Deficiency of triad junction and contraction in mutant skeletal muscle lacking junctophilin type 1

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In skeletal muscle excitation–contraction (E–C) coupling, the depolarization signal is converted from the intracellular Ca\(^{2+}\) store into Ca\(^{2+}\) release by functional coupling between the cell surface voltage sensor and the Ca\(^{2+}\) release channel on the sarcoplasmic reticulum (SR). The signal conversion occurs in the junctional membrane complex known as the triad junction, where the invaginated plasma membrane called the transverse-tubule (T-tubule) is pinched from both sides by SR membranes. Previous studies have suggested that junctophilins (JPs) contribute to the formation of the junctional membrane complexes by spanning the intracellular store membrane and interacting with the plasma membrane (PM) in excitable cells. Of the three JP subtypes, both type 1 (JP-1) and type 2 (JP-2) are abundantly expressed in skeletal muscle. To examine the physiological role of JP-1 in skeletal muscle, we generated mutant mice lacking JP-1. The JP-1 knockout mice showed no milk suckling and died shortly after birth. Ultrastructural analysis demonstrated that triad junctions were reduced in number, and that the SR was often structurally abnormal in the skeletal muscles of the mutant mice. The mutant muscle developed less contractile force (evoked by low-frequency electrical stimuli) and showed abnormal sensitivities to extracellular Ca\(^{2+}\). Our results indicate that JP-1 contributes to the construction of triad junctions and that it is essential for the efficiency of signal conversion during E–C coupling in skeletal muscle.

Introduction

The junctional membrane complex between the plasma membrane (PM)* and the endoplasmic–sarcoplasmic reticulum (ER–SR) is an important structural foundation for crosstalk between the cell surface and intracellular ionic channels (Pozzan et al., 1994; Berridge, 1998). In skeletal muscle cells, the transverse-tubule (T-tubule) as the invaginated PM and the SR membrane together form the junctional complex, called the triad junction (Flucher, 1992). The dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR) function as the cell surface voltage sensor and the SR Ca\(^{2+}\) release channel, respectively, and the proposed direct coupling between them in the triad junction converts cell surface depolarization to the intracellular Ca\(^{2+}\) signal for contraction without the requirement of Ca\(^{2+}\) influx (Tanabe et al., 1988; Schneider, 1994; Takeshima et al., 1994). The junctional membrane structure of the triad junction is probably required for functional coupling between DHPR and RyR, but neither DHPR nor RyR contributes to the constitution (Takekura et al., 1995; Ikemoto et al., 1997). In addition to the triad, junctional membrane complexes between the PM and ER–SR are shared by excitable cell types, including the diad in cardiac myocytes, peripheral coupling in smooth muscle and immature striated muscle cells, and subsurface cisternae in neurons. It is possible that these junctional membrane structures are composed by a similar molecular mechanism.

Of several transmembrane proteins found in the skeletal muscle triad junction (Fan et al., 1995; Jones et al., 1995; Nishi et al., 1998; Takeshima et al., 1998), junctophilin (JP) seems to be responsible for the formation of the junctional membrane structure. JP is composed of two major domains, a COOH-terminal hydrophobic segment spanning the...
junctional SR membrane, and the remaining cytoplasmic region interacting specifically with the PM. Moreover, the junctional membrane complex between the ER and the PM was produced by injection of JP cRNA into amphibian embryonic cells (Takeshima et al., 2000). We have identified three JP subtypes derived from different genes, namely JP-1, -2 and -3 in mammalian excitable tissues. JP-1 is found predominantly in skeletal muscle, JP-2 is expressed throughout skeletal, cardiac, and smooth muscle cells, and JP-3 is expressed specifically in the brain (Nishi et al., 2000; Takeshima et al., 2000). Mutant mice lacking JP-2 died in utero due to cardiac failure. Cardiac myocytes from the mutant embryos showed deficiency of peripheral couplings and abnormal Ca\(^{2+}\)-ATPase, the major protein component in the SR. (b) Western blot analysis of JP subtypes during muscle maturation. Total hindlimb microsomes (40 μg protein each) prepared from embryonic day 14 (E14) to postnatal day 28 (P28) mice were analyzed using the subtype-specific antibodies. Although expression of JP-1 in embryos and neonates could be detected in longer exposure (see Fig. 2), the signal densities were markedly lower than those of young adult mice. In contrast with JP-1, induction of JP-2 expression was relatively loose during muscle maturation. (C) Immunohistochemical analysis of JP subtypes in skeletal muscle. A cryosection of hindlimb muscle from adult mouse was labeled by immunofluorescence using antibodies specific to JP-1 (on 543 nm excitation) and JP-2 (on 488 nm excitation). Cytoplasmic rows immunolabeled with both antibodies are localized in identical positions (Merged). Essentially the same staining patterns were observed in all muscle fibers examined in hindlimb muscle. Bar, 10 μm.

**Results and discussion**

**Coexpression of JP-1 and JP-2 in skeletal muscle**

Our results in RNA blot hybridization have suggested that both JP-1 and JP-2 are expressed in skeletal muscle cells (Takeshima et al., 2000). To further examine the expression, we prepared bacterial fusion proteins carrying the divergent regions of JPs, in which no conserved amino acid sequences are observed among the subtypes, and subtype-specific antibodies were developed using the fusion proteins as antigens. In immunoblot analysis of microsomal preparations from adult mouse tissues, the resulting antibodies were subtype specific, and JP-1 and JP-2 were detected as protein bands showing distinguishable migrations on gel electrophoresis (Fig. 1 A). JP-1 was specifically detected in skeletal muscle, and JP-2 was found in both skeletal and cardiac muscles. The data obtained in the Northern and Western analyses were consistent. The analysis in mouse hindlimb muscle showed that the expression levels of both JP-1 and JP-2 significantly increase during muscle maturation (Fig. 1 B). The induction of JP-1 during muscle development is especially remarkable in that the expression levels were very low in embryos and neonates, but muscles from young adult mice contained abundant JP-1. The present results showed that both JP-1 and JP-2 were colocalized in the triad junction. The data obtained in the Northern and Western analyses were consistent. The analysis in mouse hindlimb muscle showed that the expression levels of both JP-1 and JP-2 significantly increase during muscle maturation (Fig. 1 B). The induction of JP-1 during muscle development is especially remarkable in that the expression levels were very low in embryos and neonates, but muscles from young adult mice contained abundant JP-1. Histochemical analysis demonstrated that both antibodies to JP-1 and JP-2 specifically reacted with the rows localized at A-I junctions in the longitudinal sections of muscle preparations (Fig. 1 C). Because all muscle cells examined in hindlimb were immunolabeled with both antibodies, the JP subtypes are coexpressed in both fast and slow fibers. Our previous results demonstrated specific localization of JP-1 in the skeletal muscle triad junction (Takeshima et al., 2000). The present results showed that both JP-1 and JP-2 are colocalized in the triad junction.
Junctophilin type 1 knockout mice | Ito et al. 1061

In both mouse and human genes, the first introns disrupt an identical position in the primary structure of JP-1 (Nishi et al., 2000). Our in situ hybridization analysis demonstrated that the JP-1 gene was mapped to mouse chromosome 1A2-5 (unpublished data). Several same genes have been mapped to the mouse chromosome 1A2-5 and human chromosome 8q21 containing the JP-1 gene, and the regions are homologous between mouse and human genomes.

The genomic DNA segments were used for constructing a targeting vector (Fig. 2A). In the vector, the region containing the first protein-coding sequence was replaced by the neomycin resistance gene (neo) and diphtheria toxin gene (DTA) for positive selection, and the diphtheria toxin gene was attached at the 3′ terminus for negative selection. ES cells were transfected with the targeting vector, and the resultant G418-resistant clones were screened by Southern blot hybridization. Several ES clones were shown to have the expected pattern of arrangement in genomic DNA for homologous recombination in Southern blot screening (unpublished data). Chimeric male mice were produced by injecting the ES cells into blastocysts, and bred to yield mutant mice detected by PCR (Fig. 2B). The chimeric mice and the heterozygous mutants could be maintained normally under our conventional housing conditions. Neonatal mice homozygous for the mutation were obtained by crossing between heterozygous mice. Western blot analyses showed an absence of JP-1 (Fig. 2C) in microsomal preparations of hindlimb from the mutant neonates, indicating that the introduced mutation is a null mutation of the gene. On the other hand, normal levels of JP-2 expression were observed in mutant muscle (Fig. 2D), and fluorescence microscopy showed no detectable difference in subcellular localization of JP-2 between the genotypes (Fig. 2E).

Perinatal lethality in JP-1 knockout mice

The JP-1 knockout neonates could be obtained by crossing between the heterozygous mutants, and therefore they survived fetal development. Mother mice delivered and nursed the knockout neonates normally, and all mutants were pink in color and showed kicking movements, demonstrating apparently normal function of the respiratory and limb motor systems.
The direct cause of death in the JP-1 knockout mice is unclear, but could be the result of undernourishment caused by the absence of milk suckling. It has been reported that mutant mice bearing suckling failure die shortly after birth but could grow by bottle feeding for several days (see, for example, Yagi et al., 1993; Kutsuwada et al., 1996). We tried to feed the JP-1 knockout neonates manually, providing artificial milk every several hours through a tube inserted into the stomach. Control mice could be maintained at least for several days, but under the same conditions we could not prolong the survival of the JP-1 knockout mice. All of the mutants seemed to suffocate due to milk regurgitation and/or infirm breathing. Thus, it is possible that the perinatal death of the JP-1 knockout mice is caused not only by suckling failure, but also other defects such as dysfunction of pharyngoesophageal or diaphragm muscles.

**Morphological abnormalities in skeletal muscle lacking JP-1**

Because JP-1 is predominantly expressed in skeletal muscle, we surveyed morphological defects in skeletal muscles from the JP-1 knockout neonates. Cell density, diameter, and the shape of mutant muscle fibers seemed to be normal, and photomicroscopic examination detected no obvious abnormalities. However, the electron microscopic examination detected ultrastructural abnormalities in the membrane systems of the mutant muscles (Fig. 4). In muscles from newborn mice, formation of the junctional complexes between the T-tubule and the SR are not yet completed, and both diads and triads partially occupy A-I junctions. In contrast to flattened T-tubular structures in mature triads, the T-tubules pinched from both sides by the SR are often elliptical in shape in immature muscles from neonates. No obvious ultrastructural abnormalities in the membrane system were observed in mutant muscles from JP-1 knockout neonates immediately after birth. However, mutant muscles from the knockout neonates that were near death (15–20 h after birth) showed the following abnormal SR features: the terminal regions of the SR were frequently swollen, longitudinal SR regions were partially vacuolated, and the orientation of SR networks was irregular. Therefore, junctional membrane complexes of both diads and triads were structurally abnormal in mutant muscles. These morphological abnormalities were detected in all muscle types examined in neonates 15–20 h after birth, and jaw muscle cells showed the most severe defects. On the other hand, mitochondria and myofibrils retained normal structures in JP-1 knockout muscles.

In mutant muscle cells prepared immediately after birth, both diads and triads were apparently normal in morphology. The T-SR gap size in the junctional membrane structures was 13 ± 1 nm in both wild-type and mutant muscles. However, statistical analysis demonstrated that the number of triad junctions was significantly reduced in all examined muscles from the JP-1 knockout neonates immediately after birth (Fig. 5). These observations clearly suggest that the loss of JP-1 affects the triad formation in skeletal muscle.

As shown in the electron microscopic observations, JP-1 knockout muscle contained few triad junctions at birth, and the SR structures worsened after birth in the mutant muscle. The observations may suggest that the triad deficiency af-
Junctophilin type 1 knockout mice | Ito et al. 1063

Effects SR structures in a stimulation activity–dependent manner. In striated muscle, disruption of the RyR genes produces abnormal SR and mitochondria showing vacuolated and swollen structures, probably due to SR Ca\(^{2+}\) overloading (Ikemoto et al., 1997; Takeshima et al., 1998). The RyR and JP-1 knockout muscles show similar structural abnormalities in the SR, although no obvious defects were developed in the mitochondria of JP-1 knockout muscle. Frequency of stimulation from motor nerves should increase after birth, and resulting Ca\(^{2+}\) signaling during excitation–contraction (E–C) coupling might produce the structurally abnormal SR in JP-1 knockout muscle. As in the case of the RyR-deficient muscle, SR Ca\(^{2+}\) overloading might underlie abnormalities in JP-1 knockout muscle because the reduced formation of triad junctions could inhibit DHPR-mediated activation of RyR.

In mutant jaw muscle, formation of both triads and diads was weak, and therefore the number of total junctional membrane complexes was significantly reduced (Fig. 5). In contrast, normal levels of diads were retained in other mus-
cle types examined. The extreme reduction of junctional complexes in jaw muscle may cause the disturbance of mouth opening in the mutant mice. The jaw muscle–specific defect on junctional membranes does not reflect different expressions of JP subtypes among muscle types. Our Western blot experiments did not find a significant difference in JP expression levels among jaw, tongue, and hindlimb muscles in neonatal mice (unpublished data). The mechanism underlying the jaw muscle–specific abnormality is unknown.

### Physiological abnormalities in skeletal muscle lacking JP-1

To survey functional abnormalities of the JP-1 knockout muscle, we examined the contraction profiles of a hindlimb muscle bundle (musculus vastus lateralis) from the mutant neonates. Fig. 6 shows significant differences in the force–frequency relationship between JP-1 knockout and wild-type muscles in normal bathing solution. Electrical stimuli at low frequencies developed less contractile forces in the mutant muscle compared with wild-type muscle. Twitch tension developed in the mutant muscle was reduced to about half the value of that in control muscle, although there is no significant difference in the maximum force between the genotypes (Table I). Thus, the force–frequency curve of the mutant muscle was shifted downward, indicating that the loss of JP-1 reduced the efficiency of E–C coupling. The low efficiency in the mutant muscle is likely due to the deficiency of triad junctions where functional coupling occurs between DHPR and RyR.

We then examined the effects of extracellular Ca$^{2+}$ levels in the JP-1 knockout muscle. In mature muscle, an increase in extracellular Ca$^{2+}$ does not obviously affect the twitch force, because contraction is produced by Ca$^{2+}$ release from the SR by functional-coupling DHPR and RyR during E–C coupling. On the other hand, when the extracellular Ca$^{2+}$ level was increased from 2.5 mM to 5 mM, twitch tension was gradually enhanced in muscle from neonatal mice (Fig. 7 A). The enhancement of twitch amplitude by high Ca$^{2+}$ in the mutant muscle was significantly higher than that of control muscle. In accordance with a previous report (Beam and Knudson, 1988), the results suggest that immature skeletal muscle cells contain a detectable population of Ca$^{2+}$ channels that are uncoupled with RyR and devoted to voltage-dependent Ca$^{2+}$ influx. The highly increased twitch by high Ca$^{2+}$, together with weak contractile responses at low-fre-

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**Figure 5.** Quantitative analysis of junctional membrane structures between wild-type and JP-1 knockout skeletal muscles. Skeletal muscles were prepared from tongue, jaw (digastric muscle), diaphragm, and hindlimb (thigh region) of newborn mice immediately after birth. The junctional membrane structures at the A-I junctions were analyzed by electron microscopic observations, and the results of random samples were compared between the genotypes. The data were obtained from at least 3,000 A-I junctions of three neonates and are shown as the mean ± SEM. Statistical differences between the genotypes are indicated by asterisks (t test, *$P < 0.05$ and **$P < 0.01$).

**Figure 6.** Force–frequency relationship in skeletal muscles from wild-type and JP-1 knockout neonates. Isometric tension of muscle preparations at several frequencies was determined in modified Krebs-Ringer solution, and typical recordings from wild-type and mutant muscles are shown in A. Force–frequency relationships in wild-type and mutant muscles are shown in B. Each value from at least n = 13 from seven mice was normalized to the maximum force and represents the mean ± SEM. Statistical differences between the genotypes are indicated by asterisks (t test, *$P < 0.05$ and **$P < 0.01$).
Published September 3, 2001

Table 1. Twitch and tetanic tension of hindlimb muscles from wild-type and JP-1 knockout neonatal mice

<table>
<thead>
<tr>
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<th>Twitch</th>
<th>Tetanus</th>
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<tr>
<td>Wild-type</td>
<td>22.4 ± 3.5</td>
<td>52.0 ± 8.3</td>
</tr>
<tr>
<td>JP-1–deficient muscle</td>
<td>11.2 ± 1.8&quot;</td>
<td>49.4 ± 7.0</td>
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Data (mean ± SEM) were derived from at least eight muscle preparations from six mice and each value was normalized by cross-sectional surface area of the muscle bundle. Twitch and tetanic responses were evoked by electrical stimuli at 0.33 and 30 Hz, respectively. "Indicates statistical difference (P < 0.01 in t test).

A faster decrease of twitch tension than control muscle. The apparent time constants of the decay in wild-type and JP-1 knockout skeletal muscles. A similar result has been observed in mitsugumin29 knockout muscles other than the jaw muscle. Alternatively, the abnormal response to extracellular Ca2+ might require dysregulation of SR Ca2+ loading in the mutant muscle.

Contraction of mature skeletal muscle is highly resistant under Ca2+-free conditions, and it is difficult to deplete the SR as the intracellular Ca2+ store. However, the high resistance is not fully established in immature muscles from neonatal mice. We examined JP-1 knockout muscle under Ca2+-free conditions (Fig. 7B). The mutant muscle showed a faster decrease of twitch tension than control muscle. The apparent time constants of the decay in wild-type and JP-1 knockout muscles were 13.7 and 7.4 min, respectively. A similar result has been observed in mitsugumin29 knockout muscle bearing structurally irregular membrane systems around the triad junction (Nishi et al., 1999). The observations on the mutant muscles lacking mitsugumin29 or JP-1 suggest that the formation of refined triad junctions is important for the Ca2+ preservation mechanism in skeletal muscle.

Proposed physiological roles of JP subtypes during triad formation

JP subtypes are composed of a COOH-terminal hydrophobic segment spanning the ER–SR membrane and the remaining cytoplasmic region interacting with the PM. Based on the biochemical properties, JP can produce junctional membrane structures between the ER and the PM in a heterologous expression system. The heart predominantly contains JP-2, and the deficiency of peripheral coupling was observed in embryonic cardiac myocytes from the JP-2 knockout mice (Takeshima et al., 2000). Despite the fact that both JP-1 and JP-2 were expressed in skeletal muscle (Fig. 1) and that JP-1 deficiency did not affect the subcellular localization of JP-2 (Fig. 2), the JP-1 knockout muscle exhibited both ultrastructural and functional abnormalities. The reduction in total JP protein level might cause the abnormalities, although JP-1 and JP-2 are functionally similar. Alternatively, JP-1 and JP-2 might be functionally different, and JP-1 is essential for the formation and/or maintenance of triad junctions. The latter theory is likely supported by several observations: (1) the formation of triad junctions was specifically reduced in muscles other than the jaw muscle from the JP-1 knockout mice; (2) the expression of JP-1 and the formation of triad junctions significantly increase during muscle maturation after birth; and (3) only a few triad-like structures are detected in cardiac myocytes predominantly expressing JP-2. However, future studies will be needed to examine whether JP-1 and JP-2 are functionally and qualitatively different during the formation of skeletal muscle triad junctions. Although the molecular basis of the triad formation is largely unknown, recent gene knockout experiments pointed out protein components involved in the construction (Fig. 8).

Why did the JP-1 knockout mice show a suckling defect and fail to raise squeaky voices in response to pain? Both the suckling response and vocalization mainly depend on reflexes, and the EMG recording indicated that the mutant mice retain a normal neural circuit for suckling response (Fig. 3). Furthermore, the tissue distribution of JP-1 indicates that primarily damaged tissues are skeletal muscle systems in the mutant mice. Therefore, one possibility is that in the mutant mice, apparently normal stimuli from motor

Figure 7. Effects of extracellular Ca2+ concentrations on twitch tension in wild-type and JP-1 knockout skeletal muscles. Control twitch tension was measured in modified Krebs-Ringer solution containing 2.5 mM CaCl2. The bathing solution was then changed to a high-Ca2+ solution containing 5 mM CaCl2 (A) or a Ca2+-free solution containing 0.1 mM EGTA (B). Twitches evoked by 0.33-Hz stimuli were monitored continuously after the replacement, and the data (at least n = 6 from four neonates in each plot) were normalized and represent the mean ± SEM. Statistical differences between the genotypes are indicated by asterisks (t test, *P < 0.05 and **P < 0.01).
neurons cannot control mouth movements for suckling or vocalization due to a low efficiency of E–C coupling in jaw muscles. Because the mutant thigh muscles (containing weak triads but retaining normal level of diads) showed several functional abnormalities in tension measurements, the significant reduction of both triads and diads might produce fatal damage in E–C coupling of mutant jaw muscles. Apparently normal respiration and limb movements in the mutant mice suggest that diaphragmatic and limb muscle motor neurons evoke excess levels of stimuli to overcome the impaired efficiencies of E–C coupling. The muscle type–specific defect proposed in the JP-1 knockout mice could be explained by differences in stimulation amplitude among innervated motor neurons, and also in reduced efficiency of E–C coupling.

Materials and methods

Immunohistochemical analyses

The mouse JP-1 cDNA fragment encoding the divergent region (amino acid residues 425–633) was cloned into pGEX4T-2 (Amersham Pharmacia Biotech) for production of a bacterial glutathione S-transferase fusion protein. Rabbits were repeatedly immunized with the fusion protein purified using glutathione-Sepharose (Amersham Pharmacia Biotech) to yield antiserum. The antibody specific to JP-1 was affinity purified using protein A–Sepharose and N-hydroxy succinimide–Sepharose (Amersham Pharmacia Biotech) conjugated with the fusion protein. The preparation of rat antibody to mouse JP-2 was described previously (Takeshima et al., 2000). Both antibodies were specific to corresponding JP subtypes and did not show cross-reactivity in the bacterial fusion proteins and mouse membrane preparations. For immunoblot analysis, total microsomal proteins were prepared from mouse tissues and analyzed as described previously (Takeshima et al., 1994). For immunohistochemical analysis, cryosections were prepared from skeletal muscles fixed in acetone. The sections were immunolabeled with the JP subtype–specific antibodies and Alexa 546– and 488–labeled secondary antibodies (Molecular Probes).

Generation of JP-1 knockout mice

A mouse genomic DNA library was screened with a JP-1 cDNA fragment as a hybridization probe to yield a phage clone carrying the 5′-terminal region of the JP-1 gene. Chromosomal mapping was carried out using fluorescence-labeled genomic fragments as probes as described previously (Shimuta et al., 1994). Our basic methods for the gene targeting were described previously (Kutsuwada et al., 1996). For immunohistochemical analysis, cryosections were prepared from skeletal muscles fixed in acetone. The sections were immunolabeled with the JP subtype–specific antibodies and Alexa 546– and 488–labeled secondary antibodies (Molecular Probes).

EMG recording during suckling response

The suckling behavior consists of nipple attachment, rhythmic suckling, and stretch response during milk letdown. Low-frequency and rhythmic movements of the jaw and tongue are observed in rhythmic suckling, and similar responses can be produced by mechanical stimulation to the lips (Westneat and Hall, 1992). EMG was recorded from a jaw muscle (musculus masseter), essentially as described previously (Kutsuwada et al., 1996). The suckling-like responses were induced by gentle stimulation of the area around the lips or tongue using a soft polyethylene tube, and the stimulation site and induced jaw movements were observed under a binocular microscope. The EMG signals evoked were picked up with bipolar electrodes (0.13-mm-diam enamel-coated copper wire) inserted into the jaw muscle, and were monitored using the electric amplifier (AB6212G; Nihon Koden) and polygraph system (PM-6000; Nihon Koden).

Morphological analyses

Skeletal muscle preparations were treated with a prefixative solution (3% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate, pH 7.4) and postfixed with a buffer containing 1% OsO4 and 0.1 M sodium cacodylate, pH 7.4. The fixed muscles were washed, dehydrated with alcohol and acetone, and embedded in Epon. For histological analysis, sections were prepared, stained with Toulidine blue, and observed under an optic microscope. For ultrastructural examination, thin sections were prepared, stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEM-200CX; JEOL).

Muscle contraction measurements

Skeletal muscle preparations (musculus vastus lateralis) were dissected from hindlimbs of newborn mice and were mounted on a force transducer in a chamber containing modified Krebs-Ringer solution (121.9 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 1.2 mM NaH2PO4, 15.5 mM NaHCO3, and 11.3 mM glucose) bubbled with 95% O2 and 5% CO2. To induce contraction, field stimulation (10 ms duration) with supramaximal voltage was given at various frequencies for 10 s, and developed force was recorded using online Chart software. The effects of extracellular Ca2+ concentrations were examined on twitches evoked at 0.33 Hz. After the experiments, the muscle preparations were fixed with 3% paraformaldehyde and were subjected to size measurements. Cross sectional surface area was determined using an image program (v. 2.3; Mitani Sangyo Co. Ltd.) based on the observations under a binocular microscope.

We thank Miyuki Kaneyama and Hiromi Sawamizu for maintaining the mutant mice.

This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health and Welfare of Japan, the Takeda Science Foundation, the Mochida Memorial Foundation, the Japan Heart Foundation, the Japanese Foundation of Metabolism and Disease, the Kimura Memorial Heart Foundation, the Kairara Morikazu Medical Science Promotion Foundation, the Toray Science Foundation, and the Inamori Foundation.
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