Sarco(endo)plasmic Reticulum Ca\(^{2+}\) ATPases (SERCA1 and -2) in Human Extraocular Muscles

Daniel Kjellgren,\(^1\) Michelle Ryan,\(^2\) Kay Ohlendieck,\(^2\) Lars-Eric Thornell,\(^3,4\) and Fatima Pedrosa-Domellof\(^5,4\)

**PURPOSE.** To investigate the composition of the fibers in human extraocular muscles (EOMs) with respect to the sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPases (SERCA)-1 and -2 and to investigate possible correlations between SERCA and myosin heavy chain (MyHC) composition.

**METHODS.** EOM samples were processed for immunocytochemistry with monoclonal antibodies specific against SERCA1 (fast isoform), SERCA2 (slow isoform), or different MyHCs. A total of 1571 fibers were analyzed. Microsomal EOM fractions were analyzed with SDS-PAGE and immunoblots.

**RESULTS.** The fast fibers, containing MyHCIIa, accounted for 79% of the fibers in the orbital layer (OL) and 74% in the global layer (GL). More than 99% of these fibers contained SERCA1, and 86% of them coexpressed SERCA1 and -2. Almost all slow fibers stained with SERCA2; 54% of those in the GL and all in the OL coexpressed SERCA1 and -2. Fifteen percent of the fibers in the GL and less than 1% in the OL were MyHCComp\(^\text{pos}\)/MyHCIIa\(^\text{neg}\) fibers. All these contained SERCA1 and in the OL also stained strongly with anti-SERCA2. Biochemically SERCA2 was more abundant than SERCA1.

**CONCLUSIONS.** The human EOMs had a very complex pattern of expression of the major protein regulating fiber relaxation rate. The coexistence of SERCA1 and -2, together with complex mixtures of MyHCs in most of the fibers provide the human EOMs with a unique molecular portfolio that allows a highly specific fine-tuning regimen of contraction and relaxation. (Invest Ophthalmol Vis Sci. 2003;44:5057-5062) DOI:10.1167/iovs.03-0218

The extraocular muscles (EOMs) are among the fastest and yet most fatigue-resistant skeletal muscles in the body. They perform very complex movements ranging from rapid saccades to slow vergence and pursuit movements, and their phenotype is so unique that they are considered a separate class of skeletal muscle (allotype).\(^1,2\) The unique character of the EOMs in small rodents has recently been confirmed at the gene level with both microarray and SAGE techniques,\(^5-5\) but extensive work is necessary before these data can be interpreted at the cell and organelle level. Corresponding genotype data are not available for human EOMs, and we have recently shown that they differ significantly from those of other species with respect to fiber composition.\(^6\)

The major determinants of the functional properties of a muscle are the force of contraction and rate of both contraction and relaxation. The contraction force and contraction velocity are dictated by the myosin heavy chain (MyHC) composition of the muscle fibers.\(^7\) We have described the unique MyHC composition of the fibers in human EOMs.\(^8\) When a muscle fiber is activated, the action potential stimulus spreads along the sarcolemma and reaches the depth of the muscle fiber through the transverse tubuli. The T-tubuli form junctions with the terminal cisterna of the sarcoplasmic reticulum (SR), depolarization triggers the release of Ca\(^{2+}\) from the SR into the sarcoplasm, and the muscle fiber contracts. The relaxation rate of a muscle fiber reflects the rate at which Ca\(^{2+}\) is transported back from the myofilbrillar space into the lumen of the SR. The Ca\(^{2+}\) ions are actively pumped from the myofilbrillar space into the lumen of the SR, and the Ca\(^{2+}\) gradient between the SR and the cytosol is restored. This is accomplished mostly by sarco(endo)plasmatic reticulum Ca\(^{2+}\) ATPases (SERCas) using adenosine triphosphate (ATP) hydrolysis as the source of energy.\(^9\) SERCas are the major protein component of the SR. Three differentially expressed genes encode SERCA proteins in human: SERCA1, -2, and -3.\(^9,10\) Differential splicing of the primary transcripts results in further variations, and at present at least seven SERCA isoforms have been described in humans.\(^11\) SERCA1 is present in fast-twitch skeletal muscle fibers, and SERCA2a is found in cardiac and slow-twitch muscle fibers. SERCA1b is present in neonatal muscles, SERCA2b in almost all muscle cells and SERCA5a/b/c in several types of nonmuscle cells. Mutations of the three genes have been associated with disease. For example, mutation of the SERCA1 gene is associated with Brody disease, a condition characterized by impairment of skeletal muscle relaxation after exercise, stiffness, and cramps.\(^12\)

Data on SERCA expression in human EOMs are not available. The distribution of SERCA1 has been reported to be rather complex in rat and rabbit EOMs.\(^13\) To elucidate further the cellular and molecular basis of the extraordinary features of the human EOMs we determined biochemically and immuno-histochemically the relative abundance and distribution of the fast (SERCA1) and slow (SERCA2) SERCA isoforms that are muscle-specific markers of the sarcoplasmic reticulum. We have also investigated the patterns of distribution of SERCA1 and -2 at the fiber level and their relation to the MyHC content of the fibers.

**MATERIAL AND METHODS**

**Muscle Samples**

Seven EOM samples obtained at autopsy from five individuals (four men and one woman), ages 17, 26, mid-thirties, 34, and 81 were used.
for immunocytochemistry. Three samples were taken from the rectus superior, two from the rectus medialis, one from the obliquus superior and one from the rectus lateralis. The samples available were taken from the middle portion of each muscle, and one sample was also taken from the anterior part of one of the rectus medialis. In addition, 11 other EOM samples were pooled together for biochemical analysis. The samples were obtained in accordance with the ethical recommendations of the Swedish Transplantation Law, with the approval of the Medical Ethics Committee, Umeå University, and adhered to the tenets of the Declaration of Helsinki for research involving human tissue.

**Immunocytochemistry**

The samples were mounted on cardboard and rapidly frozen in propane chilled with liquid nitrogen and stored at −80°C until used. Series of 80 cross sections, 5 μm thick, were cut from each muscle sample in a cryostat (Reichert-Jung, Vienna, Austria).

The sections were photographed under a microscope equipped with the objective (Carl Zeiss Meditec, Oberkochen, Germany). The overall staining pattern of each section was examined, and one area of the orbital layer and one of the global layer of each muscle sample were studied in detail. The staining pattern of each section was examined, and one area of the orbital layer and one of the global layer of each muscle sample were studied in detail. The staining pattern in 1571 fibers was analyzed.

**Biochemistry**

mAbs (mAb IID8 to the slow SERCA2 isoform of the Ca^{2+} ATPase and mAb IH11 to the fast SERCA1 isoform of the Ca^{2+} ATPase) were purchased from Affinity Bioreagents (Golden, CO). Peroxidase-conjugated secondary antibodies, acrylamide stock solutions, and protease inhibitors were from Roche Molecular Biochemicals (Leus, UK) and immunoblotting chemiluminescence substrates were obtained from Pierce & Warriner (Chester, UK). Immobilon-P nitrocellulose was purchased from Millipore Corp. (Bedford, MA). All other chemicals used in the isolation of membrane vesicles and the electrophoretic separation of proteins were of analytical grade and obtained from Sigma-Aldrich Co. (Poole, UK).

For membrane isolation, eleven individual EOM samples were combined and yielded approximately 1 g of wet tissue weight. Muscles were finely minced and homogenized at 0°C to 4°C in 10 volumes of 50 mM HEPES (pH 7.4), 10% (wt/vol) sucrose, 0.02% (wt/vol) sodium azide, and 3 mM MgCl\(_2\). Buffers were supplemented with a protease inhibitor cocktail consisting of 0.2 mM serine protease inhibitor (Pefabloc; Roche), 1.4 μM pepstatin A, 0.3 μM E64, 1 μM leupeptin, 1 mM EDTA, and 0.5 μM soybean trypsin inhibitor to avoid proteolytic degradation. A crude microsomal fraction was isolated by a standard subcellular fractionation procedure, as has been described in detail. Microsomal pellets were resuspended at the protein concentration of 10 mg/mL and used immediately for immunoblot analysis. Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard.

The gel electrophoretic separation of EOM proteins was performed with 7% (wt/vol) resolving gels using a 5% (wt/vol) stacking gel in the presence of sodium dodecyl sulfate and dithiothreitol. Due to the limited amount of material available, a electrophoresis system (Mini-PRO; BioRad Laboratories, Herts, UK) was used, whereby 30 μg protein was loaded per well and electrophoresed at 280 V/h. The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed by the method of Towbin et al. Blocking, incubation with primary and secondary antibodies, and washing of nitrocellulose sheets was performed as previously described. Immunolabeling was evaluated by the enhanced chemiluminescence technique. Densitometric scanning of enhanced chemiluminescence blots was performed on a computing densitometer (model 300S; Molecular Dynamics Sunnyvale, CA; ImageQuant, ver. 3.0 software).

### Table 1. Data on the Antibodies Used for Immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Short Name</th>
<th>Gene*</th>
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<td>SERCA1</td>
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<td>Anti-MyHCl</td>
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<td>F88§</td>
<td>MyHCo-cardiac</td>
<td>Anti-MyHCo-cardiac</td>
<td>MYH6</td>
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</tr>
<tr>
<td>A46</td>
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<td>Anti-MyHCom</td>
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<td>Anti-MyHCl+ila+com</td>
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<td>Anti-MyHCIIa</td>
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<td>31,32</td>
<td></td>
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<tr>
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<td>2B6##</td>
<td>MyHCombryonic</td>
<td>Anti-MyHCombryonic</td>
<td>MYH3</td>
<td>15,32,39</td>
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† Purchased from Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK. Anti-SERCA1 is specific against the human SERCA1 isoform expressed in type II (fast) skeletal muscle fibers and anti-SERCA2 recognizes the human SERCA2a isoform present in type I (slow) skeletal muscle fibers as well as the SERCA2b isoform. However, the staining observed with the SERCA antibodies was exclusively present in the muscle fibers, and no other cells were labeled, indicating that the affinity of the SERCA2 antibody for the 2b isoform is apparently very low and below the level of detection of our study.
‡ Obtained from The Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.
§ Gift from Jean J. Leger, National Institute of Health and Medical Research, Unit 249, Montpellier, France.
¶ Gift from Joseph F. Y. Hoh, University of Sydney, NSW, Australia.
## Gift from Donald A. Fischman, Cornell University, Ithaca, NY.
# Gift from Alan Kelly, University of Chicago, Chicago, IL.
RESULTS

Immunocytochemistry

Three major groups of fibers were distinguished according to their immunohistochemical staining patterns, as previously described: (1) fast fibers that stained with anti-MyHCIIa, (2) slow fibers that stained with anti-MyHC, and (3) MyHCComp/MyHClama fibers that stained with neither anti-MyHCIIa nor anti-MyHC but were labeled with anti-MyHCIIa + Ila+com and anti-MyHCom. Practically all slow fibers also stained with anti-MyHCom and some of them were also stained with anti-MyHCom-cardiac. Staining with the SERCA1 and -2 antibodies was restricted to the muscle fibers in all samples studied. The staining patterns of the two SERCA antibodies were different in the orbital and the global layer (Table 2).

Global Layer

Anti-SERCA1 and anti-SERCA2 stained almost all fibers in the orbital layer (Fig. 1). Anti-SERCA1 stained 85% of the fibers strongly and 15% of the fibers moderately to lightly. Anti-SERCA2 stained 36% of the fibers strongly and 63% moderately to lightly. One percent of the fibers were unstained by anti-SERCA2. The fibers were smaller and more tightly arranged than in the global layer.

The fast fibers accounted for 79% of the sampled fibers in the orbital layer. All fast fibers were strongly stained with anti-SERCA1. Anti-SERCA2 stained approximately 20% of the fast fibers strongly and 80% lightly to moderately. Less than 1% of them were unstained by anti-SERCA2.

The slow fibers accounted for 20% of the sampled fibers in the orbital layer. All of them stained with anti-SERCA2, 99% strongly and 1% lightly. Anti-SERCA1 labeled all these slow fibers, 25% strongly and 75% moderately to lightly.

The remaining fibers (MyHCComp/MyHClama fibers) accounted for less than 1% (four fibers out of 884 sampled) of the fibers in the orbital layer. These four fibers stained heavily with both anti-SERCA1 and anti-SERCA2.

Orbital Layer

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Global Layer

In general, the global layer seemed more lightly and heterogeneously stained than the orbital layer (Fig. 2). Anti-SERCA1 stained 80% of the fibers strongly and 15% moderately to lightly. Anti-SERCA2 in contrast stained 17% of the fibers strongly and 51% moderately to lightly. Only 3 of 687 sampled global fibers were unstained by both anti-SERCA1 and anti-SERCA2.

The fast fibers accounted for 74% of the fibers in the global layer. One of the 505 fast fibers sampled was unставлен by both anti-SERCA1 and anti-SERCA2. Seven percent of the fast fibers stained lightly with both anti-SERCA1 and anti-SERCA2. The remaining fast fibers stained strongly with anti-SERCA1 and were unstained (32%), lightly stained (53%), or strongly stained (8%) with anti-SERCA2.

The slow fibers accounted for 12% of the sampled fibers in the global layer. Fifty-one percent of the slow fibers sampled

<table>
<thead>
<tr>
<th>Table 2. SERCA Staining Pattern (Fiber Count)</th>
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<tbody>
<tr>
<td>SERCA Staining</td>
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<tr>
<td>----------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>orbital layer</td>
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<td>–</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>total fiber count</td>
</tr>
</tbody>
</table>

–, no staining; +, light staining; ++, strong staining.
stained strongly with anti-SERCA2 and were lightly stained with anti-SERCA1. Forty-three percent of the slow fibers were also strongly stained with anti-SERCA2 but were unstained with anti-SERCA1. Approximately 2% of the slow fibers were unstained by both anti-SERCA1 and anti-SERCA2. One percent were lightly stained by both SERCA antibodies and approximately 2% showed stronger staining with anti-SERCA1 (fast isoform) than with anti-SERCA2.

The MyHC<sup>emb</sup>/MyHC<sup>IIa</sup> fibers accounted for approximately 15% of the fibers in the global layer. Fifty-four percent of them were strongly stained with anti-SERCA1 only, 22% were lightly stained by both SERCA markers, and 24% were strongly stained with the fast isoform (SERCA1) and lightly labeled with the mAb against the slow isoform (SERCA2).

**Longitudinal Variation**

Samples from both the middle and the anterior parts of one rectus medialis muscle were available (Fig. 3). Although there was a clear difference between the anterior and midbelly parts in the staining pattern of anti-MyHC<sup>IIa</sup> in the global layer, no major difference was evident in the staining patterns of anti-SERCA1 and anti-SERCA2 (Fig. 3). There was no apparent correlation between the staining patterns observed with the SERCA markers and with anti-MyHC-embryonic (not shown).

**Biochemistry**

The relative abundance of fast and slow markers of the SERCA isoforms was determined biochemically in a microsomal fraction isolated from human EOM fibers. The Coomassie-stained gel (Fig. 4A) exhibited two major high molecular mass band regions of apparent 100 and 200 kDa, mostly probably representing the sarcoplasmic reticulum Ca<sup>2+</sup>/H<sup>+</sup> pump and MyHCs, respectively. The relative density, measured as the percentage of intensity of enhanced chemiluminescence of a control band set at 100%, of the slow isoform (SERCA2 = 95% ± 2.3% SEM) was significantly higher (P < 0.00181, unpaired t-test, two-tailed) than that of its fast counterpart (SERCA1 = 88% ± 0.7% SEM) in immunoblots (Figs. 4B, 4C).

**DISCUSSION**

The present study of the distribution of the major Ca<sup>2+</sup>/H<sup>+</sup> pump protein of the longitudinal tubules and the terminal cisternae, emphasizes the highly complex and unique structure of the human EOMs. Biochemically, the most abundant SERCA isoform in the EOMs was the slow isoform SERCA2, although the fibers containing slow MyHC were a minority (12% and 20% in the areas sampled from the global and orbital layer, respectively) in the human EOMs. The present data showed that most (99% in the orbital and 63% in the global layer) of the fibers stained strongly by anti-MyHC<sup>emb</sup>/MyHC<sup>IIa</sup> fibers, unstained by anti-MyHC<sup>IIa</sup> and anti-MyHCl, but stained by anti-MyHC<sup>IIa</sup>+<sup>com</sup>, and that these fibers were strongly stained by anti-SERCA1 and more lightly stained or unstained by anti-SERCA2. OL, orbital layer; GL, global layer.
SERCA1 and -2 in Human Eye Muscles

been observed that chronically stimulated fast-twitch muscle fibers can express SERCA1 and -2a simultaneously and even switch from SERCA1 to SERCA2a completely, if stimulated for sufficient time. A small number of fibers in the human EOMs were apparently devoid of SERCA1 and -2. These fibers were very few and may be in a transitional state. For instance, SERCAs can be significantly modified by chronic stimulation and ageing processes. Another possible explanation would be the presence of yet unidentified SERCA isoforms, possibly specific for the EOMs.

The MyHC composition of the fibers varies along the length of the EOMs in human and other species. In the rat EOMs SERCA1 is expressed in the midbelly of the muscle, but it disappears at the ends. We found no evidence of variation in the distribution of the SERCA isoforms when the middle and anterior portion of a single rectus medialis were compared. Further studies are needed to elucidate the possible heterogeneity in SERCA content along the length of the human EOM fibers.

The human EOMs differ from those of other species with respect to the SERCA composition. In rat and rabbit EOMs all fibers of the orbital layer and the singly innervated fibers of the global layer contain SERCA1, but the multiply innervated fibers of the global layer do not. We found that more than half of multiply innervated fibers of the global layer, identified by their MyHC content, in addition to SERCA2 also contain the fast isoform SERCA1 in the human EOMs. This is in agreement with data showing that there are important differences between the human EOMs and those of other species in fiber type and MyHC composition.

Acknowledgments

The authors thank Margaretha Enerstedt and Anna-Karin Olofsson for excellent technical assistance.

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