The role of the calcium channel splice variant Cav1.1dE29 in the skeletal muscle excitation-contraction coupling

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Final research report

In the current project, we investigated the role of the t-tubule membrane calcium channel dihydropyridine receptor (DHPR, the $Ca_{V1.1}$ voltage-gated Ca^{2+} channel) in various conditions. In the first period of the experimental work, we determined its sensitivity for phosphatidylinositol phosphatases, their functions in hyper muscular mice and in mice containing their splice variant ($Ca_{V1.1}\Delta E29$).

Skeletal muscle excitation-contraction (E-C) coupling is altered in several models of phosphatidylinositol phosphatase (PtdInsP) deficiency. In our first set of experiments we measured intracellular Ca^{2+} transients and intramembrane charge movement (reflecting the conformational change of $Ca_{V1.1}$ upon activation) in voltage-clamped mouse muscle fibres microinjected with a solution containing PtdIns(3,5)P_2, PtdIns(3)P, PtdIns(5)P or PtdIns. The results demonstrated that neither the voltage-sensing function of the $Ca_{V1.1}$ nor its Ca^{2+} channel function was substantially affected by PtdInsPs. In addition, no significant change was observed in the presence of either PtdIns(5)P or PtdIns in SR Ca^{2+} release while its peak was depressed by ~30 and 50% in fibres injected with PtdIns(3,5)P_2 and PtdIns(3)P, respectively. In permeabilized muscle fibers, the frequency of spontaneous Ca^{2+} release events was depressed in the presence of the three tested phosphorylated forms of PtdInsP with PtdIns(3,5)P_2 being the most effective, leading to an almost complete disappearance of Ca^{2+} release events. Our results suggest that the 3-phosphorylated PtdIns lipids active on voltage-activated Ca^{2+} release are inherently maintained at a low level, inefficient on Ca^{2+} release function in normal conditions (E. González Rodríguez, R. Lefebvre, D. Bodnár, C. Legrand, P. Szentesi, J. Vincze, K. Poulard, J. Bertrand-Michel, L. Csernoch, A. Buj-Bello, V. Jacquemond. Phosphoinositide substrates of myotubularin affect voltage-activated Ca^{2+} release in skeletal muscle. Pflughers Archive European Journal of Physiology, 466: (5) 973-985, 2014.).

We investigated E-C coupling also in non-control conditions because alterations in any of their steps – due to aging, oxidative stress, genetic modification, etc. – may lead to severe muscle dysfunction and consequently to disability. Excitation–contraction uncoupling may be

caused by alterations in expression of the voltage-dependent calcium channel $Ca_{V1.1}$ subunits, which is necessary for E-C coupling to occur. Previous studies have found that $Ca_{V1.1}$ expression declines in old rodents as an aging model. A reduction in E-C coupling may also contribute to the decrease in specific tension, as there is increasing evidence of E-C uncoupling in old age. Myostatin, a member of the transforming growth factor β (TGF-β) superfamily has emerged as a potent negative regulator of skeletal muscle growth. Myostatin is strongly expressed in skeletal muscle and MSTN−/− mice have a great increase in muscle mass demonstrating that myostatin is a muscle-specific negative regulator of skeletal muscle growth. Thus MSTN−/− mice could be a good model of un-aged mice in respect of aging because myostatin deficient (*Cmpt*) mice have a great increase in muscle mass. In voluntary wheel running control mice performed better than the mutant animals in both maximal speed and total distance covered. The pCa-force relationship, determined on chemically permeabilized fibre segments did not show any significant difference between the two mouse strains. While resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measured on single intact *flexor digitorum brevis* (FDB) muscle fibres was similar to control, the amplitude of KCl-evoked calcium transients was smaller in the mutant strain. SR calcium release flux, calculated from calcium transients evoked by tetanic stimulation showed a reduced peak with no change in the peak-to-steady ratio. The amplitude and spatial spread of calcium release events detected on permeabilized FDB fibres were also significantly smaller in mutant mice (D. Bodnár, N. Geyer, O. Ruzsnavszky, T. Oláh, B. Hegyi, M. Sztretye, J. Fodor, B. Dienes, Á. Balogh, Z. Papp, L. Szabó, G. Müller, L. Csernoch, P. Szentesi. Hypermuscular mice with mutation in the myostatin gene display altered calcium signaling. Journal of Physiology, 592: 1353-1365, 2014.). These results suggest that reduced SR calcium release underlies the reduced muscle force in *Cmpt* animals but the role of $Ca_{V1.1}$ in this process is still under investigation.

During this project we completed the experiments aimed to describe the physiological role of the newly discovered Ca^{2+} channel splice variant in skeletal muscle E-C coupling. We examined the potential physiological manifestation of the altered conducting properties of $Ca_{V1.1e}$. We accomplished the measurements concerning the role of the $Ca_{v1.1e}$ Ca^{2+} channel splice variant in muscle performance of skeletal muscle. We finalized our data concerning the distance, the duration and the speed of running of wild type and $Ca_{v1.1}\Delta E29$ mice. In addition, grip-strength tests were also performed. We found no alteration in distance and in speed (neither in average nor in maximal speed) of voluntary running. However, the knockout mice

spent significantly less time in the running wheel. In spite of their similar body weight the knockout mice were less powerful. The results of these experiments were published at the Annual Biophysical Society Meeting, in San Francisco (N. Sultana, A. Benedetti, M. Sztretye, B. Dienes, P. Szentesi, P. Tuluc, S. Quarta, G.J. Obermair, C. Schwarzer, M. Kress, L. Csernoch, B.E. Flucher: Expression of the Embryonic $Ca_{V1.1}$ Splice Variant in Adult Mice Alters Excitation-Contraction Coupling but Does not Cause Dystrophic Myotonia, Biophysical Journal, 106: 126a, 2014.).

Parameters like body and muscle weight, as well as general muscle histology were also analyzed. Reduction of SDH (immune-staining and enzyme activity) was found both in *extensor digitorum longus* (EDL) and soleus muscles. To analyze the fiber type composition of slow and fast muscles in wildtype and $Ca_{V1.1}\Delta E29$ mice, sections of soleus and EDL muscle were immune-stained with antibodies against specific myosin heavy chain isoforms. A substantial shift towards slower fiber types in both soleus and EDL muscles of $Ca_{V1.1}\Delta E29$ mice. Soleus muscles of $Ca_{V1.1}\Delta E29$ mice experienced an almost 50% increase in the fraction of type I fibers mainly at the cost of type IIA fibers. In EDL of $Ca_{V1.1}\Delta E29$ mice the fraction of type IIB fibers is reduced by 24% compared to the wild type, while the fractions of IIA, IIX, and mixed fibers increased two- to three-fold. Muscle size was not different in wildtype and $Ca_{V1.1}\Delta E29$ mice, and hematoxylin and eosin staining of muscle sections did not reveal any histological malformation of the muscles. These findings indicate that expression of the calcium-conducting $Ca_{V1.1e}$ splice variant in skeletal muscles of adult $Ca_{V1.1}\Delta E29$ mice does not lead to gross pathologic abnormalities of the muscles, but causes a shift in the fiber type composition towards slower fiber types.

Reduced endurance and increased fatigue also support this theory. Contractile force of isolated soleus and EDL muscles was recorded in response to a single electrical stimulus (twitch) and in response to high frequency trains of stimuli (tetanus). Twitch and tetanic force was significantly reduced in both muscles types of $Ca_{V1.1}\Delta E29$ mice. However, in $Ca_{V1.1}\Delta E29$ mice both muscle types display significantly reduced fatigue compared to wildtype controls.

The tetanic fusion frequency was also assessed. We reasoned that additional calcium entering the cytoplasm through the $Ca_{V1.1e}$ channel might delay calcium removal after each action potential. Thus, tetanic fusion of contractions, which is indirectly related to the speed of calcium removal, might occur at lower frequencies in $Ca_{V1.1}\Delta E29$ mice than in wildtype controls. In both muscle types the frequency at which the half-maximal tetanic force has been reached was significantly lower in $Ca_{V1.1}\Delta E29$ mice compared to wildtype controls. Together

these tests on isolated muscles demonstrate that the aberrant expression of $Ca_{V1.1e}$ in adult $Ca_{V1.1}\Delta E29$ mice alters significantly the contractile properties of isolated slow and fast muscles.

Combined patch-clamp and cytoplasmic calcium recording was performed in fibers loaded with the fluorescent calcium indicator Rhod-2. Step depolarizations to varying test potentials elicited calcium currents and cytoplasmic calcium transients in parallel. The calcium currents of $Ca_{V1.1}\Delta E29$ muscles were activated at significantly lower voltages and were significantly larger than those recorded in wildtype controls. From the calcium transients the total calcium flux (influx and SR release) during the depolarizing pulses was calculated. These calcium flux traces characteristically showed an early peak followed by a steady-state plateau phase. In control muscle the voltage-dependence of peak and plateau calcium fluxes displayed a monotonic increase both of which could be fitted with a two-state Bolzmann function. Changes in the fluorescence intensity of rhod-2 following the depolarization to intermediate voltages, especially in the range of -10 to +10 mV, indicated an extra influx of calcium into the myoplasmic space in knockout mice that is neither present at more negative nor at more positive voltages. In addition to its immediate role in E-C coupling this additional calcium influx in $Ca_{V1.1}\Delta E29$ mice may alter the calcium homeostasis in muscle cells. To examine a possible contribution of L-type calcium currents through $Ca_{V1.1}e$ to refilling of SR calcium stores we applied a protocol designed to elicit store-operated calcium entry (SOCE). These experiment provide evidence that in $Ca_{V1.1e}$ expressing muscle SR refilling is predominantly carried by calcium influx through this L-type channel, while in wildtype muscle fibers $Ca_{V1.1a}$ does not significantly contribute to SR refilling.

The manuscript summarizing all of these observations is under review (N. Sultana, B. Dienes, A. Benedetti, P.l Tuluc, P. Szentesi, M. Sztretye, M.W. Hess, C. Schwarzer, G.J. Obermair, L. Csernoch, B.E. Flucher: Restricting calcium currents by alternative splicing of $Ca_{V1.1}$ is required for correct fiber type determination in skeletal muscle. Development).

Data were partly presented at the Annual Biophysical Society Meeting, in Baltimore (B. Dienes, N. Sultana, J. Vincze, M. Sztretye, P. Szentesi, B.E. Flucher, L. Csernoch: Calcium sparklets in intact mammalian skeletal muscle fibers expressing the embryonic $Ca_{V1.1}$ splice variant, Biophysical Journal, 108: 504a., 2015.) and at the European Muscle Conference, in Warsaw (P. Szentesi, B. Dienes, J. Vincze, N. Sultana, B.E. Flucher, L. Csernoch: The calcium homeostasis may change in some myotonic dystrophies, Journal of Muscle Research and Cell Motility, 36: in press., 2015.).

In skeletal muscle E–C coupling the $Ca_{V1.1}$ voltage-sensitive Ca^{2+} channel is modulated by several intracellular factors, amongst which $[Ca^{2+}]_i$ is one of the most important. $[Ca^{2+}]_i$ modified by the Sarco-Endoplasmatic Calcium Pump (SERCA), so it was thus straightforward to investigate its effects. The neonatal SERCA1b is the major Ca^{2+} pump in myotubes and young muscle fibers. To understand its role during skeletal muscle differentiation its synthesis has been interfered with specific shRNA sequence. Quantitative analysis revealed significant alterations in CSQ, STIM1, and calcineurin expression. To examine the functional consequences of the decreased expression of SERCA1b, repeated Ca^{2+}-transients were evoked by applications of 120 mM KCl. The significantly higher $[Ca^{2+}]_i$ measured at the 20^{th} and 40^{th} seconds after the beginning of KCl application indicated a decreased Ca^{2+}-uptake capability. Furthermore, the rate of calcium release from the SR and the amount of calcium released were also significantly suppressed. These changes were also accompanied by a reduced activity of calcineurin in cells with decreased SERCA1b. In parallel, cloneC1 cells showed inhibited cell proliferation and decreased myotube nuclear numbers. SERCA1b is thus considered to play an essential role in the regulation of $[Ca^{2+}]_i$ and its *ab ovo* gene silencing results in decreased skeletal muscle differentiation.

Our results suggest that SERCA1b is required for myoblast proliferation and secondary myotube formation by having an important role in Ca^{2+} homeostasis (A. Tóth, J. Fodor, J. Vincze, T. Oláh, T. Juhász, R. Zákány, L. Csernoch, E. Zádor: The effect of SERCA1b silencing on the differentiation and calcium homeostasis of C2C12 skeletal muscle cells. PLOSone, 10(4):e0123583, 2015.).

Calcium influx through the embryonic $Ca_{V1.1e}$ substantially contributes to depolarization-induced calcium transients in fetal muscles and in cultured myotubes. In our genetically modified mouse ($Ca_{V1.1}\Delta E29$), which exclusively expresses the embryonic $Ca_{V1.1e}$ variant also in adult muscle the calcium influx component is maintained throughout life. Utilizing this mouse model, spontaneous calcium release events – calcium sparklets – were recorded. While control animals did not display such events, $Ca_{V1.1}\Delta E29$ mice spontaneously generated sparklets. The role of external calcium as the trigger was tested by either removing calcium from the external solution or by the application of 5 μM nizoldipine to block the calcium current through $Ca_{V1.1e}$. Both interventions resulted in a complete loss of the events. Identified sparklets (n=311) were characterized by an average amplitude ($\Delta F/F_0$) of 0.287±0.005, a full-width at half-maximum of 3.05±0.05 μm, and duration of 235±4 ms, clearly different from the properties of calcium sparks on saponin-permeabilized adult mammalian skeletal muscle

fibers. These findings indicate that the sustained expression of the $Ca_{V1.1e}$ splice variant gives rise to spontaneous calcium entry events (sparklets) in adult muscle fibers and that their properties are distinct from calcium sparks arising from ryanodine receptors.

To analyze such a complex calcium release events we modified the standard analysis method to high-speed confocal microscopy images. Due to the large number of points in a spark, specificity exceeds 95% in all cases, enabling automatic analysis without manual correction. As the levels of detection parameters show, the assessed toolset is highly effective in the analysis of complex muscle calcium release events. The results were published at the Annual Biophysical Society Meeting, in San Francisco (J. Vincze, L. Szabó, B. Dienes, P. Szentesi, L. Csernoch: Increased Accuracy of Calcium Spark Parameter Detection using High-Speed Confocal Microscopy, Biophysical Journal 106: 532a, 2014.) and in Baltimore (J. Vincze, B. Dienes, P. Szentesi, L. Csernoch, D.R. Laver: Effects of triad geometry and RyR gating scheme on simulated skeletal muscle sparks, Biophysical Journal, 108: 261a., 2015.).

To summarize our findings obtained in the framework of this project we published a review article about the effects of phosphoinositides (PtdInsPs) on E-C coupling. There is increasing evidence that PtdInsPs play a role in muscle Ca^{2+} signaling in differentiated muscle with SR Ca^{2+} release being a major target mechanism. PtdInsPs could directly modify the gating of RyR and $Ca_{V1.1}$, the two major Ca^{2+}-channel proteins in E-C coupling. Thus PtdInsPs could modulate muscle Ca^{2+} signaling under normal and disease conditions too (L. Csernoch, V. Jacquemond: Phosphoinositides in Ca^{2+} signaling and excitation–contraction coupling in skeletal muscle: an old player and newcomers, Journal of Muscle Research and Cell Motility, DOI 10.1007/s10974-015-9422-4, 2015.).

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