmembrane signaling in skeletal muscle.

Cholesterol has at least two fundamental and important functions in mammalian cellular membranes. First, cholesterol serves as the major structural lipid that provides strength to the membrane bilayer because of its ability to pack into the intermolecular spaces that would otherwise exist between the constituent phospholipid molecules (14, 44). Cholesterol has been termed a "rigidifying" lipid, and as the cholesterol-to-phospholipid ratio of a mammalian membrane increases, the membrane fluidity decreases (13, 15, 31, 33, 37). Paradoxically, however, the absence of membrane cholesterol results in a purely phospholipid bilayer in which the solid-to-liquid crystalline phase transition temperature is ~42°C, which is well above the normal physiological temperature (e.g., 37°C in humans) required for optimal membrane function in mammals. However, the addition of cholesterol to mammalian cell membranes reduces the solid-to-liquid crystalline phase transition temperature to ~25°C, which is well below the physiological temperature required for normal membrane function in mammals (10, 44).

A second function of cholesterol in mammalian cellular membranes is to contribute to the formation of distinct membrane microdomains, which have been referred to as "detergent-resistant membranes" (10, 35), "cholesterol-rich microdomains" (6), or "lipid rafts" (9). One apparent function of these lipid rafts is to provide a means of partitioning certain membrane proteins into cholesterol-rich or cholesterol-poor regions of the membrane. Whether proteins are incorporated directly into these cholesterol-rich domains/lipid rafts at the level of the Golgi apparatus or physically partitioned between cholesterol-rich and cholesterol-poor membrane regions dependent on lipid solubility once they enter the membrane is unclear (for review see Ref. 10). However, numerous studies in vitro and in vivo have demonstrated that the activity of certain membrane proteins is dependent on the presence and the relative concentration of cholesterol in the membrane in which the proteins reside (for review see Ref. 6). For example, increased levels of free cytosolic Ca\(^{2+}\) in otherwise intact cells can be due to a cholesterol-related disruption in the function of membrane proteins responsible for Ca\(^{2+}\) handling. For example,
increased resting cytosolic Ca\(^{2+}\) levels in arterial vascular smooth muscle cells (46), rat cardiac myocytes (5), platelets (38), monocytes (1), and erythrocytes (29) have been linked to an increase in Ca\(^{2+}\) flux across the plasma membrane due to increased membrane cholesterol content. In vascular smooth muscle cells, the activity of the L-type Ca\(^{2+}\) channel of the plasma membrane was enhanced as the membrane cholesterol content was increased (36). A similar effect was observed in cardiac myocytes, where Ca\(^{2+}\) channel activity was enhanced as membrane cholesterol content was increased (5). Conversely, the activity of the Ca\(^{2+}\)-ATPase pump found in isolated sarcosomal vesicles from cardiac tissue was inhibited by increased membrane cholesterol content, whereas it was enhanced by reduced membrane cholesterol content (30, 32). Membrane cholesterol content also appears to be important with respect to insulin and insulin-like growth factor I (IGF-I) signaling in skeletal muscle, so that myofiber membrane cholesterol content increases, so too does the insulin and IGF-I insensitivity of the muscle (27, 28, 39).

The prevailing view in the mammalian system is that the majority of membrane-associated cholesterol is located in the plasma membrane of the cell, with little or no cholesterol in intracellular membrane components such as ER membranes (2, 44). This view has been derived nearly exclusively from cell fractionation studies, in which linear sucrose gradients have been utilized to separate cellular membrane components on their respective densities, followed by biochemical analysis of the lipid constituents of these membrane fractions. This approach in skeletal muscle has led to the view that the majority of membrane-associated cholesterol is found in the sarclemma and T tubule membrane fraction of adult skeletal muscle (amphibian and mammalian), with little or no cholesterol being detected in the sarcoplasmic reticulum (SR) membrane fraction (19, 20, 23, 34). More recent studies have challenged this finding with biochemical demonstration that significant amounts of cholesterol are detectable in the SR membrane fractions of mammalian skeletal muscle (8, 43) and that the relative amount of cholesterol in these membranes increases with increasing age (25). Because of the potentially far-reaching effects of increased membrane cholesterol content on the biomechanical properties and the transmembrane signaling function of membrane proteins located within the lipid matrix of skeletal muscle membranes, this study was undertaken to develop an immunohistochemical method that would allow the precise in situ cellular localization of cholesterol in frozen sections of normal adult rat and human skeletal muscle tissue.

**MATERIALS AND METHODS**

**Antibodies**

An anti-cholesterol mouse IgM monoclonal antibody (MAb) was derived from a hybridoma cell line purchased from American Type Culture Collection (HB-8995, designation MAb 2C5-6) and grown according to the supplier's instructions. This antibody has been shown to detect cholesterol in synthetic phospholipid vesicles in a concentration-dependent fashion and to specifically bind to the surface of purified crystalline cholesterol at the ultrastructural level by electron microscopy (3, 41). The anti-dihydropyridine (DHP) receptor (anti-pan \(\alpha_{\text{subunit}}\) rabbit IgG polyclonal antibody was obtained from Almone Labs (Jerusalem, Israel). The anti-SR Ca\(^{2+}\)-ATPase mouse IgG MAb (SERCA2), which recognizes only the SR Ca\(^{2+}\)-ATPase found in type I myofibers, was obtained from Research Diagnostics (Flanders, NJ). The rhodamine-conjugated F(\(\text{Ab}\)\(^{\text{b}}\))\(_{2}\) fragment of goat anti-mouse IgG (\(\mu\)-chain) polyclonal antibody was obtained from ICN Pharmaceuticals (Aurora, CA). The Alexa 350-conjugated goat anti-mouse IgG and Alexa 488-conjugated goat anti-rabbit IgG antibodies were purchased from Molecular Probes (Eugene, OR). The aqueous antifade mounting medium FluoroMount G was purchased from Electron Microscopy Supplies (Fort Washington, PA).

**Frozen Sectioning**

Frozen (10-\(\mu\)-m-thick) sections of perfusion-fixed (formaldehyde) or snap-frozen rat soleus and triceps muscles were obtained as previously described (12). Frozen (10-\(\mu\)-m-thick) sections of needle biopsy samples from the vastus lateralis of human test subjects were obtained as previously described (4).

**Immunostaining Protocol**

All incubations and washes were carried out at room temperature (i.e., 25°C). Frozen sections were washed in PBS containing 1 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\) (D-PBS) (pH 7.2) and then incubated with D-PBS (pH 7.2) containing 50 mM ammonium chloride for 4 h to reduce autofluorescent signal in the tissue. The sections were again washed once with D-PBS over a period of 5 min and then incubated in blocking buffer consisting of 50% (vol/vol) heat-inactivated goat serum (HIGS) for 1 h. Alternatively, those sections destined for localization of the DHP receptor were incubated in D-PBS containing 50%, 25%, or 12.5% HIGS for 2 h as follows: the anti-cholesterol mouse IgM monoclonal antibody MAb 2C5-6 was used at a 1:4 dilution of neat hybridoma supernatant, the anti-DHP receptor (anti-pan \(\alpha_{\text{subunit}}\) rabbit IgG polyclonal antibody was used at a 1:60 dilution of manufacturer's stock solution, and the anti-SR Ca\(^{2+}\)-ATPase mouse IgG monoclonal antibody (SERCA2) was used at a 1:400 dilution of the manufacturer's stock solution. Those sections destined for double or triple labeling of cholesterol, the DHP receptor, and/or the SR Ca\(^{2+}\)-ATPase in the same section were incubated for 2 h in a mixture of primary antibodies at the dilutions noted above. After primary antibody incubation, the sections were washed five times over a period of 30 min with D-PBS and then incubated for 1 h with the relevant secondary antibody alone or a mixture of secondary antibodies, all of which were used at a 1:400 dilution of the manufacturer's stock solutions, and the anti-SR Ca\(^{2+}\)-ATPase mouse IgG monoclonal antibody (SERCA2) was used at a 1:400 dilution of the manufacturer's stock solution. Those sections destined for double or triple labeling of cholesterol, the DHP receptor, and/or the SR Ca\(^{2+}\)-ATPase in the same section were incubated for 2 h in a mixture of primary antibodies at the dilutions noted above. After primary antibody incubation, the sections were washed five times over a period of 30 min with D-PBS and then incubated for 1 h with the relevant secondary antibody alone or a mixture of secondary antibodies, all of which were used at a 1:400 dilution of the manufacturer's stock solutions diluted in D-PBS containing 1% (vol/vol) HIGS. All primary and secondary antibody staining solutions were centrifuged at 10,000 g for 2 min immediately before use to remove any particulate matter. The sections were then washed over a period of 30 min with five changes of D-PBS, mounted in FluoroMount G antifade aqueous mounting medium, coverslipped, and sealed with clear nail polish before microscopic examination. Immunostained sections were viewed using a Zeiss Axiosplan2 fluorescence microscope or a Zeiss scanning confocal microscope. Epifluorescent images were captured...
using a COHU charge coupled device camera attached to the Axioplan 2 microscope controlled by MacProbe image capture software (PSI, League City, TX). Confocal images were captured using the Zeiss image analysis software integral to the scanning confocal microscope.

**Demonstration of MAb 2C5-6 Specificity for Cholesterol in Frozen Sections**

Cross-reaction of MAb 2C5-6 with skeletal muscle membrane-associated proteins. Crude membrane fractions were prepared from rabbit skeletal muscle as previously described (18) (membrane fractions were kindly provided by Dr. Susan Hamilton, Baylor College of Medicine, Houston, TX). Crude membrane fractions were solubilized in standard Laemmli solubilization buffer by boiling for 5 min at a concentration of 1 µg of total protein per microliter of buffer. The solution was then centrifuged at 10,000 g to remove any insoluble material, and the resulting supernatant was collected. The supernatant was then dot blotted onto 0.2-µm supported nitrocellulose in 5-µl aliquots and allowed to dry. Subsequent aliquots of supernatant were reapplied to each dot blot to create a concentration gradient of solubilized membrane-associated protein of 5, 10, 15, and 30 µg/dot blot, respectively. Negative controls containing 1 µg of BSA per microliter of Laemmli buffer were applied in a similar fashion to the nitrocellulose membrane. The dot blot was blocked with D-PBS containing 4% (vol/vol) HIGS and 0.1% (vol/vol) Tween 20 and then incubated with a 1:8 dilution of MAb 2C5-6 hybridoma supernatant made up in D-PBS containing 1% (vol/vol) HIGS and 0.1% (vol/vol) Tween 20 for 1 h at room temperature with gentle agitation. The blot was washed three times with D-PBS containing 0.1% (vol/vol) Tween 20 over a period of 20 min and then incubated with a 1:1,000 dilution of an alkaline phosphatase-conjugated F(AB')₂ fragment of goat anti-mouse IgM (μ-chain) polyclonal antibody made up in D-PBS containing 1% (vol/vol) HIGS and 0.1% (vol/vol) Tween 20 for 1 h at room temperature with gentle agitation. The blot was again washed, and secondary antibody binding was disclosed using 5-bromo-4-chloro-3-indolylphosphate-p-toluidine-nitro blue tetrazolium substrate (Vector Laboratories, Burlingame, CA).

**Acetone extraction.** Frozen sections were incubated in acetone (previously dehydrated with calcium chloride) for 1 h at 4°C. This procedure has previously been shown to extract all lipids, including cholesterol, from fixed and snap-frozen tissues (124). The sections were then stained for cholesterol with MAb 2C5-6 as described above.

**Cold ethanol-Triton X-100 extraction.** Frozen sections were incubated in four changes of pure ethanol containing 5% Triton X-100 for 1 h at 4°C. Triton X-100 and/or cold ethanol have previously been shown to extract a variety of lipids and proteins, excluding cholesterol, from fixed and snap-frozen tissues (10, 24). The sections were then stained for cholesterol with MAb 2C5-6 as described above.

**Treatment with saponin.** Frozen sections were incubated in four changes of Ca²⁺- and Mg²⁺-free PBS containing 0.1% saponin for 1 h at room temperature. Saponin treatment of cells has previously been shown to “chelate” or “complex” cholesterol within the matrix of the membrane (7, 17, 45). The sections were then stained for cholesterol with MAb 2C5-6 as described above.

**Specificity of MAb 2C5-6 for Cholesterol on the Basis of Histological Criteria**

The cells of Ito are cells that occasionally line the bile canaliculi of the liver. These cells are in intimate contact with bile fluid and contain large amounts of cholesterol. In addition, hepatocytes synthesize and process large amounts of cholesterol. Frozen sections of perfusion-fixed rat liver were stained with MAb 2C5-6, with and without the cholesterol extraction treatments described above before staining.

**Preadsorption of Primary Antibody Against Purified Crystalline Cholesterol and Closely Related Compounds**

Purified crystalline cholesterol (99.9% pure; Sigma Chemical, St. Louis, MO), dihydrocholesterol (cholesterol dimer, 95% pure; Sigma Chemical), 4-cholesten-3-one (cholesterol oxidation product, 95% pure; Sigma Chemical), cholesteryl oleate (cholesterol ester, 99% pure; Sigma Chemical), or brain lipid extract (type VII from bovine brain, acetone precipitated Folch extract of whole bovine brain containing all the major brain phospholipids and glycolipids; Sigma Chemical) were each dissolved in 200 µl of chloroform at a concentration of 200 mg/ml and dispensed into 15-ml glass bottles that had been rinsed with chloroform. The glass bottles were then rolled along their long axes to coat the internal surface of the bottles with the chloroform-lipid solution. As the chloroform evaporated from the bottles, a thin, even coating of crystalline lipid was deposited on the internal surface of the glass bottles. The bottles were dried in an oven at 65°C for 1 h, filled with D-PBS containing 4% (vol/vol) HIGS (i.e., frozen section blocking buffer), and then incubated for 1 h at room temperature and gently washed with D-PBS. After nonspecific protein blocking of the crystalline lipid layer, 4 ml of MAb 2C5-6 hybridoma supernatant (diluted 1:4 in D-PBS containing 1% (vol/vol) HIGS) were added to each bottle. The bottles were then gently rotated for 16 h at 4°C, thereby ensuring efficient contact of the antibody solution with the crystalline lipid layer. The preadsorbed antibody solutions were removed from each bottle, centrifuged at 15,000 g for 15 min to remove any particulate matter, and used instead of primary antibody to stain frozen sections as described above.

**RESULTS**

When frozen sections of rat soleus or human vastus lateralis were stained with MAb 2C5-6, which was previously shown to recognize purified cholesterol in synthetic phospholipid vesicles and purified crystalline cholesterol (3, 41), distinct staining of intramyofiber membranes could clearly be seen (Fig. 1). In cross section, the MAb 2C5-6 staining pattern exhibited a “rosette” appearance around individual myofibrils that was morphologically reminiscent of the pattern expected if the terminal cisternae of the SR membrane component in cross section had been stained. In longitudinal sections, the MAb 2C5-6 staining appeared as an intermittent banding pattern around individual myofibrils. Surprisingly, no evidence of any staining at the light-microscopic level of the sarcolemma of individual myofibrils in human or rat muscle sections was observed. Because the prevailing view is that cholesterol is not found in the SR membranes, but in the sarcolemma/T tubule membranes of skeletal muscle, the specificity of the MAb 2C5-6 staining for cholesterol in frozen tissue sections was verified by additional experiments.

The first step in this process was to determine whether MAb 2C5-6 cross-reacted with protein components of skeletal muscle membranes. When SDS-solubilized crude muscle membrane fractions were dot-
Fluorescent micrograph of MAb 2C5-6 staining in a frozen cross section of human vastus lateralis (A) and a longitudinal section of rat soleus muscle (B). Note the “rosette” appearance of the staining pattern (A) around individual myofibrils in cross section and the intermittent banding pattern (B) around individual myofibrils in longitudinal section. Scale bars, 50 and 10 μm in A and B, respectively.

blotted and immunostained with MAb 2C5-6, no reactivity was observed, indicating that this antibody does not recognize an epitope found in reduced skeletal muscle membrane-associated protein samples. The second step in this process was to determine whether MAb 2C5-6 stained cellular structures definitively known to contain cholesterol. With use of frozen sections of perfusion-fixed rat liver, MAb 2C5-6 was shown to heavily stain the cells that occasionally line the bile canaliculi (Fig. 2). These cells, known as the cells of Ito, are responsible for the uptake and concentration of cholesterol from the surrounding hepatocytes before secretion of cholesterol into the bile canaliculi as a constituent of the bile fluid. In addition, MAb 2C5-6 also stained membranous and nonmembranous components of the hepatocytes, the major cholesterol-producing cells of the liver. When liver sections were extracted with acetone before they were stained with MAb 2C5-6, all staining was abolished (Fig. 2B). A similar decrease in staining intensity was observed if liver sections were stained with MAb 2C5-6 preadsorbed with purified crystalline cholesterol (Fig. 2C). When liver sections were extracted with a mixture of cold ethanol and Triton X-100 (neither of which extract cholesterol at room temperature or below) before they were stained with MAb 2C5-6, staining intensity of the cells of Ito and hepatocytes was enhanced (Fig. 2D). If liver sections were treated with saponin before they were stained with MAb 2C5-6, the staining pattern changed from a discrete to a diffuse particulate pattern (Fig. 2E). This alteration in staining pattern is consistent with the cholesterol chelating or complexing action of saponin in mammalian membranes (7). No significant staining was observed in liver sections if a mixture of fresh hybridoma growth medium containing 1% (vol/vol) HIGS was substituted for primary antibody solution (Fig. 2F).

Similar effects on MAb 2C5-6 staining were observed in human vastus lateralis sections (Fig. 3) when these samples were treated in a similar fashion to the liver sections, namely, staining was abolished if sections were acetone extracted before they were stained (Fig. 3B), staining was reduced if sections were stained with MAb 2C5-6 preadsorbed against purified crystalline cholesterol (Fig. 3C), staining was enhanced if sections were treated with cold ethanol-Triton X-100 (Fig. 3D), and MAb 2C5-6 staining was redistributed if the sections were treated with saponin before they were stained (Fig. 3E). No significant staining was observed in skeletal muscle sections if a mixture of fresh hybridoma growth medium containing 1% (vol/vol) HIGS was substituted for primary antibody solution (Fig. 3F). Similar results were observed if frozen sections of perfusion-fixed or snap-frozen rat soleus muscle were treated in a similar manner (data not shown).

To further demonstrate the specificity of MAb 2C5-6 for cholesterol, antibody supernatant was preadsorbed against a number of purified lipid molecules closely related to cholesterol in structure, namely, dihydrocholesterol (cholesterol dimer), cholesteryl oleate (cholesterol ester), and 4-cholesten-3-one (cholesterol ketone, 95% pure; Sigma Chemical). In addition, antibody supernatant was also preadsorbed against a complex mixture of lipids (i.e., brain extract, fraction VII) that contains all the major phospholipids and glycolipids of brain tissue but is cholesterol depleted by virtue of being an acetone precipitation of a Folch extraction. When frozen sections of rat soleus muscle were stained with MAb 2C5-6 preadsorbed against these purified lipids, a significant reduction in staining was observed if the antibody was preadsorbed with cholesterol (Fig. 4B) or its dimer dihydrocholesterol (Fig. 4C) but not with cholesteryl oleate (Fig. 4D), 4-cholesten-3-one (Fig. 4E), or brain extract (fraction VII; Fig. 4F).

On the basis of previously published results that demonstrated the specificity of MAb 2C5-6 for cholesterol in synthetic membranes, as well as at the electron-microscopic level for purified crystalline cholesterol (3, 41), combined with the immunofluorescent staining results obtained in frozen liver and muscle sections reported above, we conclude that MAb 2C5-6 can be used to detect cholesterol biochemically, as has previously been reported (3, 41), but also to specifically localize cholesterol immunohistochemically at the cellular level in situ.
As illustrated in Fig. 1, the cellular distribution of cholesterol in skeletal muscle appears on morphological criteria at the light-microscopic level to be localized almost exclusively to the SR membrane component. To verify this result, frozen sections of rat soleus muscle were double immunolabeled for cholesterol and either the \( \alpha_1 \)-subunit of DHP receptor (40) found exclusively in the sarcolemma/T tubule membranes (Fig. 5) or the \( \mathrm{Ca}^{2+} \)-ATPase of type I myofibers (SERCA2) (22) found exclusively in SR membranes (Fig. 6). Cholesterol staining (red, Fig. 5A) and DHP receptor staining (green, Fig. 5B) do not exhibit a similar pattern within the same section. When a composite image of cholesterol and DHP receptor staining in the same section is viewed at a higher magnification (Fig. 5C), the DHP receptor staining is spatially oriented above or beside the membrane component stained for cholesterol, indicating that cholesterol does not colocalize at the light-microscopic level with a protein (i.e., DHP receptor) found exclusively in the sarcolemma/T tubule membranes of skeletal muscle. Conversely, if the section is double immunolabeled for cholesterol (red, Fig. 6A) and the SERCA2 protein (green, Fig. 6B), the cholesterol and SERCA2 staining patterns at the light-microscopic level are identical. When a composite image of cholesterol and SERCA2 staining in the same section is viewed at a higher magnification (Fig. 6C), both antibodies recognize antigens localized to the same membrane structures within an individual myofiber. Confocal microscopy of frozen cross sections of human vastus lateralis stained for cholesterol and the DHP receptor showed a clear spatial delineation of the T tubule marker (i.e., the DHP receptor) and cholesterol staining (Fig. 7). When longitudinal frozen sections of rat soleus were triple immunolabeled for cholesterol (red, Fig. 8A), the DHP receptor (green, Fig. 8B), and SERCA2 (blue, Fig. 8C), the composite overlay image (Fig. 8D) clearly indicates that DHP receptor staining (green) could be distinguished from the cholesterol and SERCA2 staining signal, which appeared as a magenta color (red-and-blue mix).

**DISCUSSION**

The most commonly perceived function for the membranes of living cells is that of a physical barrier
between the extracellular and intracellular environment (i.e., plasma membrane) or as “partitioning” structures between specific regions of the intracellular environment (e.g., nuclear membrane and endoplasmic reticulum). Historically, cellular membranes have been considered an inert “lipid scaffold structure,” in which the elements essential for transmembrane signaling (i.e., proteins) are housed and supported while they carry out their signal transduction function (10). However, it seems unlikely that the large heterogeneity observed in the lipid components of the plasma membrane or intracellular membranes would be required if they simply served only a physical barrier function. It is the growing body of experimental evidence which suggests that the lipid composition of a membrane, specifically its cholesterol content, can alter the biomechanical properties of the membrane and the transmembrane signaling function of membrane proteins embedded within the three-dimensional matrix of the membrane that has driven this study (for review see Refs. 9, 6, 44).

Utilizing a novel immunostaining procedure for detecting cholesterol in frozen sections, we have localized cholesterol, at the fluorescent light-microscopic level, exclusively to the SR membrane of normal skeletal muscle rather than to the sarcolemma/T tubule membrane. This is not to say that cholesterol is completely absent from other myofiber membranes (i.e., sarcolemma/T tubule or mitochondrial membranes) but rather indicates that the vast majority of cholesterol found in skeletal muscle is present in the SR membrane component. Utilizing a series of selective solvent and/or detergent extraction protocols (i.e., acetone extraction of all lipids including cholesterol, ethanol-Triton X-100 extraction of some lipids and some proteins without extraction of cholesterol, and chelation/complexing of cholesterol using saponin) and preadsorption of the primary antibody against purified crystalline cholesterol immobilized on a solid support, we have demonstrated that the epitope recognized by MAb 2C5-6 is specific to cholesterol and its dimer and that it is capable of recognizing native cholesterol residing in skeletal muscle membranes of frozen sections in situ. In addition, utilizing known

Fig. 3. Fluorescent micrographs of MAb 2C5-6 staining in snap-frozen cross sections of human vastus lateralis after MAb 2C5-6 staining without pretreatment (A), MAb 2C5-6 staining after acetone extraction (B), MAb 2C5-6 staining after preadsorption of the primary antibody against purified crystalline cholesterol immobilized on a solid support (C), MAb 2C5-6 staining after extraction with a mixture of ethanol and 1% (vol/vol) Triton X-100 (D), MAb 2C5-6 staining after saponification of the tissue (E), and immunostaining associated with secondary antibody binding only (F). All images were acquired under identical photographic conditions. Scale bars, 10 μm.
histological criteria, we have demonstrated that MAb 2C5-6 recognizes cellular structures, membranous and otherwise, definitively known to contain large quantities of cholesterol in vivo, namely, hepatocytes and cells of Ito in frozen liver sections. Furthermore, utilizing double and triple immunolabeling protocols with antibodies that recognize proteins already shown to be exclusively localized to the sarcolemma/T tubule membranes (i.e., the DHP receptor) (40) or the SR membranes (i.e., SR Ca\(^{2+}\)-ATPase pump) (22), we have demonstrated at the light-microscopic level that cholesterol is localized to the same membrane component as the SR Ca\(^{2+}\)-ATPase pump (i.e., the SR membrane) rather than to the sarcolemma/T tubule component in human and rat skeletal muscle.

Because MAb 2C5-6 does not significantly stain other membrane structures within the same muscle section (i.e., sarcolemma, T tubule, or mitochondrial membranes) or show significant cross-reactivity with a cholesterol-depleted complex lipid mixture containing phospholipids and glycolipids (Fig. 4F), it appears unlikely that MAb 2C5-6 recognizes the phospholipids or glycolipids presumably common to all mammalian membranes. In addition, extraction of skeletal muscle sections with cold acetone abolishes MAb 2C5-6 staining (Figs. 2 and 3). Inasmuch as acetone extracts only hydrophobic lipids, rather than hydrophilic lipids, phospholipids and glycolipids being hydrophilic lipids (24), these data, in combination with a lack of cross-reactivity with a complex lipid mixture (Fig. 4), confirm that MAb 2C5-6 recognizes neither phospholipids nor glycolipids in addition to cholesterol. It is possible that MAb 2C5-6 may recognize several other hydrophobic lipid species that may be present in skeletal muscle SR membranes, including free fatty acids, terpenes, neutral fats, waxes, cholesterol esters, and steroid hormones that have a hydrophobic nature similar to that of cholesterol (i.e., are soluble in acetone). However, the lack of cross-reactivity of MAb 2C5-6 with purified cholesteryl oleate or 4-cholestene-3-one (Fig. 4) indicates that MAb 2C5-6 recognizes an epitope common to cholesterol and its dimer, but not its ester or ketone.

The localization of the majority of cholesterol (and potentially its dimer dihydrocholesterol) present in skeletal muscle to the SR membranes rather than to the sarcolemmal/T tubule membranes in vivo raises many interesting physiologically relevant questions as to the importance of relative membrane cholesterol content and the localization of cholesterol in a particular membrane. For example, we and others have previously shown that a large increase in membrane cholesterol content (as occurs in endothelial cells after exposure to serum containing hypercholesterolemic levels of low-density lipoproteins) increases cell susceptibility to mechanically induced membrane damage or “membrane wounding” (13, 42). These effects have been attributed to cholesterol’s ability to increase membrane order (i.e., decrease membrane fluidity or increase membrane rigidity), re-
Fig. 5. Double immunofluorescent labeling of snap-frozen longitudinal sections of rat soleus with MAb 2C5-6 [tetramethylrhodamine isothiocyanate (TRITC)-linked secondary antibody, red; A] and an anti-dihydropyridine (DHP; α₁-chain) antibody (Alexa 488-linked secondary antibody, green; B) and a composite overlay of both staining patterns (C). MAb 2C5-6 and the anti-DHP (α₁-chain) antibody do not exhibit a similar staining pattern within the same section, and, in a composite overlay of the same section viewed at a higher magnification (C), DHP receptor staining is spatially oriented above or beside the MAb 2C5-6 staining. Scale bars, 10 μm.

Fig. 6. Double immunofluorescent labeling of snap-frozen longitudinal sections of rat soleus with MAb 2C5-6 (TRITC-linked secondary antibody, red; A) and an anti-SERCA2 antibody (Alexa 488-linked secondary antibody, green; B) and a composite overlay of both staining patterns (C). MAb 2C5-6 and the anti-SERCA2 antibody exhibit a similar staining pattern within the same section, and, in a composite overlay image of the same section viewed at a higher magnification (C), it appears that the SERCA2 and MAb 2C5-6 staining patterns are identical. Scale bars, 10 μm.
resulting in a membrane that is less capable of withstanding the imposition of mechanical force (15). Evidence to support this contention comes from the rapid (i.e., 2 min) reversal of this effect by the addition of a membrane-disordering lipid to the cholesterol-enriched endothelial cell membrane (13).

In addition, cholesterol-induced increases in membrane order can affect the transmembrane signaling function of membrane proteins in a variety of different systems. First, increased amounts of cholesterol in the plasma membrane of a variety of different cell types, in vitro and in vivo, result in a concomitant increase in membrane order and alteration in the fidelity of numerous transmembrane signaling events, including those associated with neuronal transmission and intracellular Ca\(^{2+}\) regulation (reviewed in Ref. 6). Second, increased cholesterol content and the subsequent increase in membrane order of skeletal muscle sarcolemma detected after feeding rats a high-fat diet results in a reduction in the sensitivity of this tissue to circulating anabolic factors, such as insulin and IGF-I (27, 28, 39). Decreased sensitivity of skeletal muscle to these growth factors has been associated with the inability of the growth factor receptor to undergo the appropriate conformational changes required for efficient transmembrane signaling of the growth factor stimulus. This effect has been referred to as “molecular freezing” of the receptor protein in the three-dimensional lipid matrix of the sarcolemma. This phenomenon is directly related to a number of alterations in the lipid matrix of the plasma membrane, including increased cholesterol content, increased saturated fatty acid content, and increased cross-linking of the lipid membrane matrix due to free radical-induced lipid peroxidation, all of which result in an increase in membrane order (11, 27, 39). More importantly, the decrease in skeletal muscle sensitivity to insulin and IGF-I observed after an increase in membrane order due to cholesterol enrichment of the sarcolemma was
reversed if the animals were fed a diet high in n–3 fatty acids. The n–3 fatty acids were shown to substitute into the sarcolemma and bring about a decrease in membrane order (i.e., an increase in membrane fluidity) that was paralleled by a return to normal levels of insulin and IGFI sensitivity in the muscle (27, 28). Aged muscle has been reported to contain elevated levels of cholesterol compared with young muscle (25, 43), and Ca2+ uptake is decreased in skeletal muscle SR vesicles isolated from aged rodent (16, 26) and human (21) muscle.

Historically, the prevailing view has been that the majority of cholesterol found in skeletal muscle was located in the sarcolemma and contiguous T tubule membrane system. This view has been supported by lipid analysis of skeletal muscle membranes isolated and purified by differential centrifugation by use of linear sucrose gradients (19, 23, 34). These studies suggested that the majority of cholesterol was associated with the sarcolemma and T tubule membrane fractions rather than the SR membrane fractions of purified skeletal muscle membranes. However, several studies utilizing the same technology have challenged this view, suggesting that skeletal muscle SR membranes contain a significant amount of cholesterol (8, 25, 43) and that incomplete separation/purification of sarcolemma/T tubule and SR membrane fractions by density centrifugation may have led to this discrepancy.

Our results utilizing MAb 2C5-6 to spatially localize cholesterol in frozen sections indicate that SR membranes contain the vast majority of cholesterol present in rat and human skeletal muscle. However, the possibility that cholesterol may behave as a "cryptic" antigen in natural and synthetic membranes has been suggested theoretically and partially demonstrated experimentally. The inability of anti-cholesterol antibodies to react with the surface of synthetic liposomes containing <50% cholesterol, coupled to the theoretical analysis of the size of an antibody molecule relative to the membrane-embedded antigen (i.e., cholesterol intercalated into a phospholipid bilayer) (reviewed in Ref. 3), could potentially explain why we did not observe cholesterol staining with MAb 2C5-6 in sarcolemma or T tubule membranes. Specifically, the relative cholesterol concentration in the sarcolemma or T tubule membranes was below the "detectable" limit for our antibody because of antigen-antibody inaccessibility, whereas the relative concentration of cholesterol in the SR membranes was above the detectable limit by utilization of immunofluorescent localization techniques. However, unlike a phospholipid liposome where the cholesterol molecule is embedded within the lipid matrix and may be inaccessible to an antibody below a certain concentration threshold, the experiments detailed here were carried out on transversely sectioned membrane structures in situ. As such, exposure of the central regions of sarcolemma/T tubule and SR membranes by frozen sectioning will reduce antigen-antibody accessibility problems to a minimum. Our results suggest that if cholesterol is present in membrane components of normal skeletal muscle other than SR membranes, such as sarcolemma/T tubules or mitochondrial membranes, then the absence of staining at the immunofluorescent microscopic level is due to the presence of cholesterol in very small amounts relative to the amount found in SR membranes. The ability to spatially immunolocalize cholesterol in frozen sections by use of MAb 2C5-6 will provide a useful tool in the study of the cellular localization of this important molecule in normal and disease states and will further our knowledge of the specific spatial interactions of cholesterol with proteins embedded in the three-dimensional matrix of the membrane.

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