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Caveolin-3 is a direct molecular partner of the $Ca_v1.1$ subunit of the skeletal muscle L-type calcium channel

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ABSTRACT

Caveolin-3 is the striated muscle specific isoform of the scaffolding protein family of caveolins and has been shown to interact with a variety of proteins, including ion channels. Mutations in the human *CAV3* gene have been associated with several muscle disorders called caveolinopathies and among these, the P104L mutation (Cav-3^{P104L}) leads to limb girdle muscular dystrophy of type 1C characterized by the loss of sarcolemmal caveolin. There is still no clear-cut explanation as to specifically how caveolin-3 mutations lead to skeletal muscle wasting. Previous results argued in favor of a role for caveolin-3 in dihydropyridine receptor (DHPR) functional regulation and/or T-tubular membrane localization. It appeared worth closely examining such a functional link and investigating if it could result from the direct physical interaction of the two proteins. Transient expression of Cav-3^{P104L} or caveolin-3 specific siRNAs in C2C12 myotubes both led to a significant decrease of the L-type Ca^{2+} channel maximal conductance. Immunolabeling analysis of adult skeletal muscle fibers revealed the colocalization of a pool of caveolin-3 with the DHPR within the T-tubular membrane. Caveolin-3 was also shown to be present in DHPR-containing triadic membrane preparations from which both proteins co-immunoprecipitated. Using GST-fusion proteins, the I–II loop of $Ca_v1.1$ was identified as the domain interacting with caveolin-3, with an apparent affinity of 60 nM. The present study thus revealed a direct molecular interaction between caveolin-3 and the DHPR which is likely to underlie their functional link and whose loss might therefore be involved in pathophysiological mechanisms associated to muscle caveolinopathies.

1. Introduction

Caveolin is a 150-residue-long protein which acts as a structural determinant of plasma membrane caveolae. It is thought to operate as a multifunctional scaffold through associations with a wide range of partners. In skeletal muscle, caveolin-3, the striated muscle isoform (Tang et al., 1996; Way and Parton, 1996), has been shown to interact with a number of proteins including beta-dystroglycan (Sotgia et al., 2000), dysferlin (Matsuda et al., 2001), nNOS (García-Cardeña et al., 1997; Venema et al., 1997), phosphatidylinositol 3-kinase (Smythe and Rando, 2006), Src-kinase (Smythe et al., 2003) and phosphofructokinase (Scherer and Lisanti, 1997), and is thought to play a role in developmental processes (Biederer et al., 2000; Nixon et al., 2005; Parton et al., 1997). However the physiopathological role of caveolin-3 in skeletal muscle is still misunderstood. Several mutations in the gene encoding for caveolin-3 have been identified in patients that invariably cause caveolin-3 deficiency but lead to highly heterogeneous myopathic phenotypes (see Gazzerro et al., 2010). Although it remains to be clarified, a correlation could exist between such diverse clinical features and the multiple signaling pathways in which caveolin-3 is thought to be involved.

Recently, we examined the consequences of expressing in primary cultured myotubes and skeletal muscle fibers the P104L dominant negative mutant form of caveolin-3 (Cav-3^{P104L}), responsible for human type 1C limb-girdle muscular dystrophy. We observed an almost complete depletion of endogenous caveolin-3 and a strong reduction of the density of the voltage-dependent L-type Ca^{2+} current (Couchoux et al., 2007; Weiss et al., 2008). These results are complementary to our previous observations on membrane cholesterol-depleted skeletal muscle cells (Pouvreau et al., 2004).

In mammalian skeletal muscle, L-type Ca^{2+} currents are supported by the dihydropyridine receptor (DHPR), a molecular complex particularly enriched in the T-tubular system and composed of three membranous subunits, $Ca_v1.1$ (or α_{1S}), $\alpha_2\delta$ and γ, and of a

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cytoplasmic one, β_{1a}. The $Ca_v1.1$ subunit carries both the pore and the voltage sensor domains of the protein. Transmembrane folding model of $Ca_v1.1$ sequence highlights four homologous domains (I to IV), each one carrying 6 alpha helices, linked together by cytoplasmic loops named I–II, II–III and III–IV respectively (Tanabe et al., 1987; Catterall, 1988). The β_{1a} subunit was shown to interact with the I–II loop of $Ca_v1.1$ on a domain called Alpha Interacting Domain (AID) and such an interaction is thought to control the targeting of $Ca_v1.1$ to the T-tubule membrane and some of its Ca^{2+} channel gating properties (Pragnell et al., 1994; Bichet et al., 2000).

DHPR also exerts the critical role of voltage sensor in the excitation-contraction coupling process via a physical interaction with the type 1 ryanodine receptor (RyR1). This process takes place in structures called triads corresponding to the apposition of one T-tubule and SR cisternae and leads to a transient increase in the cytoplasmic Ca^{2+} concentration that directly triggers muscle contraction (Bannister, 2007).

The decrease of L-type Ca^{2+} current we previously observed in dominant negative Cav-3^{P104L} expressing myotubes could at least partially be explained by a defective membrane targeting or tethering of DHPR, as suggested by the reduced density of $Ca_v1.1$ subunits. Indeed, caveolin-3 and the non-muscle isoform caveolin-1 have been suggested to be involved in trafficking and/or membrane anchoring of proteins such as dysferlin, store-operated Ca^{2+} channels and K^+ channels (Brazer et al., 2003; Alioua et al., 2008; McEwen et al., 2008). Also, caveolae and caveolin-3 have been proposed to participate to T-tubules development during myogenesis (Ishikawa, 1968; Franzini-Armstrong, 1991; Nixon et al., 2005; Parton et al., 1997) and altered tubular biogenesis consecutive to caveolin-3 deficiency could indirectly alter DHPR distribution. In contrast, transient expression of Cav-3^{P104L} in adult skeletal muscle did not significantly modify the density of intramembrane charge movement (Weiss et al., 2008) suggesting that the total amount of functional voltage-sensing DHPRs was unchanged.

Based on electrophysiological recordings and immunolabeling of cultured C2C12 myotubes and on immunoprecipitation, pull-down and co-fractionation assays, the present study further validates our previous functional conclusions. More importantly, it demonstrates that caveolin-3 is not only restricted to caveolae, but that it can also be immunolocalized within T-tubules and detected in triadic membrane fractions where it interacts with the I–II loop of $Ca_v1.1$. This direct interaction of caveolin-3 with the DHPR likely represents the molecular basis of the regulation of the DHPR Ca^{2+} channel activity by caveolin-3.

2. Materials and methods

All experiments were carried out at room temperature unless otherwise stated.

2.1. C2C12 culturing and plasmid transfection

C2C12 murine myoblasts were cultured at 37 °C in DMEM-based proliferation medium containing 15% fetal calf serum and 4.5 g L^{-1} of glucose. Myoblasts were seeded at 50,000 cells per 35-mm culture dish and transfected the following day with a plasmid encoding YFP-tagged Cav-3^{P104L} (see Couchoux et al., 2007 for details) using FuGene HD (Roche Diagnostics). Myoblasts were allowed to fuse into myotubes by switching to DMEM-based differentiation medium containing 3% horse serum the same day as transfection.

2.2. Small interfering RNA transfection

A mixture of 4 predesigned small interfering RNAs (siRNAs) was used for caveolin-3 expression knockdown and validated non-targeting siRNAs were used as negative controls (On-target plus Smartpool, Dharmacon). C2C12 myoblasts were transfected with siRNAs (25 nM) using Dharmafect 1 reagent (Dharmacon) and differentiation medium was added the following day. Subsequent experiments were performed on myotubes at about 110 h post-transfection. Protein extracts were prepared in lysis buffer containing 1% NP40, 0.1% Triton X-100 and 0.1% SDS and protease inhibitors cocktail (ThermoScientific). After centrifugation (10,000 × *g*, 20 min, 4 °C), supernatants were collected and protein content was determined. Equal amounts of protein were separated by SDS–PAGE as described below.

2.3. Electrophysiology

Ca^{2+} currents were measured on C2C12 myotubes after 4–6 days in differentiation medium with the standard whole-cell configuration as previously described (Couchoux et al., 2007). Briefly, after a 10-min period after establishment of the whole-cell configuration as to ensure intracellular equilibration of the pipette solution, voltage-activated Ca^{2+} currents were recorded in response to 2.5-s-long depolarizing steps to values ranging between −50 and +80 mV with a 10 mV increment delivered every 30 s from a holding potential (HP) of −80 mV. Cell capacitance was used to calculate current densities. Mean capacitances did not significantly differ between myotubes groups (results not shown). Individual voltage dependence curves of the L-type Ca^{2+} current density were fitted with the following equation: $I(V) = G_{max}(V - V_{rev})/\{1 + \exp[(V_{1/2} - V)/k]\}$, where $I(V)$ is the density of the current measured, V, the test pulse, G_{max}, the maximum conductance for the peak current, V_{rev}, the apparent reversal potential, $V_{1/2}$, the potential that elicits the half-maximun increase in conductance, and k, a steepness factor. The voltage dependence of the macroscopic conductance $G(V)$ was obtained by dividing $I(V)$ by $G_{max}(V - V_{rev})$.

Data were analyzed using pCLAMP and GraphPad software packages and are presented as means ± s.e.m. Values were statistically analyzed using unpaired Student's *t* test and considered significant when $P < 0.05$.

2.4. Immunolabeling of C2C12 myotubes and adult muscle fibers

Cav-3^{P104L}-expressing C2C12 myotubes were immunolabeled for caveolin-3 as previously described for primary cultured myotubes (Couchoux et al., 2007).

For double immunolabelling of si-RNA transfected C2C12 myotubes, cells were incubated in a mix of rabbit polyclonal anti-caveolin-3 antibody (PA1-066, Affinity BioReagents) and of mouse monoclonal anti-$Ca_v1.1$ antibody (1:500, mAb427, Chemicon International). Secondary antibodies were then applied (Alexa488-conjugated secondary antibody and biotin-conjugated goat anti-mouse IgG; Jackson Immunoresearch), followed by a treatment with Cy3-conjugated streptavidin (1:500, Jackson Immunoresearch).

Skeletal muscle fibers were isolated from Flexor Digitorum Brevis (FDB) muscles from adult mice (Swiss OF1, Charles River, France) using enzymatic treatment as previously described (Jacquemond, 1997). The protocol used for immunolabeling was adapted from (Friedrich et al., 2008) with some modifications. Isolated fibers were dispersed in laminin/collagen coated glass-bottom dishes (Mattek). After 20 min, Tyrode solution was slowly exchanged for relaxing solution (in mM: 10 HEPES, 135 KCl, 2.5 $CaCl_2$ and 1 $MgCl_2$) and cells were fixed with 5% paraformaldehyde in PBS (2 min) and permeabilized for 5 min with 0.05% Triton X-100-50 mM glycine in PBS. Non-specific sites and endogenous biotins were blocked as described above for myotubes and all antibodies were diluted in blocking buffer. Fibers were sequentially labeled with anti-