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REVIEW ARTICLES

Connexin- and Pannexin-Based Channels in Normal Skeletal Muscles and Their Possible Role in Muscle Atrophy

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Abstract Precursor cells of skeletal muscles express connexins 39, 43 and 45 and pannexin1. In these cells, most connexins form two types of membrane channels, gap junction channels and hemichannels, whereas pannexin1 forms only hemichannels. All these channels are low-resistance pathways permeable to ions and small molecules that coordinate developmental events. During late stages of skeletal muscle differentiation, myofibers become innervated and stop expressing connexins but still express pannexin1 hemichannels that are potential pathways for the ATP release required for potentiation of the contraction response. Adult injured muscles undergo regeneration, and connexins are reexpressed and form membrane channels. In vivo, connexin reexpression occurs in undifferentiated cells that form new myofibers, favoring the healing process of injured muscle. However, differentiated myofibers maintained in culture for 48 h or treated with proinflammatory cytokines for less than 3 h also reexpress connexins and only form functional hemichannels at the cell surface. We propose that opening of these hemichannels contributes to drastic changes in electrochemical gradients, including reduction of membrane potential, increases in intracellular free Ca^{2+} concentration and release of diverse metabolites (e.g., NAD^{+} and ATP) to the extracellular milieu, contributing to multiple metabolic and physiologic alterations that characterize muscles undergoing atrophy in several acquired and genetic human diseases. Consequently, inhibition of connexin hemichannels expressed by injured or denervated skeletal muscles might reduce or prevent deleterious changes triggered by conditions that promote muscle atrophy.

Keywords Gap junction · Cell–cell channel · Physiology of calcium channels in muscle · Pharmacology of muscle diseases

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Connexin- and Pannexin-Based Channels

Connexins (Cxs) and pannexins (Panxs) constitute two families of integral membrane proteins that, in mammals, are composed of about 20 and 3 members, respectively. In most cells studied thus far, the pattern of Cx expression varies according to the species, cell type and physiological state (Gorbe et al. 2005; Račkauskas et al. 2010; Bedner et al. 2011). Similarly, Panxs are expressed in many different cell types, but Panx2 has been detected preferentially in the nervous system of vertebrate animals (Bruzzone et al. 2003; Li et al. 2011; Ray et al. 2006).

Since several investigators have been unable to find evidence of gap junctions formed by Panxs, it was recently proposed that Panxs only form hemichannels (HCs); thus, it was recommended to call them Panx channels (Sosinsky et al. 2011). However, Panx gap junction channels (GJCs) have been observed in exogenous expression systems, including *Xenopus* oocytes and mammalian cells, as well as

in endogenous expression systems such as osteoblasts (Baranova et al. 2004; Peñuela et al. 2007; Söhl and Willecke 2004), which suggests that further efforts to find GJCs made of Panxs are required. In spite of this discrepancy, it is clear that Cxs and Panxs present four transmembrane domains, one cytoplasmic and two extracellular loops, and the $–NH_2$ and –COOH termini are located at the cytoplasmic side of the cell membrane. Both Cxs and Panxs can form HCs, and at least Cxs can form GJCs (Bruzzone and Dermietzel 2006; Ishikawa et al. 2011; Solan and Lampe 2009); HCs are also called connexons or pannexons, if composed of connexins or pannexins, respectively. HCs are composed of six subunits (except for Panx2 HCs, which form octamers [Ambrosi et al. 2010]), and if they are constituted of the same or different subunits, they are homomeric or heteromeric, respectively.

HCs can be found either at unapposed cell surfaces or at the vicinity of gap junction plaques at cellular interfaces, where they serve as precursors of GJCs (Gaietta et al. 2002). GJCs commonly allow communication between the cytoplasms of adjacent cells, but in a few exceptions they can provide communication means within the cytoplasm of the same expressing cell, in which they are called reflexive gap junctions.

Transfer of ions and small molecules (e.g., amino acids, peptides, sugars and some lipophilic compounds such as PGE_2) across the cellular membrane was believed to take place mainly via selective ion channels and/or transporters or by simple diffusion due to their physicochemical features. However, data reported during the last decade indicate that HCs, which present rather low selectivity, particularly when compared with ion channels, may also serve as membrane pathways for diffusional transfer of ions and small molecules between the intra- and extracellular compartments (Sáez et al. 2005). HCs have been shown to mediate the release of ATP, NAD^+ and/or PGE_2 from cells (Bruzzone et al. 2001; Cherian et al. 2005; Orellana et al. 2012). Interestingly, several HCs are permeable to Ca^{2+} (Sánchez et al. 2009, 2010; Schalper et al. 2010; Vanden Abeele et al. 2006) and, thus, can contribute to diverse cell responses activated by rises in free intracellular Ca^{2+} concentration. However, kinetic permeability properties have been studied for only a few HC types (Orellana et al. 2011), and further studies are needed to understand what distinguishes HCs with different subunit compositions and/or covalent modifications.

HCs play relevant roles in autocrine and paracrine cell–cell signaling. Opening of Cx HCs can be enhanced by diverse conditions, including the reduction of extracellular divalent cation concentrations, positive membrane potentials, some polyunsaturated fatty acids and changes in covalent modifications (e.g., oxidation or dephosphorylation) (Retamal et al. 2011; Schalper et al. 2012), whereas opening of Panx1 HCs can be enhanced by membrane depolarization, mechanical and metabolic stress (Domercq et al. 2010; Thompson et al. 2006) and activation of purinergic $P2X_7$ or $P2Y_1$ and $P2Y_2$ receptors (Locovei et al. 2006b; Thompson et al. 2006). Cx and Panx HCs present some pharmacological differences. For example, Cx HCs are inhibited by octanol and La^{3+} and resistant to probenecid, while Panx HCs are resistant to octanol and La^{3+} and inhibited by probenecid (Schalper et al. 2008). Moreover, low carbenoxolone concentrations (<10 μM) inhibit Panx1 HCs but not Panx2 or Cx HCs (Ambrosi et al. 2010), and high carbenoxolone concentrations (>50 μM) inhibit Panx1, Panx3 and Cx HCs (Ambrosi et al. 2010; Ishikawa et al. 2011; Schalper et al. 2008); probenecid inhibits Panx1-based HCs but not Panx2-based HCs (Ambrosi et al. 2010). Notably, some cells do not form either Cx or Panx GJCs, such as erythrocytes, and the main functional role of Panx1 is as HCs (Locovei et al. 2006a). A similar situation might occur in adult skeletal muscles (see below). These and other demonstrations in normal cells (Orellana et al. 2011; Schalper et al. 2008) indicate that HC opening can be handled by normal cells. Moreover, several cellular functions previously thought to be mediated by GJCs need to be reevaluated because they might be partially or totally explained by changes in the functional state of HCs, as recently demonstrated in astrocyte death induced by hypoxia reoxygenation in high glucose (Orellana et al. 2010).

GJCs facilitate direct communication between adjacent cells (Solan and Lampe 2009) because they allow intercellular transfer of electrical and metabolic signals without leakage to the extracellular space and, thus, participate in numerous functions of most cell communities (Račkauskas et al. 2010). To date, GJCs formed by endogenously expressed Cxs are rapidly inhibited (from seconds to a few minutes) by most Cx HC blockers. Peptides that react with cytoplasmic domains of Cx43 block Cx43 HCs but do not affect Cx43 GJCs (Ponsaerts et al. 2010). Similarly, La^{3+} blocks Cx43 and other Cx HCs (Schalper et al. 2008) but does not affect Cx43 GJCs (Contreras et al. 2002). The available information on Panx GJCs is limited mostly to studies performed in exogenous expression systems (Bruzzone et al. 2005), and their pharmacological sensitivity is likely to be similar to that of Panx HCs. On the other hand, like HCs, GJCs are permeable to ions and small molecules; and to date, the permeability properties of these two channel types have been assumed to be similar, but no empirical demonstration has been reported.

Possible Role of GJCs and HCs in Myoblast Commitment Acquisition and Differentiation during Ontogeny

Myogenic development, from the acquisition commitment of mesenchymal stem cells to contractile myofiber

formation, is highly regulated and coordinated. Ultrastructural, functional and molecular studies suggest the involvement of gap junctions. Skeletal muscle ontogeny occurs via a series of cellular and molecular steps that lead to the formation of multinucleated myofibers. Members of the MyoD transcription factors family, including MyoD, Myf5, myogenin and MRF4/herculin/Myf6, are key regulators in skeletal muscle development (Molkentin and Olson 1996). Each myogenic determination factor is expressed at different stages during skeletal muscle differentiation and becomes a marker of the corresponding stage (Pownall et al. 2002). For example, MyoD and Myf5 are markers of myogenic commitment (Braun et al. 1992; Rudnicki et al. 1993; Weintraub 1993).

Gap junctions were detected by electron microscopy in myoblast cultures and during embryonic development of rat myofibers derived from diaphragm and extensor digitorum longus (EDL) muscles (Kalderon et al. 1977; Ling et al. 1992). From a functional point of view, communication between developing myofibers was found to expand, and even if one myofiber was stimulated, the response propagated to other fibers of the muscle (Dennis et al. 1981). Consequently, gap junctions were proposed to allow the passage of molecules and metabolites required for myogenic development (Kalderon et al. 1977; Dennis et al. 1981). Later, Cxs were detected in embryonic skeletal myoblasts (monucleated muscle cells) (Duxson and Usson 1989). So far, Cx43 has been found to participate in terminal differentiation of skeletal muscles because myoblasts deficient in Cx43 ($Cx43^{Cre\text{-}ER(T)/fl}$) are associated with a relevant reduction in MyoD and myogenin expression (Araya et al. 2005). Moreover, rat L6 myoblasts and primary cultures of mouse myoblasts express Cx43 at 24 and 48 h of differentiation (Araya et al. 2005; Balogh et al. 1993), and consistently, the coupling index between myoblasts of the C_2C_{12} cell line (mouse myoblast cell line) increases at 24 and 48 h of differentiation (Araya et al. 2005). In addition, the expression of dominant negative Cx43, which reduces intercellular coupling through GJCs, inhibits the formation of myotubes (multinucleated muscle cells) in primary cultures of myoblasts obtained from newborn rats, suggesting that Cx43 is relevant in the fusion process of myoblasts (Gorbe et al. 2007). In agreement with this interpretation, blockers of GJCs inhibit both the fusion of myoblasts and the increase in levels of transcription factors that regulate myogenesis, e.g., myogenin (Araya et al. 2003a; Proulx et al. 1997). Therefore, Cx GJCs have been proposed to play a relevant role in several physiological processes of skeletal muscle ontogeny, such as cell growth, acquisition of myogenic commitment and differentiation (Araya et al. 2004; Söhl and Willecke 2004; von Maltzahn et al. 2004).

Cx39 (orthologue of hCx40.1) is expressed in murine myotubes but not in myoblast cells (von Maltzahn et al. 2006), suggesting that this connexin is involved in skeletal muscle differentiation but not in the process of myogenic commitment acquisition. Myotubes also express Cx43 and Cx45 (Araya et al. 2003a) and form gap junctions with myoblasts (Araya et al. 2004). However, gap junctions are reduced near birth and are absent in 1-week postnatal mice (Ling et al. 1992; von Maltzahn et al. 2004). At that time, only the remaining mononucleated cells that correspond to resident stem cells (satellite cells) express Cxs. Both Cx43 and Cx45 are known to form functional GJCs and HCs (Barrio et al. 1997; Li et al. 1996; Moreno et al. 1995; Steinberg et al. 1994), but evidence of whether Cx39 forms functional GJCs and/or HCs is yet to be reported. Moreover, the expression of Panxs in myoblasts remains unknown.

The acquisition of myogenic commitment is triggered by increases in $[Ca^{2+}]_i$, which promotes activation of calcineurin, a Ca^{2+}-dependent protein phosphatase that induces expression of the transcription factor myf-5 (Friday and Pavlath 2001). Two main Ca^{2+} mobilizing systems coexist in the cell: (1) Ca^{2+} release from internal stores (Zhang et al. 2011) and (2) Ca^{2+} influx from the extracellular medium. Upon extracellular stimulation by various receptor agonists, including ATP, phospholipase-C (PLC) is activated and phosphatidylinositol 4,5-bisphosphate is hydrolyzed, generating IP_3. The latter binds to its receptors (IP_3Rs) located in the membrane of the endoplasmic reticulum, leading to Ca^{2+} release (Zhang et al. 2011). The Ca^{2+}-release activity of the IP_3R channel is regulated by many intracellular modulators such as ATP, Ca^{2+}, IP_3R-binding proteins and protein kinases (Berridge 1993; Foskett et al. 2007; Zhang et al. 2011). On the other hand, Ca^{2+} influx is not mediated by voltage-sensitive Ca^{2+} channels (Araya et al. 2003b) but could be mediated by other Ca^{2+}-permeable channels, including stretch-activated Ca^{2+} channels (Sonobe et al. 2008), capacitive Ca^{2+} channels (Okon et al. 2002), TRP channels like TRPV4 (Pritschow et al. 2011), P2X receptors (Banachewicz et al. 2005) and possibly by Cx and Panx HCs. Since most Cxs also form HCs, the relative role of HCs and GJCs in these events remains unknown.

In L6 cells, a cell line derived from rat myoblasts, treatment with β-glycyrrhetinic acid, a blocker of Cx GJCs and Cx or Panx HCs (Schalper et al. 2008), inhibits the expression of myogenin and MRF4, two transcription factors that promote myogenesis. Such treatment also inhibits the cellular fusion process that leads to myotube formation (Proulx et al. 1997). P2X receptors ($P2X_{1-7}$) are ionotropic and activated by purine triphosphate nucleotides. P2Y receptors ($P2Y_{1,2,4,6,11-14}$) are metabotropic, and most of them are coupled to G proteins activated by both di- and triphosphate purine or pyrimidine nucleotides (Araya et al. 2004; Burnstock 2007; North 2002). In addition, C_2C_{12} cells, a cell line derived from satellite myoblasts, express $P2Y_1$, $P2Y_2$,

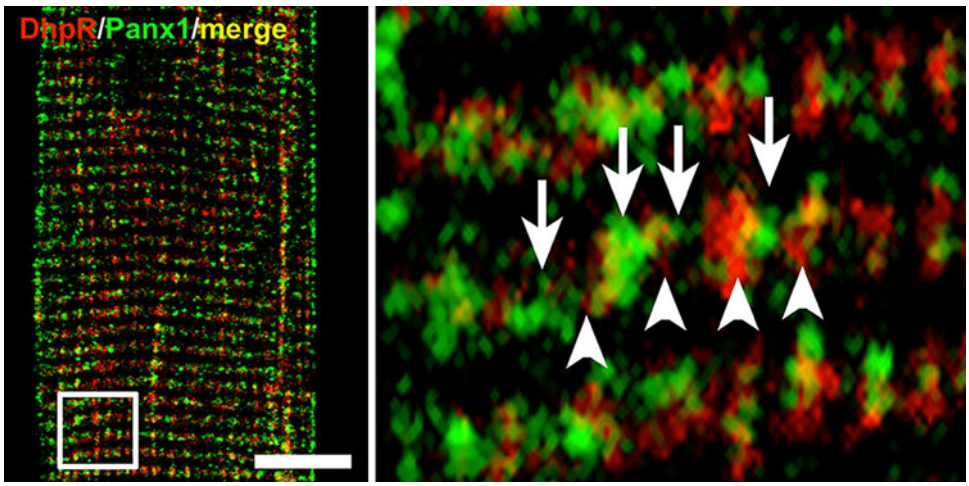

Fig. 1 Panx1 reactivity in rat skeletal muscle. Myofibers freshly isolated from flexor digitorum brevis (FDB) muscle were fixed with formaldehyde 4 % for 30 min, permeabilized (0.025 % Triton X-100) and double-labeled with rabbit anti-Panx1 antibody (*green*) designed by our group and directed to the nonconserved region of the C terminus of human, mouse and rat Panx1 (amino acid residues CNLGMIKMD). The antidihydropiridine receptor 1 (DhpR, *red*) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology (Iowa City, IA). *Left* Merged image of Panx1 (*green*, FitC) and DhpR (*red*, rhodamine) immunoreactivity. *Right* Magnification of the boxed region. Panx1 (*arrows*) was intercalated with DhpR (*arrowheads*) reactivity along transverse domains in the myofiber with little colocalization ($n = 4$). *Scale bar* = 10 μm

$P2Y_4$, $P2Y_6$ and $P2Y_{12}$ as well as $P2X_4$, $P2X_5$ and $P2X_7$ receptors (Banachewicz et al. 2005). Additionally, inhibition of P2 receptors prevents myoblast differentiation (Araya et al. 2004; Ryten et al. 2002). These findings suggest that myogenic commitment acquisition and differentiation require GJCs for intercellular propagation of a feed-forward mechanism generated in cells that express more P2 receptors. Immunohistochemical and in situ localization studies demonstrated that P2X receptors are expressed during skeletal muscle formation in vivo (Meyer et al. 1999; Ruppelt et al. 2001; Ryten et al. 2002). Moreover, it is possible that myoblasts express Panx or Cx HCs as membrane pathways for ATP release that modulates the intracellular free Ca^{2+} concentration required for muscle ontogeny. During late ontogeny, rat myotubes present functional Panx1 HCs (Buvinic et al. 2009) and nucleotide receptors ($P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_4$), and their activation of P2 receptors with exogenous nucleotides evokes Ca^{2+} transients (Buvinic et al. 2009; Deli et al. 2007). Moreover, tetanic stimuli (45 Hz, 400 1-ms pulses) evoke Ca^{2+} transients in myotubes that are inhibited by suramin, an inhibitor of P2 receptors, and apyrase, an ectonucleotidase that hydrolyzes ATP (Buvinic et al. 2009).

Possible Participation of HCs in Healthy Adult Skeletal Muscles

In rats, electrical coupling between skeletal myofibers disappears 1 week after birth (Dennis et al. 1981; Ling et al. 1992). In agreement, Cx expression has not been detected in normal adult skeletal muscles (Račkauskas et al. 2010). However, the presence of Panx1 mRNA (Baranova et al. 2004) and protein (Dvoriantchikova et al. 2006) has been demonstrated, suggesting that innervated myofibers might form Panx1 HCs. Moreover, we found that adult innervated myofibers present Panx1 reactivity side by side with dihydropyridine receptors (unpublished observation by Riquelme, Cea and Sáez; preliminary findings were presented at the Annual Meeting of the American Society for Cell Biology 2009) (Fig. 1), suggesting that Panx1 plays a relevant functional role in muscle physiology. Thus, it is possible that direct interaction between Panx1 HCs and P2 receptors plays a critical role in transduction processes, such as excitation–transcription coupling (Araya et al. 2003b). In support of this possibility, the dihydropyridine receptor coprecipitates with both the $P2Y_2$ receptor and Panx1 in homogenates of skeletal myotubes (Buvinic et al. 2009). However, similar studies in innervated skeletal muscles have not been reported yet. The above findings also indicate that the Panx1 expression of myotubes is not abrogated by innervation, but whether innervation up- or downregulates Panx1 expression remains to be studied.

The absence of Cx expression by adult rat skeletal muscles is in agreement with the proposal that Cx-based channels are not relevant for physiological muscle responses. This interpretation is consistent with the absence of functional Cx HCs in freshly isolated rat myofibers (Fig. 2). However, freshly isolated mouse myofibers show dye uptake through a membrane pathway inhibited by La^{3+} (Fig. 2). This finding suggests that normal mouse skeletal muscles might present Cx HCs on their surfaces. Clearly, a comparative study on Cx expression in skeletal muscles of

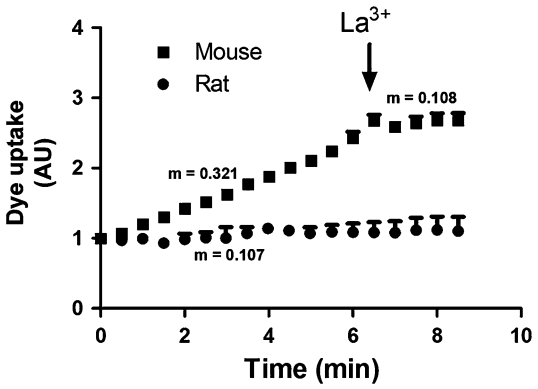

Fig. 2 Comparison of basal hemichannel activity between mouse and rat skeletal muscle fibers. We extracted the FDB skeletal muscle from rat and mouse and isolated the myofibers with collagenase type Ia (Sigma, St. Louis, MO). Then, we used the fibers for ethidium (5 μM) uptake assays in real time ($n = 6$). Fibers from mouse muscles showed a higher dye uptake rate than fibers from rats, and this increase was totally inhibited by La^{3+} (200 μM), an inhibitor of Cx-HCs

different mammalian species would contribute to resolving this apparent controversy.

As mentioned above, possible membrane receptors involved in $[Ca^{2+}]_i$ increments are P2 receptors activated by extracellular ATP/ADP (Araya et al. 2004; Buvinic et al. 2009; North 2002). Differentiated cells obtained from human skeletal muscle treated with exogenous P2Y receptor agonists show Ca^{2+} transients dependent on the IP_3 pathway but independent of ryanodine receptors (RyRs), which leads to ERK1/ERK2 activation (May et al. 2006).

P2Y receptors are likely to be expressed in fast skeletal muscles because potentiation of the contraction response occurs in the absence of extracellular Ca^{2+} (Louboutin et al. 1996). $P2X_4$ receptors are expressed in fast and slow skeletal muscles (Sandona et al. 2005). However, they participate in potentiation of muscle contraction only in slow muscles, where ectonucleotidases abrogate this response (Sandona et al. 2005). In the same line of thought, Panx1 HCs are permeable to Ca^{2+} (Vanden Abeele et al. 2006) and ^{10}panx1, a mimetic peptide that blocks Panx1 HCs, inhibits both the intracellular Ca^{2+} transients and the release of ATP to the extracellular milieu induced by tetanic stimulation in rat myotubes (Buvinic et al. 2009). The possible role of Panx1 HCs in ATP release and potentiation of muscle contraction needs to be demonstrated in fully differentiated (innervated) fast and slow skeletal muscles.

During repetitive skeletal muscle stimulation, extracellular ATP levels rise, causing activation of purinergic receptors that increase Ca^{2+} influx, potentiating the contraction force (Sandona et al. 2005; Zhi et al. 2005). In slow muscles, the potentiation response depends on the activation of purinergic $P2X_4$ receptors present in the T-tubule membrane as well as on the entry of extracellular Ca^{2+} (Sandona et al. 2005). In fast muscles, $P2X_4$ receptors are not found and potentiation occurs in the absence of extracellular Ca^{2+} (Louboutin et al. 1996; Sandona et al. 2005), suggesting the involvement of Ca^{2+} released from intracellular stores. Accordingly, potentiation of fast muscle contraction is blocked by extracellular ATPases, suggesting that extracellular ATP activates metabotropic P2 receptors linked to G proteins, leading to Ca^{2+} release from intracellular stores (Sandona et al. 2005). The three cloned IP_3Rs (IP_3R1, IP_3R2, IP_3R3) (Mikoshiba 2007) are present in adult muscles but in different intracellular locations (Casas et al. 2010). The rise in $[Ca^{2+}]_i$ increases the amount of calmodulin bound to Ca^{2+}, which activates calmodulin kinase II (CaMKII) and myosin light chain kinase. The latter phosphorylates the myosin regulatory protein kinase, enhancing Ca^{2+} affinity of the contractile machinery and increasing the twitch force (Zhi et al. 2005).

Skeletal muscle fibers release ATP during contraction (Cunha and Sebastião 1993; Sandona et al. 2005). In other cell types, ATP can be released via vesicles (Pangrsic et al. 2007) or through ATP permeable channels, including Cx and Panx HCs (Bao et al. 2004; Buvinic et al. 2009; Lu et al. 2012). In differentiated myoblast cultures, ATP release is inhibited by treatment with the Panx1 inhibitor ^{10}panx1, providing pharmacological evidence for a role of Panx1-based HCs in ATP release (Buvinic et al. 2009; Wang et al. 2007). It remains unknown, however, if innervation affects the expression of Panx1 in differentiated myofibers. Nevertheless, it is likely that innervated myofibers also express Panx1 because adult skeletal muscles express Panx1 and its transcript (Dvoriantchikova et al. 2006). Moreover, Panx1 might be located in T tubules because it can be coimmunoprecipitated with dihydropyridine receptors from total homogenates of myotubes (Buvinic et al. 2009). Since P2 receptors are present in adult myofibers, repetitive electrical stimulation could induce accumulation of intracellular Ca^{2+}. This would lead to activation of Panx1 HCs via a cytoplasmic mediator such as CaMKII, followed by ATP release via Panx1 HCs and activation of P2 receptors known to activate Panx1 HCs (Locovei et al. 2006b). This would then further increase ATP release and favor Ca^{2+} influx, which could contribute to potentiating the contraction force.

Cx Expression in Regenerating Skeletal Muscles

During skeletal muscle regeneration, satellite cells acquire myogenic commitment, reflected by the expression of myogenic determination factors, such as MyoD, Myf-5 and myogenin, transforming these cells into proliferative

myoblasts (Chargé and Rudnicki 2004). Moreover, adult regenerating mouse skeletal muscles present an increase in Cx43 and Cx45 levels after injection of $BaCl_2$, with highest expression at 5 and 3 days postinjection, respectively. At least, the absence of Cx43 delays the regeneration response (Araya et al. 2005). This process also occurs in adult rats, and the increase of Cx43 is accompanied by the synchronized exit of myoblasts from the cell cycle (Gorbe et al. 2005). In addition, a transient increase in Cx39 immunoreactivity was found through immunofluorescence analysis in regenerating adult skeletal muscle 2–10 days after injection of $BaCl_2$ into the tibialis anterior muscle of 6-week-old mice (von Maltzahn et al. 2004). However, muscles from mice deficient in Cx39 present an earlier myogenesis and accelerated regeneration after injection of $BaCl_2$, accompanied by higher levels of Cx43, probably compensating for the absence of Cx39 (von Maltzahn et al. 2011).

The above findings suggest that Cx-based channels expressed during regeneration play roles similar to those performed during the ontogeny of skeletal muscles.

Skeletal Muscle Atrophy and Hemichannels

Skeletal muscle atrophy can be defined as a decrease in mass, due to muscle wasting. It can occur in a variety of diseases associated with inflammatory responses and/or disuse (see below), trauma (tenotomy or denervation), prolonged immobilization, extended unloading (microgravity) or starvation or as a natural progression of aging. Denervation-induced atrophy involves the total disruption of trophic factors and nerve conduction, thus representing a model of acute acquired myopathy, which is frequently used in the study of muscle atrophy (Goldspink 1976; Goldspink et al. 1983).

Currently, it is difficult to establish the timing of different muscle changes associated with atrophy, mainly because the rate of atrophy varies among species, among individuals of the same species and among muscles of the same individual. For example, the weight of a rat muscle may be reduced by 50 % after 2 weeks of denervation, while in humans the same muscle suffers only a small loss of weight (Winlow and Usherwood 1975). Therefore, for practical purposes, variations in different muscle features are divided into early and late events linked to atrophy.

In the short term after skeletal muscle denervation, functional and structural changes can be observed. The distal segment of the motor neuron begins with Wallerian degeneration (Coleman 2005; Raff et al. 2002). Whereas muscles show a reduction in resting membrane potential (Albuquerque et al. 1971; Finol et al. 1981), there are increases in acetylcholine sensitivity at the extrajunctional

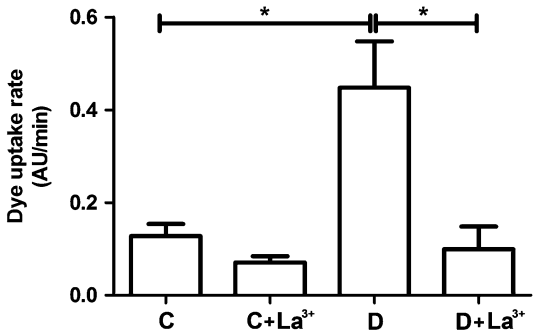

Fig. 3 Denervation induces an increase in membrane permeability in fast skeletal muscle fibers. At 6 days postdenervation we extracted the rat FDB skeletal muscle and isolated the myofibers. Then, we used the myofibers for ethidium (5 μM) uptake assays in real time ($n = 4$). Myofibers from denervated muscles showed a higher dye uptake rate (AU/min) than control fibers, and this increase was totally inhibited by La^{3+} (200 μM), an inhibitor of Cx-HCs. $^*P < 0.05$, ANOVA with posttest. *C* control, *D* denervated

membrane (Lomo and Westgaard 1975), proliferation of satellite cells (Ontell 1974; Snow 1983) and increases in the expression of myogenic regulatory factors (MRFs) (Buonanno et al. 1992; Legerlotz and Smith 2008).

Since the mechanism that explains the reduction in membrane potential after denervation remains unidentified (Kotsias and Venosa 2001; Lenman 1965), we evaluated membrane permeability in skeletal muscle fibers under different experimental conditions that induce muscular atrophy, e.g., denervation and proinflammatory cytokine exposure. We isolated skeletal muscle fibers from rat denervated flexor digitorum brevis muscle from 6 days postdenervation and found that HC activity was higher in denervated fibers (approximately sixfold) (Fig. 3). We blocked the increase in permeability with La^{3+}, which is a Cx HC blocker. Additionally, we evaluated the influence of proinflammatory cytokines that induce muscular atrophy, such as TNF-α and IL-1β, in the membrane permeability of skeletal muscle fibers. We found that TNF-α itself induces an increase in ethidium uptake rates and that the combination of TNF-α and IL-1β induces a higher increase in membrane permeability, which is also blocked by La^{3+} (Fig. 4).

With regard to the mechanism that could explain the reduction in resting membrane potential, it is relevant to mention that intracellular levels of Na^+ and extracellular concentrations of K^+ are elevated compared to control muscles (Kotsias and Venosa 2001). However, Na^+ and K^+ channels show properties similar to normal muscles (Escobar et al. 1993; Kotsias and Venosa 2001). In contrast to a possible increase in Na^+ current, the number of TTX-sensitive Na^+ channels is reduced 3–10 days after denervation in rat skeletal muscles (Schmid et al. 1984). Moreover, a reduction in Na/K-ATPase activity has also been ruled

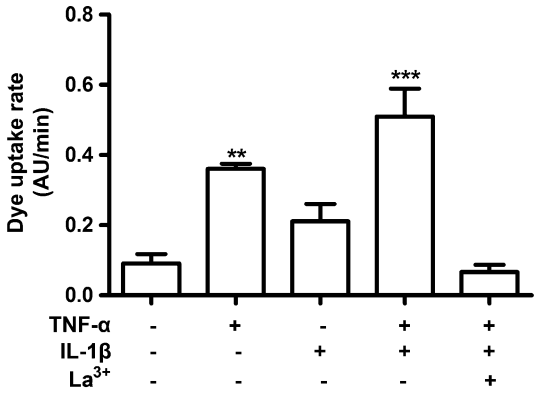

Fig. 4 Proinflammatory cytokines increase membrane permeability in fibers of fast skeletal muscles. Freshly isolated myofibers from rat FDB muscle were incubated for 3 h with either TNF-α (10 ng/ml) or IL-β (10 ng/ml) or with both cytokines. Membrane permeability was evaluated by ethidium (5 μM) uptake in time-lapse measurements ($n = 4$). TNF-α and IL-1β alone increased dye uptake, and treatment with both cytokines induced the summation response. Dye uptake induced by the cytokine mix was inhibited by La^{3+}. $^{**}P < 0.05$, $^{***}P < 0.001$ with respect to control uptake, ANOVA with posttest

out (Schmid et al. 1984). Since the increase in Cx HC levels occurs early after denervation, it is attractive to propose that a simultaneous increase in Na^+ influx and K^+ efflux mediated by Cx HCs might contribute to the reduction in resting membrane potential observed after denervation. Despite the low selectivity of HCs, it is conceivable that a new and lower resting membrane potential is established, mainly because intracellular negatively charged proteins are retained within the cells and, thus, reduce K^+ efflux.

The maintenance of muscle mass is regulated by a balance between protein synthesis and protein degradation pathways, which shift toward protein degradation during atrophy induced by denervation (Goldspink 1976, 1978). In addition, a net efflux of amino acids, such as glutamine, was detected after denervation (Hundal et al. 1990). Since Cx43 HCs, and most likely other Cx HCs (e.g., Cx39 and Cx45 HCs), are permeable to amino acids such as glutamate (Jiang et al. 2011), it is possible that they could disfavor protein synthesis by avoiding increases in intracellular concentrations of free amino acids required for protein synthesis, being that they would allow amino acid diffusion to the extracellular milieu.

Protein degradation is mainly mediated by four pathways: (1) calpains (Dargelos et al. 2008), (2) caspasas (Du et al. 2004), (3) cathepsins (Bechet et al. 2005), and (4) ubiquitin proteasome (Kandarian 2008). Many studies suggest that the latter plays a more significant role in protein degradation during muscle atrophy (Cao et al. 2005; Lecker 2003; Masiero et al. 2009; Ventadour and Attaix 2006). The major genes regulated during atrophy are *Fbxo32* and *Trim63*, which correspond to the ubiquitin ligases atrogin-1 and MuRF-1, respectively (Bodine et al. 2001), These genes show the greatest increase around 3 days after denervation, which is the time at which weight loss rates are higher in muscle (Sacheck et al. 2007). Overexpression of MuRF-1 in myotubes produces atrophy, whereas mice deficient in any of these ligases exhibit reduced atrophy after denervation (Bodine et al. 2001; Gomes et al. 2001; Rommel et al. 2001). During the second month of denervation, type II fibers show greater atrophy levels than type I fibers. From that point on, the observed atrophy is similar in all fibers and larger amounts of interstitial collagen are deposited (Lu et al. 1997).

Intracellular Ca^{2+} levels are higher in denervated than in normal skeletal muscles (Kirby and Lindley 1981; Picken and Kirby 1976; Shimizu and Kuriaki 1960). In addition, Ca^{2+} transport is slightly diminished in the sarcoplasmic reticulum of fast muscles, while Ca^{2+} transport of slow muscles is markedly increased (Marcreth et al. 1972). Accordingly, proteins involved in reducing intracellular Ca^{2+} levels are reduced; a reduced expression of fast SERCA1 Ca^{2+}-ATPase isoform, fast calsequestrin and fast parvalbumin occurs after denervation (Donoghue et al. 2004; Müntener et al. 1985) and possibly contributes to the persistent increase in free intracellular Ca^{2+} levels. Moreover, the denervated muscle contractile response depends on extracellular Ca^{2+} concentration (Picken and Kirby 1976). Since Cx43 HCs are permeable to Ca^{2+} (Schalper et al. 2010), it is possible that the higher free intracellular Ca^{2+} levels measured in denervated muscles are, in part, the consequence of enhanced Ca^{2+} influx via Cx HCs. Interestingly, protein degradation pathways can be activated by elevated free intracellular Ca^{2+} (Hussain et al. 1987), and thus, an increase in Cx HC activity in denervated muscles might be relevant in the development of muscle atrophy.

Other Pathological Conditions Associated with Muscular Atrophy

Other muscle diseases (i.e., myasthenia gravis) and different pathological conditions associated with inflammation (i.e., diabetes and septic shock) or chronic disuse (i.e., cancer) also develop muscle atrophy. However, all of these conditions may share some, but not all, etiological factors; and muscle atrophy is just a common characteristic developed by yet unknown factors.

Diseases of Neural Origin

Several diseases affect motor neurons and finally result in muscular atrophy. In this review, we focus on the most prevalent diseases, including spinal muscular atrophy. This is a neurodegenerative disease caused by mutations of the

survival motor neuron gene (D'Amico et al. 2011) that affects lower motor neurons, yielding progressive limb muscle weakness and paralysis. Motor neurons are also affected in spinal and bulbar muscular atrophy, which is a chromosome X–linked disease that affects lower motor neurons. It is caused by the expansion of CAG repeats, encoding the polyglutamine (polyQ) tract in the androgen receptor gene (Kennedy et al. 1968; Sobue et al. 1989). A normal individual possess 9–36 CAG repeats, and an affected individual has 38–62 repeats.

Another disease is amyotrophic lateral sclerosis, which is a progressive condition affecting upper and lower motor neurons and can either be of genetic origin or occur sporadically (Hama et al. 2012). A number of potential mechanisms have been proposed, including superoxidase dismutase type 1–mediated toxicity, excitotoxicity, cytoskeletal derangements, RNA processing and microglial activation (Rowland and Shneider 2001).

A classic denervation disease that affects lower motor neurons is Guillain-Barré syndrome. This is a group of acute immune-mediated polyneuropathies with several clinical forms. The most common variant is acute inflammatory demyelinating polyradiculoneuropathy, in 85–90 % of cases (Ropper 1992). Cardinal symptoms are progressive ascending weakness, which can compromise facial, oropharyngeal, oculomotor and respiratory muscles (Alshekhlee et al. 2008).

Diseases of Genetic Origin

There are several genetic diseases that induce skeletal muscle atrophy. The most prevalent of these is Duchenne muscular dystrophy (DMD), which is produced by a spontaneous mutation in the dystrophin gene, leading to muscular atrophy. Humans and dogs primarily show muscle atrophy. However, Mdx mice progress through an initial phase of muscle hypertrophy, followed by atrophy; and cats show persistent muscle hypertrophy (Kornegay et al. 2012). Dystrophin deficiency produces different responses depending on the species, individual and muscles. The reasons for phenotypic variation are not clear and raise questions about primary versus secondary effects of dystrophin deficiency. Other dystrophin mutations lead to attenuated expression of truncated protein and result in a milder phenotype referred to as Becker muscular dystrophy. Dystrophin is a member of the dystrophin-glycoprotein complex (DGC), a structural complex that anchors the cytoskeleton to the extracellular matrix. Alterations of dystrophin destabilize the DGC, thus compromising the cellular integrity and, finally, the structural and functional integrity of skeletal muscles. DMD patients report first weakness in the proximal lower limbs and then progress to the remaining ones. Onset of symptoms in humans occurs between 2 and 3 years of age. Muscle biopsies reveal myofiber necrosis, inflammatory cell infiltration, early muscle regeneration and fibrosis, which finally evolve to atrophy (Kornegay et al. 2012).

Acute Inflammatory Diseases

Sepsis and respiratory failure are two conditions of major risk for acquired weakness in critically ill patients. As a complication of critical illness, weakness of limb and respiratory muscles frequently slows and even dominates the course of recovery. Long-term consequences such as exercise limitation and low health-related quality of life sometimes persist for years (Herridge et al. 2003). Patients manifest both neuropathy and myopathy but with a common feature of muscle atrophy (Angel et al. 2007). There is a decrease in skeletal muscle protein synthesis and enhancement of catabolic degradation (Helliwell et al. 1998; Vary and Kimball 1992). However, sepsis induces early changes in contraction fiber properties, even without modifications in muscle mass (Callahan and Supinski 2009).

Chronic Inflammatory Diseases

Several chronic inflammatory diseases manifest a state called *cachexia*, which is defined as "a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment" (Fearon et al. 2001). The molecular mechanism involves TNF-α, which activates NFκB, which in turn increases the levels of atrogin (Judge et al. 2007) and MurF1 (Adams et al. 2008; Cai et al. 2004), two E3 ligase proteins that belong to the ubiquitin-proteasome system, and favors a catabolic state leading to muscular atrophy (Foletta et al. 2011).

One of the most prevalent chronic diseases is diabetes (types 1 and 2). It presents serious complications including neuropathies, retinopathies, cardiovascular disease and muscular atrophy. Skeletal muscle wasting is one of the most common alterations, in which protein breakdown increases and protein synthesis decreases (Smith et al. 1989).

Another highly prevalent chronic disease is cancer, in which there is an increase in TNF-α levels (Tisdale 2008), which in turn activates NFκB, leading to muscular atrophy. However, other cytokines, such as IL-1β and IL-6, and several tumor-derived substances are involved in cancer cachexia (Davis et al. 2004). Cachexia is a common state in patients with advanced cancer and is associated with less treatment tolerance, reduction of response to therapy and lower survival rates (Fearon 2011). It occurs with reduced

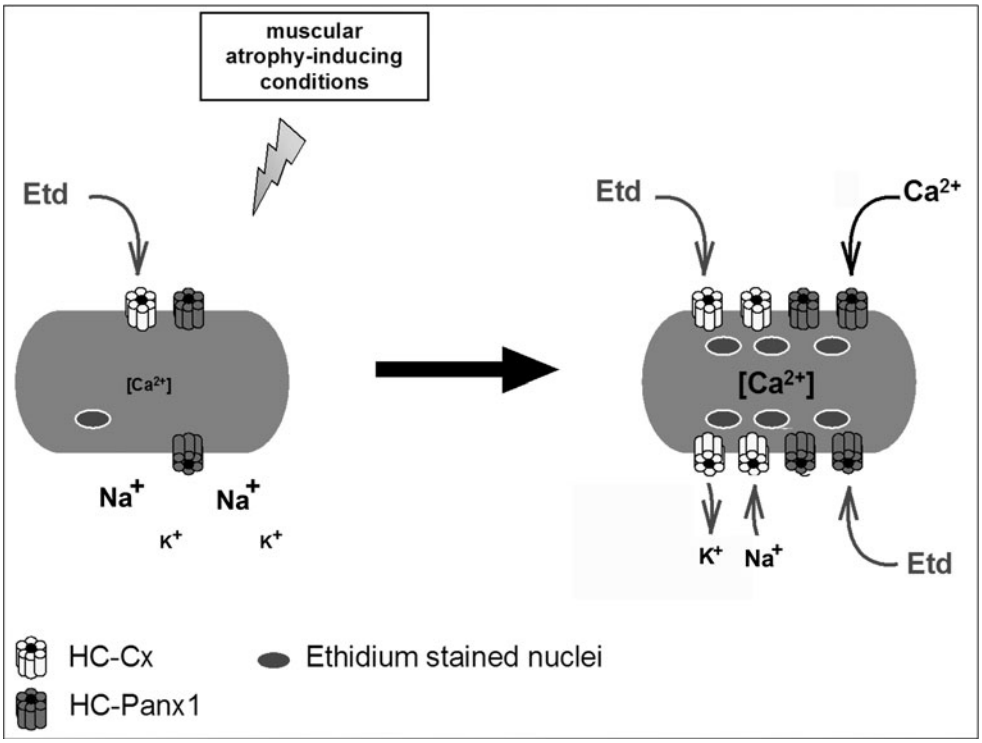

Fig. 5 Scheme showing possible membrane permeability changes in adult skeletal muscle undergoing atrophy. *Left* Normal skeletal muscle fibers have no or very low hemichannel activity. *Right* Conditions that induce muscular atrophy (i.e., proinflammatory cytokines) induce functional expression of connexin-based hemichannels that increase the membrane permeability to ions such as Na^+, K^+ and Ca^{2+}. Panx1 HCs are present in normal muscles, where most likely they are activated via extracellular ATP/P2 receptors and could also favor ion and small molecule fluxes in fibers undergoing atrophy

food intake and abnormal metabolism, which leads to a negative balance of energy and proteins. Moreover, this state is influenced by systemic inflammation (Zhou et al. 2010).

Muscular wasting is also present in nearly 20 % of patients with HIV/AIDS (Wanke et al. 2000). AIDS wasting syndrome is an involuntary weight loss >10 % of baseline body weight during the previous 12 months or a 5 % weight loss in the previous 6 months. Inflammatory myopathy resembling idiopathic polymyositis can occur in patients with HIV infection, as well as myopathy induced by treatment with nucleoside reverse transcriptase inhibitors, such as ziduvudine, lamivudine, abacabir and tenofovir (Moyle 2005).

In general, the paradigms of chronic inflammatory diseases that compromise muscle functions are the inflammatory myopathies, which share the common feature of autoimmune–mediated muscle injury. The most important subtypes include dermatomyositis, inclusion-body myositis, idiopathic polymyositis and overlap syndromes (with another systemic rheumatic disease). The exact pathogenic process differs, but they are all characterized by autoantibody production, inflammation, muscle weakness and immunity-independent NFκB activation such as endoplasmic reticulum stress by overload (Nagaraju et al. 2005).

Metabolic Myopathies

This group of diseases combines infrequent metabolic defects in energy production to muscle requirements. These include defects in lipid, glycogen and glucose metabolism as well organelle defects, namely, mitochondrial and lysosomal pathologies. Most patients report exercise intolerance, muscle pain and cramps rather than a fixed sense of weakness. However, some patients may develop muscular atrophy when experiencing fixed weakness. This evolution can occur in patients with acid maltase deficiency (Shea and Raben 2009), mitochondrial myopathies by mutations involving cytochrome *b*, cytochrome *c* oxidase (cox), MELAS A3260G mutation (Kollberg et al. 2005) and fatty acid oxidation defects (Hale and Bennett 1992). In the latter group, free fatty acids cannot be metabolized due to the existing metabolic block. As a result, they are stored in the cytoplasm as triglycerides, thereby resulting in progressive lipid storage myopathy with weakness,

hypertrophic and/or dilated cardiomyopathy and fatty liver (Treem 2000).

Concluding Remarks

Cx GJCs and HCs in ensemble with several membrane receptors, including P2 receptors, orchestrate numerous relevant steps in the muscle ontogeny process, including cell commitment to skeletal muscle lineage and differentiation. Moreover, in normal adult skeletal muscles Panx1 HCs may be key players in tissue responses, such as potentiation of muscle contraction. This is an example where Panx1 HCs do not coexist with Cx HCs.

Cxs are not expressed by normal adult skeletal muscles. However, under pathological conditions, such as denervation and inflammation, differentiated myofibers express functional Cx HCs (Fig. 5). Since Cx HCs are low-resistance membrane pathways with low selectivity, their functional expression in the cell membrane might lead to partial dissipation of the electrochemical gradient across the cellular membrane. Consequently, the increase in Cx HC activity offers a partial explanation for a number of cellular events that precede muscle atrophy, such as reduction in resting membrane potential and increases in protein degradation. Therefore, Cx HC blockers are expected to prevent or reduce skeletal muscle atrophy caused by different etiologies.

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