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THE Ca_v 1.2 Ca²⁺ CHANNEL IS EXPRESSED IN SARCOLEMMA OF TYPE I AND IIA MYOFIBERS OF ADULT SKELETAL MUSCLE

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Abstract

Although Ca²⁺-dependent signaling pathways are important for skeletal muscle plasticity, the sources of Ca²⁺ that activate these signaling pathways are not completely understood. Influx of Ca²⁺ through surface membrane Ca²⁺ channels may activate these pathways. We examined expression of two L-type Ca²⁺ channels in adult skeletal muscle, the Ca_v 1.1 and Ca_v 1.2, with isoform-specific antibodies in Western blots and immunocytochemistry assays. Consistent with a large body of work, expression of the Ca_v 1.1 was restricted to skeletal muscle where it was expressed in T-tubules. Ca_v 1.2 was also expressed in skeletal muscle, in the sarcolemma of type I and IIA myofibers. Exercise-induced alterations in muscle fiber types cause a concomitant increase in the number of both Ca_v 1.2 and type IIA-positive fibers. Taken together, these data suggest that the Ca_v 1.2 Ca²⁺ channel is expressed in adult skeletal muscle in a fiber type-specific manner, which may help to maintain oxidative muscle phenotype.

Keywords

calcineurin; exercise; fiber type specificity; L-type voltage-gated calcium channel; muscle plasticity

Voltage-gated Ca²⁺ channels comprise a multigene family, and individual members serve specific functions within the cell.^{10,41} The L-type Ca²⁺ channels, Ca_v 1.1–1.4, are closely related to each other and share sensitivity to the dihydropyridines. The Ca_v 1.1 channel is restricted to skeletal muscle, where it is expressed in the T-tubules and provides the gating charge movement that triggers the ryanodine receptor to release Ca²⁺ from the sarcoplasmic reticulum and initiate muscle contraction.^{20,31} The Ca_v 1.2 channel is broadly expressed in the surface membranes of cardiac, smooth muscle, nerve, and neuroendocrine cells, where it generates Ca²⁺ action potentials that initiate intracellular signaling events such as excitation–contraction coupling in heart and smooth muscle and downstream signaling cascades through calcineurin and Ca²⁺/calmodulin-dependent kinase (CAMK) pathways in all these cell types.^{10,12,23,44,52,54} The broad expression and importance of the Ca_v 1.2 channel was recently

demonstrated by a human mutation that causes Timothy syndrome, a disorder characterized by arrhythmia, autism, and other problems.⁵¹ Although the authors of this study looked at Ca_v 1.2 expression in a number of tissues, they did not directly characterize expression or defects in skeletal muscle.

The other L-type Ca²⁺ channels, Ca_v 1.3 and 1.4, are expressed in a number of cell types, but especially in neurons and neuroendocrine cells, where mutations in these channels cause deafness and night blindness, respectively.¹⁰ More distantly related voltage-gated Ca²⁺ channels include the Ca_v 2.1–2.3 channels, which initiate neurotransmitter release, and the Ca_v 3.1–3.3 channels, which are responsible for T-type currents.¹⁰ Taken together, the voltage-gated Ca²⁺ channel gene family plays critical roles in many aspects of cell physiology.

In this study, we addressed expression of the Ca_v 1.2 Ca²⁺ channel in skeletal muscle because it is the predominant L-type Ca²⁺ channel expressed in other excitable cell types.^{10,17,41} In addition, we compared its subcellular distribution to that of the well-known T-tubular Ca²⁺ channel, the Ca_v 1.1.^{20,31} Our findings suggest that the Ca_v 1.2 channel is expressed in the surface membrane of type I and IIa skeletal muscle myofibers, where it may allow entry of Ca²⁺ to activate downstream signaling pathways.

METHODS

Preparation of Membrane Fractions and Western Blot Analysis

Rat hind-leg skeletal muscle (mixed muscles from the entire hind-leg region), heart, liver, and brain were harvested and membrane fractions prepared as described previously,⁵³ using a comprehensive panel of phosphatase and protease inhibitors (Catalog Nos. 539134, 208733, and 524625; Calbiochem, San Diego, California) effective against acid and alkaline phosphatases and all classes of proteases, including calpains. A Lowry protein assay was used to normalize protein content between samples, with 200 µg of membrane protein used per lane on a 5%–15% gradient sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel for the Ca_v 1.1 Ca²⁺ channel and a 10% SDS-PAGE gel for the Ca_v 1.2 Ca²⁺ channel. Following electrophoretic transfer to nitrocellulose membranes, the Ca_v 1.1 Ca²⁺ channel was detected with a monoclonal antibody (Abcam, Cambridge, MA) and the Ca_v 1.2 Ca²⁺ channel was detected with a rabbit polyclonal antibody (Alomone, Jerusalem, Israel). To confirm that the detected Ca_v 1.2 Ca²⁺ channel signal was specific, a competition assay with the immunizing peptide was carried out using 5 µg peptide/1 µg of antibody. Primary antibodies were visualized using the Western Star detection kit (Tropix, Bedford, Massachusetts).

To prepare membranes from the superficial white vastus lateralis, a muscle enriched in type IIB fibers,³⁶ and tibialis anterior muscles, a muscle that contains type I, type IIa, and type IIb fibers,² four muscles of each were harvested and pooled for the membrane preparation. Otherwise, the procedure followed was the same as described earlier.

RNAse Protection Assays

Total RNA from hind-leg muscles, heart, liver, and brain were obtained using standard protocols.¹³ RNAse protection assays were carried out as described previously³⁷ using a complementary probe specific to nucleotides 3306–3638 of the Ca_v 1.2 Ca²⁺ channel, which yields an expected band of 333 bp.²⁶ As a control, protection of the 28S RNA was also assessed, using a commercially available complementary probe, pTRI-RNA-28S (Ambion, Austin, Texas). In RNAse protection assays with total RNA, this 28S probe yields two closely spaced protection products around 100 bp.

Preparation and Immunocytochemistry of Muscle Sections

Tibialis anterior muscles were removed from rats, fixed in 4% paraformaldehyde, cryoprotected in 15% sucrose, and frozen in liquid nitrogen-cooled isopentane. Cross-sections or longitudinal sections (10 μm) were cut on a Microm cryostat and mounted on chilled glass microscope slides. Sections were stained with the same antibodies to the Ca_v 1.1 or Ca_v 1.2 Ca^{2+} channels used in the Western blot analyses or with antibodies to caveolin-3 (Catalog No. sc-28828; Santa Cruz Biotechnology, Santa Cruz, California), dystrophin (Catalog No. sc-7461; Santa Cruz), and type I (Catalog No. M8421; Sigma, St. Louis, Missouri) or IIa (Catalog No. N2.261; Iowa Hybridoma Bank, Iowa City, Iowa) myosin. Competitions for the Ca_v 1.2 Ca^{2+} channel were carried out in the same manner used for the Western analysis. For staining with the antibody to type IIb myosin (Catalog No. BF-F3; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), it was necessary to use fresh-frozen sections (5 μm), which were obtained from plantaris muscles as described previously.² To visualize primary antibodies, the sections were counterstained with fluorescein-isothiocyanate (FITC) or tetramethyl rhodamine-isothiocyanate (TRITC)-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania). Immunostained sections were analyzed by confocal microscopy using an Olympus Fluoview (20 $\times$, air 0.7 NA and 60 $\times$ oil 1.4 NA objectives; Olympus, Tokyo, Japan). The images shown are from single planes.

To assess changes in fiber types induced by exercise, C57Bl/6J mice were allowed to exercise freely by long-term voluntary running (4 weeks), as described previously.⁵⁵ Control C57Bl/6J mice were maintained in cages with exercise wheels in locked position. For quantification of fiber types from these exercised or control mice ($n = 3$ for each group), 200- μm^2 regions from plantaris muscle cross-sections were scored for fibers positive to the Ca_v 1.2 Ca^{2+} channel and type IIa myosin, stained as described above. Data are reported as averages $\pm$ SEM. Data were analyzed by two-way analysis of variance (ANOVA), and a post hoc Tukey's comparison carried out to determine which samples were statistically different. Different letters are used to indicate samples that were statistically different from each other ($P < 0.05$).

All animal protocols accorded with the NIH *Guide for Care and Use of Laboratory Animals*, and were approved by the animal care and use committees at the institutions in which the studies were carried out.

RESULTS

Both Ca_v 1.1 and Ca_v 1.2 Ca^{2+} Channels Are Expressed in Skeletal Muscle

Using isoform-specific antibodies to the L-type voltage-gated calcium channels, Ca_v 1.1 and Ca_v 1.2, we examined expression of these channels in brain, heart, liver, and skeletal muscle membranes using Western blot analyses (Fig. 1A). The Ca_v 1.1 Ca^{2+} channel, also known as the dihydropyridine receptor or α_{1s} , was expressed only in skeletal muscle, consistent with previous reports.^{10,17,31,41} In contrast, the Ca_v 1.2 Ca^{2+} channel, also known as α_{1c} , was expressed in brain, heart, liver, and skeletal muscle. The slightly different size observed for this channel in different tissues is consistent with a previous finding that different splice variants of the Ca_v 1.2 Ca^{2+} channel are expressed in different tissues.^{10,17} To confirm the identification of the Ca_v 1.2 channel in skeletal muscle, the peptide used to generate the Ca_v 1.2 antibody was used as a competitor to displace binding in both brain and skeletal muscle (Fig. 1B).

To confirm the expression of the Ca_v 1.2 Ca^{2+} channel with a different approach, RNase protection assays were carried out using RNA from these same tissues. A probe complementary to nucleotides 3306–3638 of the Ca_v 1.2 channel yielded a band of approximately 300 bp in all of these tissues (Fig. 1C). Surprisingly, the level of Ca_v 1.2 mRNA was similar in all of

these tissues, although there was more robust expression of the protein in brain and skeletal muscle, suggesting that post-transcriptional mechanisms contribute to the level of protein expression. Taken together, these data confirm the novel finding that the $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel is expressed in skeletal muscle.

Distinct Distribution of $\text{Ca}_v 1.1$ and $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ Channels in Skeletal Muscle

To address the issue of Ca^{2+} channel distribution in skeletal muscle, we analyzed muscle cross-sections and longitudinal sections stained with both antibodies (Fig. 2). In muscle cross-sections, both antibodies exhibited a somewhat mosaic pattern of expression. The $\text{Ca}_v 1.1$ antibody stained most fibers, although there was some heterogeneity. Because this heterogeneity could arise from the fact that this Ca^{2+} channel is present in the T-tubule membranes and cross-sections were taken parallel to T-tubules, it was important to analyze staining in longitudinal sections as well. At either low or higher magnification, the $\text{Ca}_v 1.1 \text{ Ca}^{2+}$ channel staining in longitudinal sections gave rise to a uniformly striped pattern, identical to that found previously for this channel due to its localization in the T-tubular membranes.³¹ There was little, if any, fiber-to-fiber variation in this pattern.

The staining pattern of the $\text{Ca}_v 1.2$ antibody was far more complex than that of the $\text{Ca}_v 1.1$, especially in the longitudinal sections. The mosaic pattern found in the muscle cross-sections with the $\text{Ca}_v 1.2$ antibody was also found in the longitudinal sections at both low and higher magnification. This antibody gave rise to a robust staining of the outer rim of some muscle fibers, consistent with a sarcolemmal location for this channel.

That the $\text{Ca}_v 1.2$ staining was present in some fibers and not others suggests that this surface $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel was expressed in some muscle fiber types and not others. However, there was also a striped pattern present in all fibers, consistent with a portion of the $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel being localized within the T-tubular membranes of all muscle fibers. Competition with the peptide used to generate the $\text{Ca}_v 1.2$ antibody completely displaced all staining. Taken together, our data suggest that the $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel has a pattern of expression in skeletal muscle that is different from that of the well-known T-tubular $\text{Ca}_v 1.1$ channel.

$\text{Ca}_v 1.2 \text{ Ca}^{2+}$ Channel Expressed in Surface Membrane of Type I and IIa Fibers, But Not Type IIb Fibers

To address the issue of fiber type-specific expression of the surface $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel, muscle cross-sections were stained with both the $\text{Ca}_v 1.2$ antibody and antibodies directed at the specific myosin heavy chain (MHC) isoforms that define the different fiber types, MHC I, MHC IIa, and MHC IIb (Fig. 3A). Both MHC I- and IIa-stained fibers were also clearly positive for surface membrane staining of the $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel, whereas the MHC IIb-stained fibers lacked surface $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channels. There were also $\text{Ca}_v 1.2$ -positive fibers that stained for neither MHC I nor IIa (see also Fig. 4). These additional fibers could correspond to MHC IIc/x fibers, but we lacked an antibody against this myosin isoform and therefore could not directly confirm whether this was true. Nevertheless, our data clearly indicate that expression of the $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel in the surface membrane was fiber type-specific.

To confirm that expression of $\text{Ca}_v 1.2$ is diminished in type IIb fibers, individual muscles rich in type IIb fibers (superficial white vastus lateralis, 95% IIb fibers³⁶) were compared to a muscle of mixed fiber type (tibialis anterior), the same muscle used for the immunostaining (Fig. 3B). Although both muscles express the T-tubular $\text{Ca}_v 1.1$ channel robustly, there was reduced expression of the $\text{Ca}_v 1.2$ channel in the type IIb-rich muscle, consistent with the immunostaining results.

Expression of Type IIa fibers and Ca_v 1.2–Positive Fibers Concomitantly Increased by Exercise

To determine whether there might be a relationship between expression of the Ca_v 1.2 Ca²⁺ channel in the surface membrane and fiber type switching, we applied stimuli known to change fiber type specificity and determined whether there was a corresponding change in Ca_v 1.2 expression (Fig. 4). As our stimulus, we chose exercise, which is known to increase the proportion of type IIa fibers in plantaris muscle.⁵⁵ In plantaris muscles from control mice, there were more Ca_v 1.2–positive fibers than type IIa fibers. Exercise changed this pattern in that both the number of type IIa fibers and Ca_v 1.2 fibers increased concomitantly, with the end result being that there were the same proportions of type IIa and Ca_v 1.2–positive fibers. Taken together, these data suggest that some fibers express the Ca_v 1.2 Ca²⁺ channel prior to becoming type IIa fibers.

Markers of Sarcolemma Indicate Ca_v 1.2 Ca²⁺ Channel Expression in Both Sarcolemma and a Subsarcolemmal Region

To confirm that the Ca_v 1.2 was expressed in the sarcolemma, co-staining with the Ca_v 1.2 antibody and an antibody to dystrophin, a marker of the surface membrane,¹⁴ was carried out (Fig. 5, top row). As shown most clearly in the overlay, there was considerable overlap in the staining pattern with the dystrophin and Ca_v 1.2 antibodies (Fig. 5, yellow arrow 1). However, immediately beneath this region, there was further extension of the Ca_v 1.2 staining (Fig. 5, red arrow 2). The surface membranes of fibers that were negative for Ca_v 1.2 channel were stained only by the dystrophin antibody (Fig. 5, green arrow 3).

Recently, it has been reported that the Ca_v 1.2 channel in cardiac muscle is expressed in lipid rafts, as shown by co-localization with the lipid raft protein caveolin.⁵ To determine whether the thick staining observed with the Ca_v 1.2 antibody corresponded to these lipid rafts, we also carried out co-staining with a caveolin-3 antibody. However, the pattern of co-staining with the caveolin-3 and Ca_v 1.2 antibodies was similar to that observed with the dystrophin and Ca_v 1.2 staining. Collectively, these data indicate that the Ca_v 1.2 Ca²⁺ channel is expressed in both the sarcolemma and a subsarcolemmal region.

DISCUSSION

The results of this study present the novel finding that the Ca_v 1.2 Ca²⁺ channel is expressed in the surface membrane of type I and IIa fibers in adult skeletal muscle and perhaps also expressed on a subset of fibers that later switch to type IIa as a result of stimuli such as exercise. The apparent molecular weight (M_r) of both the Ca_v 1.1 and Ca_v 1.2 Ca²⁺ channel in our Western blot analysis is similar to the high-M_r 212-kDa band in previously published reports^{15,16,25,35}; we attribute the appearance of only this high-M_r band to the utilization of calpain inhibitors, as degradation of this larger M_r band due to calpains is a well-documented phenomenon.¹⁶ The differences in M_r of the Ca_v 1.2 Ca²⁺ channel in different tissues may well be due to the splice variants expressed in different tissues.^{10,17} To confirm the expression of the Ca_v 1.2 Ca²⁺ channel using an independent method, RNase protection assays were used. The expression of Ca_v 1.2 mRNA in all these tissues is consistent with a previous report that this Ca²⁺ channel is broadly expressed in many cell types, including multiple brain regions, cardiac regions, and liver,⁵¹ and now, as shown in this report, in skeletal muscle.

The spatial expression of the Ca_v 1.2 Ca²⁺ channel is quite distinct from that of the T-tubular Ca_v 1.1 Ca²⁺ channel. Whereas the latter channel is expressed uniformly in the T-tubules of all muscle fibers, consistent with earlier work,³¹ the Ca_v 1.2 Ca²⁺ channel is expressed predominantly in the surface membrane of type I, IIa, and possibly IId/x fibers, but not in IIb fibers.

One important role of L-type Ca^{2+} channels is as a source of Ca^{2+} to regulate downstream signaling pathways.^{6,7,11,27,29,40} One set of pathways are the Ca^{2+} /calmodulin-dependent kinase (CAMK) pathways. Different isoforms of CAMK are known to regulate muscle plasticity and expression of the oxidative muscle phenotype through transcriptional regulators that include serum response factor (SRF), activator protein-1 (AP-1), and peroxisome proliferators activator gamma (PPAR γ) coactivator-1 α (PGC-1 α). A second pathway involves the Ca^{2+} /calmodulin-activated phosphatase, calcineurin. Calcineurin ultimately activates several downstream transcription factors, including Id, myocyte-enhancing factor 2 (MEF2), and nuclear factor of activated T cells (NFATs), to regulate both initial differentiation and subsequent maturation of myofibers.^{21,22,28,30,32} In adult skeletal muscle, calcineurin is involved in specification of different fiber types.^{43,45,46}

One manifestation of muscle plasticity is switching of fiber types. There are four different fiber types in adult skeletal muscle that are categorized by the predominant expression of different isoforms of MHC.^{6,7} MHC I is highly expressed in slow-twitch fiber types, which predominate in postural muscles, such as soleus, that are characterized by slow tonic contraction, fatigue resistance, and relatively high levels of resting cytosolic calcium.^{1,6,7} MHC IIa, IIc/x, or IIb are expressed in fast-twitch fiber types with type IIa fibers being oxidative, type IIb fibers being glycolytic, and type IIc/x fibers being in between.⁴⁷ Muscles that are rich in fast-twitch glycolytic fibers are characterized by fast and powerful force generation, greater fatigability, and relatively low levels of resting cytosolic calcium.^{6–8,18,19} Alterations in neuromuscular activity, such as that induced by nerve stimulation or exercise, can induce a switch from one type to another,^{2,9,24,48,55} with a concomitant change of other cellular proteins. For example, plantaris muscles from mice have an increased proportion of MHC IIa-positive fibers after long-term voluntary running.^{2,55} In this study, we have demonstrated that there is a concomitant increase in both IIa and Ca_v 1.2-positive fibers in response to running, suggesting that calcium influx through this channel is important for specifying fiber type.

One possibility is that the Ca_v 1.2 Ca^{2+} channel is an entry pathway in skeletal muscle for the Ca^{2+} that activates the calcineurin signaling pathway, shown by other investigators to activate fiber type switching and specification.^{6,7,27} There are several lines of evidence, both in vivo and in vitro, implicating calcineurin signaling in muscle plasticity. First, in vitro experiments have shown that cultured muscle cells express a mixture of fiber types, with MHC IIa, IIc/x, and IIb predominating, but that an electrical stimulation pattern simulating that of a slow fiber type induces an increased proportion of MHC I in these cells.^{33,34,38,39} In these same experiments, addition of the calcium ionophore, A23187, directly induced expression of MHC I and the calcineurin blocker, cyclosporine, blocked this induction. In vivo, transgenic mice that had a constitutively active form of calcineurin driven by the muscle creatine kinase promoter had an increased proportion of MHC I-positive fibers,⁴³ whereas mice lacking the catalytic subunit of calcineurin had reduced numbers of MHC I-positive fibers⁴⁵ and those lacking the regulatory subunit of calcineurin had deficiencies in expression of both MHC I- and IIa-positive fibers.⁴⁶ Taken together, these data suggest prominent roles for calcineurin signaling in both type I and IIa fiber types.

The results with the calcineurin knockout mice are consistent with studies focused on analyzing the promoters that drive MHC genes, in that calcineurin signaling pathways stimulate the MHC I promoter^{12,50} and also the fast MHC promoters in the order IIa > IIc/x > IIb.^{3,4} Our finding that the Ca_v 1.2 Ca^{2+} channel is expressed preferentially in the sarcolemma of these same fiber types establishes the possibility that influx of Ca^{2+} through this channel may activate the calcineurin signaling pathway, as it does in other excitable cell types such as neurons and smooth muscle.^{10,23,44,52}

Establishing a direct causal link between influx of Ca^{2+} through surface L-type Ca_v 1.2 Ca^{2+} channels and activation of the calcineurin signaling pathway will require further work. Current pharmacological agents directed at L-type Ca^{2+} channels do not distinguish between these channel isoforms. However, conditional knockout of the Ca_v 1.2 Ca^{2+} channel isoform in skeletal muscle may be possible, given that such lines have been established for neuronal specific expression of this channel isoform.^{41,42,49} Nevertheless, the data presented in this study clearly indicate that the Ca_v 1.2 Ca^{2+} channels are present in selected fiber types and likely play an important and unique role in the biology of adult skeletal muscle.

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Abbreviations

AP-1, activator protein-1; CAMK, calmodulin-dependent kinase; FITC, fluorescein-isothiocyanate; MEF2, myocyte-enhancing factor 2; MHC, myosin heavy chain; NFAT, nuclear factor of activated T cells; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SRF, serum response factor; PGC-1 α , peroxisome proliferators activator gamma (PPAR γ) coactivator-1 α ; TRITC, tetramethyl rhodamine-isothiocyanate.

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FIGURE 1.

The Ca_v 1.1 Ca²⁺ channel is restricted to skeletal muscle, whereas the Ca_v 1.2 Ca²⁺ channel is expressed in brain, heart, liver, and skeletal muscle. (A) Isoform-specific antibodies to the Ca_v 1.1 Ca²⁺ channel or the Ca_v 1.2 Ca²⁺ channel were used in Western blot analyses of membranes prepared from brain (Br), heart (H), liver (L), or skeletal muscle (SkM). The Ca_v 1.1 antibody detects a Ca²⁺ channel only in skeletal muscle, whereas the Ca_v 1.2 antibody detects Ca²⁺ channels in brain, heart, liver, and skeletal muscle. The slightly different size of the Ca_v 1.2 Ca²⁺ channel detected in different tissues is consistent with the known expression of alternatively spliced forms of Ca_v 1.2.^{10,17} (B) Competition with the peptide used to generate the Ca_v 1.2 antibody displaced binding of the antibody to the Ca_v 1.2 Ca²⁺ channel

in both brain and skeletal muscle. (C) RNase protection, with a probe complementary to the $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel, yielded an expected product of approximately 300 bp in all tissues, indicating that the $\text{Ca}_v 1.2$ channel was expressed in all tissues. As a loading control, protection of the 28S RNA was found to be the same in all lanes.

FIGURE 2.

The $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel is only expressed in a subset of skeletal muscle fibers. Muscles analyzed in cross-sections with the two antibodies indicate that there was a mosaic of some fibers stained with the $\text{Ca}_v 1.2$ antibody, whereas others were not stained. A thick outer membrane staining was found in $\text{Ca}_v 1.2$ -positive fibers. The scale bar in these panels indicates $50 \mu\text{m}$. To more fully visualize the T-tubular membrane system, longitudinal sections of muscle were also analyzed, both at low power and high power. Staining with the $\text{Ca}_v 1.1$ antibody was fairly uniform throughout the muscle at both low and high power, but staining with the $\text{Ca}_v 1.2$ antibody demonstrated a mosaic, with some fibers having intense surface membrane staining. Competition with the peptides used to generate the $\text{Ca}_v 1.2$ antibody displaced binding to the sections, as shown at high power. The scale bar for the low-power images indicates $50 \mu\text{m}$ and that for the high power indicates $20 \mu\text{m}$.

FIGURE 3.

The Ca_v 1.2 Ca²⁺ channel is expressed in type I and IIa fibers, but not IIb fibers. **(A)** To determine whether specific fiber types expressed the Ca_v 1.2 Ca²⁺ channel, muscle cross-sections were analyzed with both the Ca_v 1.2-specific antibody and antibodies that are specific for myosin heavy chain isoforms (see Methods). Both type I- and type IIa-positive fibers stained with the Ca_v 1.2 antibody, whereas the type IIb-positive fibers did not stain with the Ca_v 1.2 antibody. The scale bar in each panel indicates 50 μ m. **(B)** Western blot analysis was carried out on membrane proteins prepared from superficial white vastus lateralis muscles (WVA), a muscle type composed of 95% type IIb fibers,³⁶ and tibialis anterior (TA), the muscle of mixed fiber type used for the immunostaining. Although both muscles expressed the Ca_v

1.1 robustly, expression of $Ca_v 1.2$ was greatly reduced in the muscle enriched in type IIb fibers, consistent with the immunostaining results.

FIGURE 4.

There is a concomitant increase in type IIa- and Ca_v 1.2-positive fibers in the plantaris muscles from animals that exercise. Plantaris muscles from control animals or animals that were allowed to exercise were isolated and stained with antibodies to type IIa MHC and Ca_v 1.2 Ca²⁺ channels. The number of positive fibers for each group (type IIa- or Ca_v 1.2-positive) is indicated per 200 μm^2 . Consistent with previous results, the number of type IIa fibers increased in muscle cross-sections taken from animals that were allowed to exercise.⁵⁵ In control muscles, there were significantly more Ca_v 1.2-positive fibers than type IIa fibers and, in the exercised muscles, there was a concomitant increase in the number of both type II- and Ca_v 1.2-positive fibers. Different letters indicate significant differences between numbers of fibers in each sample set ($P < 0.05$).

FIGURE 5.

The $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel is expressed in both the sarcolemma and a subsarcolemmal region of the muscle fiber. To determine whether the $\text{Ca}_v 1.2$ channel was expressed in the surface membrane, co-staining with the sarcolemmal marker dystrophin was carried out (top row). As indicated by yellow arrow 1 in the overlay, there is considerable overlap in the staining of the $\text{Ca}_v 1.2$ and dystrophin antibodies, but as indicated by red arrow 2, the $\text{Ca}_v 1.2$ staining extends into a subsarcolemmal region. In fibers that are negative for $\text{Ca}_v 1.2$ staining, only the dystrophin staining is observed, as indicated by green arrow 3. To determine whether the denser staining observed with the $\text{Ca}_v 1.2 \text{ Ca}^{2+}$ channel corresponded to lipid rafts, co-staining with a caveolin-3 antibody was also carried out, but the pattern of co-staining was similar to that observed with the dystrophin antibody. The scale bar in each panel indicates $50 \mu\text{m}$.