Published in final edited form as:
Aging Cell. 2009 September ; 8(5): 584–594. doi:10.1111/j.1474-9726.2009.00507.x.

Increased $Ca_V\beta_{1a}$ Expression with Aging Contributes to Skeletal Muscle Weakness

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Summary

Ca^{2+} release from the sarcoplasmic reticulum (SR) into the cytosol is a crucial part of excitation-contraction (E-C) coupling. E-C uncoupling, a deficit in Ca^{2+} release from the SR, is thought to be responsible for at least some of the loss in specific force observed in aging skeletal muscle. E-C uncoupling may be caused by alterations in expression of the voltage-dependent calcium channel α_{1s} ($Ca_V1.1$) and β_{1a} ($Ca_V\beta_{1a}$) subunits, both of which are necessary for E-C coupling to occur. While previous studies have found $Ca_V1.1$ expression declines in old rodents, $Ca_V\beta_{1a}$ expression has not been previously examined in aging models. Western blot analysis shows a substantial increase of $Ca_V\beta_{1a}$ expression over the full lifespan of FVB mice. To examine the specific effects of $Ca_V\beta_{1a}$ overexpression, a $Ca_V\beta_{1a}$ -YFP plasmid was electroporated *in vivo* into young animals. The resulting increase in expression of $Ca_V\beta_{1a}$ corresponded to decline of $Ca_V1.1$ over the same time period. YFP fluorescence, used as a measure of $Ca_V\beta_{1a}$ -YFP expression in individual fibers, also showed an inverse relationship with charge movement, measured using the whole-cell patch-clamp technique. Specific force was significantly reduced in young $Ca_V\beta_{1a}$ - YFP electroporated muscle fibers compared to sham-electroporated, age-matched controls. siRNA interference of $Ca_V\beta_{1a}$ in young muscles reduced charge movement, while charge movement in old was restored to young control levels. These studies imply $Ca_V\beta_{1a}$ serves as both a positive and negative regulator $Ca_V1.1$ expression, and that endogenous overexpression of $Ca_V\beta_{1a}$ during old age may play a role in the loss of specific force.

Keywords

Sarcopenia; Calcium channel; DHPR; E-C Coupling; Specific Force; Calcium

INTRODUCTION

Depolarization of the sarcolemma leads to muscle fiber contraction and the generation of mechanical force in a process called excitation-contraction (E-C) coupling. Two key proteins

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AUTHOR CONTRIBUTIONS
O.D. designed research; J.R.T., Z-M.W., Z.Z., M.L.M., A.M.P. and O.D. performed research; J.R.T., Z-M.W., Z.Z., A.M.P. and O.D. analyzed data, and J.R.T. and O.D. wrote the paper.

involved in E-C coupling are: dihydropyridine receptor (DHPR) and ryanodine receptor (RyR). DHPR serves as a modest L-type Ca^{2+} channel but is primarily known for its function as a voltage sensor. DHPRs are located within invaginations of the sarcolemma called t-tubules, and are arranged into clusters of four known as tetrads. Each tetrad is positioned directly across from a single RyR, which are embedded within the membrane of the adjacent sarcoplasmic reticulum (SR). DHPRs contain four domains, each composed of six-transmembrane spanning segments. The S4 segment of each domain contains charged amino acid residues, and these residues respond to membrane depolarization by undergoing a conformational shift. This shift results in a proposed physical interaction of DHPR with RyR, causing RyR to open and release intracellular Ca^{2+} stores from the SR, allowing muscle contraction to occur (for review, see Melzer *et al.* 1995).

The decline in muscular strength with age, known as sarcopenia, is caused largely by a loss of total muscle mass - but also a disproportionate loss of strength. This loss of specific force (total force/cross sectional area) in old age (Brooks & Faulkner 1988; Gonzalez *et al.* 2000) is characterized in part by a deficit in Ca^{2+} release following depolarization (Delbono *et al.* 1995; Jimenez-Moreno *et al.* 2008), a phenomenon known as E-C uncoupling. E-C uncoupling is not a result of decreased Ca^{2+} stores or RyR release function (Jimenez-Moreno *et al*, 2008), and therefore may be caused by alterations in the functionality and expression of DHPR and its subunits with aging. The primary DHPR subunit in skeletal muscle is $Ca_V1.1$, previously known as DHPRα_{1s} (Catterall *et al.* 2005). $Ca_V1.1$ is a large transmembrane protein which contains both the Ca^{2+} conducting pore and the voltage sensing S4 domain. Four other auxiliary subunits bind $Ca_V1.1$ to make up DHPR (for review, see Flucher *et al.* 2005), with the most widely studied being the cytosolic $Ca_V\beta_{1a}$ subunit. $Ca_V\beta_{1a}$, a muscle specific member of the $Ca_V\beta$ family of proteins, binds to a region of the I–II intracellular loop of $Ca_V1.1$ known as the alpha interaction domain (AID) (Chen *et al.* 2004). $Ca_V\beta_{1a}$ is classically described by its role in chaperoning $Ca_V1.1$ to the plasma membrane and regulating L-type Ca^{2+} current (Gregg *et al.* 1996; Strube *et al.* 1996; Beurg *et al.* 1997; Neuhuber *et al.* 1998). Most notably, E-C coupling cannot occur without $Ca_V\beta_{1a}$ (Gregg *et al.* 1996). $Ca_V\beta_{1a}$ binds to charged residues on RyR (Cheng *et al.* 2005) and neutralization of these residues reduces E-C coupling, suggesting a direct interaction with RyR. The correct organization of $Ca_V1.1$ into tetrads within the t-tubule membrane is also a specific function of the $Ca_V\beta_{1a}$ isoform (Schredelseker *et al.* 2005).

Although classically known for augmenting the expression and function of Ca_V1 subfamily of calcium channels, the $Ca_V\beta$ family of subunits may contribute to the down-regulation of Ca_V1 as well. A family of Ras-related G-proteins (RGKs) mediate the down-regulation of several Ca_V1 isoforms in a $Ca_V\beta$ dependent manner (Beguin *et al.* 2001). Additionally, the previously uncharacterized SH3 domain of $Ca_V\beta$ was shown to bind dynamin and mediate endocytosis of $Ca_V1.2$ (Gonzalez-Gutierrez *et al.* 2007).

As previous studies have shown that the $Ca_V1.1$ subunit declines in old rodents (Renganathan *et al.* 1997; Moreno *et al.* 2006; O'Connell *et al.* 2008) and this causes an impairment of E-C coupling (Renganathan *et al.* 1997), we wanted to investigate what effects aging had on $Ca_V\beta_{1a}$ expression, as this subunit is also critical for E-C coupling. We have found that $Ca_V\beta_{1a}$ expression is highly increased in old mice, and that experimental overexpression of $Ca_V\beta_{1a}$ reduces both the expression of $Ca_V1.1$ and specific force in dissociated single fibers of young mice. Additionally, siRNA inhibition of $Ca_V\beta_{1a}$ restores charge movement in aged muscle. These findings suggest that overexpression of $Ca_V\beta_{1a}$ with aging contributes to E-C uncoupling by reducing the level of $Ca_V1.1$.

RESULTS

$Ca_V\beta_{1a}$ subunit expression increases with age

As our laboratory had earlier shown that $Ca_V1.1$ expression declines in old animals (Renganathan *et al.* 1997), we investigated whether $Ca_V\beta_{1a}$ might also change with age. Hindlimb muscles from FVB mice were harvested at 2, 6, 14, and 24 months of age and subjected to a membrane extraction protocol (see Methods) for use in western blot analyses. Gluteus and hamstring muscles (designated "pool") contain a mixture of type I (slow) and type II (fast) fiber types (Fig. 1A), while tibialis anterior (TA) and extensor digitorum longus (EDL) muscles contain only type II fibers (Manttari and Jarvilehto, 2005; Augusto *et al*., 2004) (Fig. 1B). Actin was used as a loading control as it appears to remain relatively stable throughout the lifespan of FVB mice. In order to precisely compensate for any variations in loading or possible global alterations in protein expression with aging, the optical density of each $Ca_V1.1$ band was divided by that of the corresponding actin band. The resulting values confirm a significant increase of $Ca_V\beta_{1a}$ with age. Relative $Ca_V\beta_{1a}$ expression increased continuously during aging, approximately doubling at each time point. This culminated in a substantial increase between very young and very old age (Fig. 1C) in both muscle groups, though the effect was less pronounced in the TA/EDL muscle groups (~7-fold), than in pool (~10-fold). The mean normalized intensity values (arbitrary units, AU) for each age group (n=4) are as follows: 0.20 ± 0.02 (2 months), 0.49 ± 0.06 (6 months), 1.14 ± 0.25 (14 months), and 2.34 ± 0.49 (24 months) for pool muscles and 0.29 ± 0.09 (2 months), 0.51 ± 0.1 (6 months), 0.84 ± 0.27 (14 months), and 1.81 ± 0.41 (24 months) for TA/EDL muscles.

Overexpression of $Ca_V\beta_{1a}$ results in decreased $Ca_V1.1$ expression

We next sought to determine if the observed increase in $Ca_V\beta_{1a}$ expression was directly involved in the age-related decline in $Ca_V1.1$. In order to separate the effects of $Ca_V\beta_{1a}$ overexpression from all other age-related changes, a $Ca_V\beta_{1a}$-YFP plasmid was electroporated into the TA muscle of young (4–5 month old) FVB mice *in vivo*. For these experiments we used TA because it is a superficial muscle suitable for electroporation *in vivo*, and large enough to provide tissue for protein analysis. Western blots of membrane fractions from electroporated muscles at 3 days (n=4), 7 days (n=3), and 2 weeks (n=4) confirm an increase in expression of endogenous $Ca_V\beta_{1a}$, which continued to rise during the two weeks following *in vivo* electroporation (Fig 2A). Conversely, $Ca_V1.1$ expression levels from the same samples declined steadily over the two week time course. Quantification of $Ca_V1.1$ band optical density shows an approximately 50% decrease compared to control at two weeks following $Ca_V\beta_{1a}$-YFP electroporation (Fig 2B). Sham electroporated muscles (n=4 for each time point) exhibited no significant changes in $Ca_V1.1$ or $Ca_V\beta_{1a}$ expression relative to non-electroporated controls. Additionally, quantitative real time RT-PCR was used to assess $Ca_V1.1$ transcript levels at 2 weeks after $Ca_V\beta_{1a}$-YFP electroporation (Fig 2C). No difference was seen in $Ca_V\beta_{1a}$-YFP electroporated (n=4) vs. controls (n=3), suggesting that $Ca_V1.1$ down regulation during $Ca_V\beta_{1a}$ overexpression occurs only at the protein level. These results are significant for two reasons. First, they show use of the $Ca_V\beta_{1a}$-YFP plasmid to artificially overexpress endogenous $Ca_V\beta_{1a}$ *in vivo*. Second, $Ca_V\beta_{1a}$ overexpression directly correlates to a decline in $Ca_V1.1$ protein levels in young mice, thus providing evidence for $Ca_V\beta_{1a}$ involvement in $Ca_V1.1$ down-regulation; namely when present in high levels such as those observed during senescence.

$Ca_V\beta_{1a}$ -YFP intensity corresponds to reduced charge movement

The intensity of YFP fluorescence (F, arbitrary units) varied between individual FDB fibers following *in vivo* electroporation with the $Ca_V\beta_{1a}$-YFP plasmid. Thus, the relative level of YFP intensity can be used to represent differences in individual fiber's $Ca_V\beta_{1a}$-YFP expression level. Similarly, charge movement (Q) is a measure of single cell $Ca_V1.1$ expression at the plasma membrane (Wang *et al.* 2000; for review, see Rios & Pizarro 1991). Maximal charge

movement (Q_{max}) was plotted against the corresponding fiber's YFP intensity (Fig 3A). The resulting plot shows an inverse relationship between $Ca_V\beta_{1a}$-YFP fluorescence level and $Ca_V1.1$ membrane expression (r=0.91, n=10), where electroporated fibers with high YFP fluorescence (F > 40 AU) show increasingly impaired Q_{max}. Fibers with relatively low YFP fluorescence (F ≤ 40 AU) are presumed to exhibit minimal overexpression of $Ca_V\beta_{1a}$ -YFP, and subsequently showed no loss of Q_{max}. Fibers from muscles electroporated with a GFP plasmid (n=10) showed no difference in Q_{max}, regardless of relative F intensity. Figure 3B and Figure 4 display an example of two fibers with different YFP intensity, and the resulting difference in Q. Fiber D0912, a fiber with lower $Ca_V\beta_{1a}$ -YFP expression (F=42 AU) exhibited unimpaired maximal charge movement (Q_{max} =52.6 nC/μF). Conversely, fiber C0925 showed high levels of $Ca_V\beta_{1a}$ -YFP (F=71.3 AU) and substantially reduced maximal charge movement (Q_{max} =26.6 nC/μF). While the absolute Q-V relationship was reduced in fiber C0925 and others showing high AU (Fig. 4D), the relative Q-V relationship was unchanged (Fig. 4E). Therefore overexpression of $Ca_V\beta_{1a}$ -YFP does not shift the Q-V curve in the voltage axis, reaffirming the notion that the effects of $Ca_V\beta_{1a}$-YFP are on $Ca_V1.1$ membrane expression and not other alterations of the channel's function.

$Ca_V\beta_{1a}$ -YFP causes a decline in specific force

Loss of muscle strength with aging is thought to be partially caused by reduction of $Ca_V1.1$, specifically those coupled to RyR in the T-tubule membrane, thereby resulting in an impairment of E-C coupling. As $Ca_V\beta_{1a}$ overexpression lead to a marked reduction $Ca_V1.1$, shown by both western blot and charge movement studies, it seemed likely that $Ca_V\beta_{1a}$ overexpression would also result in a loss of specific force. Tetanic specific force (kPa) was measured 14 days after single fibers from FDB muscles were electroporated *in vivo* with either $Ca_V\beta_{1a}$-YFP (n=7), GFP (n=7), or non-electroporated (n=3) (Fig 5A). The electroporation process itself causes short-term damage to the muscle fiber in the days immediately following the procedure (Schertzer *et al.* 2006) and as such the GFP group showed a significant reduction in specific force compared to controls. However, the $Ca_V\beta_{1a}$-YFP group showed an even greater and statistically significant decline in specific force compared to the YFP electroporated fibers. Figure 5B shows representative tetanic contraction traces from $Ca_V\beta_{1a}$-YFP, GFP, and control groups. These results support the idea that overexpression of $Ca_V\beta_{1a}$ impairs single fiber specific force by reducing the amount of $Ca_V1.1$ expressed at the plasma membrane. No significant difference was found on average time to peak torque (ms), or half relaxation time (ms), between control, GFP, and $Ca_V\beta_{1a}$-YFP electroporated groups (data not shown), indicating no alterations in calcium buffering activity or rate of cross bridge formation. Twitch specific force (kPA) appeared to decline in $Ca_V\beta_{1a}$-YFP electroporated fibers compared to GFP and non-electroporated controls, although the reduction did not reach significance (p=0.081).

siRNA inhibition of $Ca_V\beta_{1a}$

If overexpression of $Ca_V\beta_{1a}$ is responsible for muscle impairment with aging, then inhibition of $Ca_V\beta_{1a}$ may provide a way to reverse these effects. We created several siRNA sequences against $Ca_V\beta_{1a}$ (Fig 6A). The effectiveness of these sequences was determined by transfecting each of them into separate C2C12 cell cultures and performing western blot analyses on the harvested cell lysates. Each sample was probed for $Ca_V\beta_{1a}$, and the optical density of each band was compared to that of a control, untransfected sample. Clones 69051 and 69052 caused the greatest decline in $Ca_V\beta_{1a}$ expression (37% and 28% decline, respectively). Both of these clones were then electroporated *in vivo* into the FDB muscle of young and old FVB mice (Fig. 6B). In young animals, siRNA against $Ca_V\beta_{1a}$ caused a significant reduction of charge movement in dissociated FDB cells, recorded 7–11 days post electroporation. As demonstrated in Fig 3, electroporation of GFP alone does not result in a loss of charge movement. Old (24 month) mice naturally exhibit reduced charge movement, which was confirmed by our results.

Interestingly, siRNA against $Ca_V\beta_{1a}$ restored charge movement of old mice to young control levels. Sham electroporation of old muscles did not result in a further decline in charge movement, suggesting that the deleterious effect of electroporation on force reported above is due primarily to damage to contractile proteins and not due to reduced Cav1.1 expression.

DISCUSSION

Here we show that $Ca_V\beta_{1a}$ is significantly overexpressed in aging muscle, and present a model by which this phenomenon may contribute to loss of specific force with aging. In young mice, overexpression of $Ca_V\beta_{1a}$ corresponds to decreased $Ca_V1.1$ expression at the sarcolemma, as shown by both western blot and charge movement studies. $Ca_V\beta_{1a}$ overexpression also results in a loss maximal specific force, presumably by reducing the number of $Ca_V1.1$ channels in the t-tubule membrane coupled to RyRs. Because $Ca_V1.1$ subunits are critical for the transduction of sarcolemmal depolarizations into Ca^{2+} release from the SR, a decreased number of $Ca_V1.1$ subunits in the membrane would cause less Ca^{2+} to be released from intracellular stores, resulting in weakened contractions (Delbono, 2002). To further implicate overexpression of $Ca_V\beta_{1a}$ directly with decreased $Ca_V1.1$, we show that using siRNA to partially inhibit $Ca_V\beta_{1a}$ in old muscle restores charge movement to young control levels. Expectedly, inhibition of $Ca_V\beta_{1a}$ in young muscle fibers significantly reduced charge movement. Thus, both the under and overexpression of $Ca_V\beta_{1a}$ results in reduced expression of $Ca_V1.1$.

$Ca_V\beta_{1a}$ Overexpression with Aging

While the most striking increase in $Ca_V\beta_{1a}$ expression appears to be between middle aged and very old mice, the rate of $Ca_V\beta_{1a}$ increase is surprisingly uniform, with the normalized expression level roughly doubling at each time point. Relative $Ca_V\beta_{1a}$ level in very young mice is substantially lower than the level seen even at 6 months of age (young adulthood). Thus, increasing $Ca_V\beta_{1a}$ expression may also be a necessary component of muscle development. It is therefore tempting to speculate that the extreme levels of $Ca_V\beta_{1a}$ seen in very old animals may reflect a developmental program hyperfunction, a common attribute of aging (Blagosklonny, 2006). Interestingly, the rate of increase of $Ca_V\beta_{1a}$ expression did not seem to be as high in the TA/EDL muscle group compared to that of the Pool group. TA and EDL muscles are of exclusively type II composition, while there is a mixture of type I and type II fibers found in the gluteus and hamstring muscles used for our pool group. The possible resistance to $Ca_V\beta_{1a}$ overexpression seen in the type II muscle group is perplexing, as type I fibers are thought to be more resistant to age related changes such as denervation (for review, see Larsson, 1995). One possible explanation of this could be due to the increased cell turnover seen in type II fibers, as indicated by increased apoptotic signaling (Phillips and Leeuwenburgh, 2005) and depletion of satellite cells with age (Verdijk et al., 2007) compared to type I fibers, thus somewhat limiting the accumulation of $Ca_V\beta_{1a}$ with age. However, the mouse is not a preferred model to examine fiber type due to the lack of pure type I muscles as seen in the rat, and thus any conclusions on the fiber type-specific rate of $Ca_V\beta_{1a}$ increase are limited. Still, the notion that $Ca_V\beta_{1a}$ is highly overexpressed in both fiber types is strongly supported by these experiments.

The two most likely explanations of $Ca_V\beta_{1a}$ overexpression with age are an increase in its transcription levels, or a failure in its ability to be properly degraded. In regards to the latter, impairment of proteolysis is already known to be a hallmark of aging muscle (for review, see Combaret *et al.* 2009). Further experiments examining $Ca_V\beta_{1a}$ mRNA levels during aging should shed light on whether transcriptional upregulation plays any role. These explanations are not mutually exclusive and indeed it would seem probable that both contribute to the accumulation of $Ca_V\beta_{1a}$ in old muscle. Although we present a model by which $Ca_V\beta_{1a}$

overexpression causes deleterious effects on muscle function by reducing the number of voltage sensing $Ca_V1.1$ subunits in the t-tubule membrane, the relative expression of $Ca_V\beta_{1a}$ in old muscle is so high that it may also interfere with other key processes in a nonspecific manner. $Ca_V\beta$ subunits contain two conserved protein interaction domains: GK and SH3 (Chen *et al.*, 2004) and have recently been shown to interact with several other proteins besides Ca_V1, such as RGKs (kir/Gem, Rad, Rem), dynamin (for review, see Hidalgo and Neely, 2007), chromobox protein 2/heterochromatin protein 1γ (Hibino *et al.*, 2003), and Akt (Catalucci *et al.*, 2009). It is therefore possible that $Ca_V\beta_{1a}$ may interact with additional proteins in a currently uncharacterized manner.

Potential mechanisms of $Ca_V\beta_{1a}$ involvement in $Ca_V1.1$ down-regulation

The notion that $Ca_V1.1$ expression declines during old age is supported by several studies (Renganathan *et al.*1997; Ryan *et al.* 2000; Moreno *et al.* 2006; Wang *et al.* 2007; O'Connell *et al.* 2008). Importantly, $Ca_V1.1$ mRNA does not decrease significantly with age (Zheng *et al.* 2001), implicating some other mechanism responsible for the decline in its protein level. Here we demonstrate that $Ca_V1.1$ expression also declines following induced overexpression of $Ca_V\beta_{1a}$. Thus, $Ca_V\beta_{1a}$ overexpression, both during natural aging and experimental overexpression in young cells, coincides with a decline in $Ca_V1.1$ expression. While we present evidence that $Ca_V\beta_{1a}$ overexpression causes a decline of $Ca_V1.1$ at the protein level, the precise mechanism behind this occurrence is not known. $Ca_V1.1$ mRNA does not decline in young mice following $Ca_V\beta_{1a}$ overexpression, suggesting $Ca_V\beta_{1a}$ does not regulate $Ca_V1.1$ gene expression, in agreement with the aforementioned aging studies on $Ca_V1.1$ mRNA. Due to the multiple regulatory functions that $Ca_V\beta_{1a}$ exerts on $Ca_V1.1$, both classically and those discovered more recently, there are several hypothetical mechanisms by which overexpression of $Ca_V\beta_{1a}$ could lead to a reduction of $Ca_V1.1$. The two most important classical functions of $Ca_V\beta_{1a}$ in skeletal muscle are increasing the trafficking of newly formed $Ca_V1.1$ subunit to the t-tubule membrane (Neuhuber *et al.* 1998; Bichet *et al.* 2000), and arranging DHPRs into orthogonal arrays, or tetrads (Schredelseker *et al.* 2005), which are presumed to be necessary for skeletal muscle E-C coupling. Interfering with tetrad formation is one possible way that $Ca_V\beta_{1a}$ overexpression could reduce $Ca_V1.1$ insertion into the membrane. Tetrads are critical for the precise alignment of DHPRs with RyR, which is required for proper transduction during E-C coupling. Because $Ca_V\beta_{1a}$ also binds to RyR (Cheng *et al.* 2005), it seems possible that overexpression of $Ca_V\beta_{1a}$ could interfere with the alignment and coupling of DHPR tetrads to RyR. Also, our group in collaboration with others has found that $Ca_V\beta_{1a}$ interacts with the junctional protein JP-45 (Anderson *et al.* 2006). Like $Ca_V\beta_{1a}$, JP-45 is localized to the t-tubule/SR junction (triad) and interacts with $Ca_V1.1$ via the AID. In co-immunoprecipitation experiments, treatment with exogenous purified $Ca_V\beta_{1a}$ reduces the ability of JP-45 to pull down $Ca_V1.1$, suggesting $Ca_V\beta_{1a}$ interferes with this interaction. As JP-45 KO mice have impaired muscle strength due to reduced levels of $Ca_V1.1$ (Delbono *et al.* 2007), disruption of the JP-45-$Ca_V1.1$ interaction associated with excess $Ca_V\beta_{1a}$ is another possible mechanism by which $Ca_V\beta_{1a}$ overexpression reduces the level of $Ca_V1.1$ at the membrane. Disruption of the DHPR tetrad – RyR complex, through any of the mechanisms mentioned above, could in turn make $Ca_V1.1$ more susceptible to endocytosis and proteolytic degradation.

Much attention has been given recently to the involvement of the RGK family of Ras-related GTP-binding proteins in Ca_V1 channel function and expression. $Ca_V\beta$ isoforms are necessary for the RGK (kir/Gem) mediated down-regulation of $Ca_V1.2$ (Beguin *et al.* 2001), and $Ca_V\beta_{2a}$ forms a trimeric complex with the RGK Rem and the Ca_V1 AID (Finlin *et al.*, 2006). In skeletal muscle, overexpression of Rem leads to a decline in the number of $Ca_V1.1$ channels in the membrane (Bannister *et al.* 2008). Thus overexpression of $Ca_V\beta_{1a}$ may contribute to $Ca_V1.1$ down-regulation via interaction with RGKs, although the necessity for up-regulation of $Ca_V\beta_{1a}$ itself is unclear. Interestingly, Beguin and colleagues (2006) have recently shown

that the RGKs Rad and Rem may sequester $Ca_V\beta 3$ subunits into the nucleus in a calmodulin and 14-3-3 regulated manner. A previous study by Colecraft *et al.* (2002) also showed the presence of $Ca_V\beta_{1b}$, $Ca_V\beta_{2a}$, and $Ca_V\beta_4$ isoforms in the nucleus of cardiomyocytes. In additional to nuclear localization, Hibino *et al.* (2003) demonstrated that the $Ca_V\beta_4$ splice variant $Ca_V\beta_{4c}$ acts as a transcriptional regulator by binding to and inhibiting the gene silencing ability of the nuclear protein CHCB2/HP1γ. Although we found no influence of $Ca_V\beta_{1a}$ on $Ca_V1.1$ mRNA levels, based on these observations it is worth speculating on the possibility of $Ca_V\beta_{1a}$ acting as transcription factor, perhaps even in a self regulating fashion.

The GK domain of $Ca_V\beta$ subunits binds with high affinity to the AID of Ca_V1 channels (Chen *et al.*2004). While this is the consensus binding site of the classically defined Ca_V1- $Ca_V\beta$ complex, there is evidence that an additional $Ca_V\beta$ subunit can bind to a region on the C-terminus of Ca_V1 channels (in addition to the high affinity AID) and that this secondary binding modulates the functional properties of Ca_V1 (Qin *et al.* 1996; Birnbaumer *et al.* 1998; Gerster *et al.* 1999; Canti *et al.* 2001; García *et al.* 2005). The question of Ca_V1 containing multiple $Ca_V\beta$ binding sites is particularly relevant to our present results. Colecraft's group present model (Dalton *et al.* 2005) in which $Ca_V\beta$ initially traffics Ca_V1 to the plasma membrane, then either remains bound to the AID or dissociates, producing two populations of Cav1 with either high ($Ca_V\beta$ associated) or low ($Ca_V\beta$-less) gating activity. However Colecraft's model does not exclusively rule out the possibility of one or more additional binding sites on Ca_V1. The possibility of Ca_V1 containing multiple $Ca_V\beta$ binding sites of differing affinities offers a logical explanation for the pleiotropic effect of $Ca_V\beta_{1a}$ shown here. If $Ca_V\beta_{1a}$ is present in high concentrations (eg; exogenously applied or due to overexpression with aging), it may then be able to bind the proposed secondary, low affinity binding site with increasing frequency. In additional support of this hypothesis, the SH3 domain of $Ca_V\beta 2$ has recently been shown to interact with dynamin to promote endocytosis of $Ca_V1.2$ (Gonzalez-Guiterrez *et al*,. 2007). Interestingly, this group found that disruption of the AID was necessary for the $Ca_V\beta 2$-dynamin mediated endocytosis, further supporting the possibility that the presence of $Ca_V\beta$ at a secondary binding site results in Ca_V1 endocytosis. Alternatively, rather than directly mediating channel endocytosis via protein-protein interactions, $Ca_V\beta$ overexpression may alter Ca_V1 expression indirectly via Ca^{2+} dependent mechanisms. Garcia *et al.* (2005) showed that pressure injection of purified $Ca_V\beta_{1a}$ into dissociated muscle fibers produces a rapid increase in both L-type Ca^{2+} current and intracellular Ca^{2+} release, without altering charge movement. Endogenous overexpression of $Ca_V\beta_{1a}$ in the short term may produce similar physiological effects. In turn, a chronic increase in intracellular Ca^{2+} may activate some form of negative feedback, perhaps culminating in $Ca_V1.1$ endocytosis and proteolysis. Indeed, Sanchez's group has also shown that long-term increase of L-type Ca^{2+} current in skeletal muscle leads to proteolytic down-regulation of $Ca_V1.1$, likely via a local Ca^{2+}-dependent protease such as calpain (Carrillo *et al.* 2004).

Sarcopenia is a major cause of loss of independence in the elderly and presents a substantial public health cost (Janssen *et al.* 2004). As sarcopenia is caused in part by muscle dysfunction beyond the obvious loss in mass, E-C uncoupling may be a significant contributor to sarcopenia in aging humans. E-C uncoupling appears to be primarily caused by a decline in functional $Ca_V1.1$ subunits, which leads to an impairment of the mechanical coupling between neural signals and Ca^{2+} release necessary for contraction. Here we have shown that another protein, $Ca_V\beta_{1a}$, with several regulatory influences on $Ca_V1.1$, also exhibits significant changes in expression level with aging. Combined with evidence that $Ca_V\beta_{1a}$ overexpression causes $Ca_V1.1$ decline in young muscle, our findings present a potentially novel and physiologically significant contributing factor to the loss of skeletal muscle strength with aging.

Experimental Procedures

Animals

Muscles were dissected from FVB (Friend Virus B, our colony) mice between 1.5 and 24 months of age. FVB mice have a maximum lifespan of 25 months and have been used previously as a model of aging skeletal muscle in our laboratory (Renganathan *et al.* 1998; Payne *et al.* 2004). Animals were housed at Wake Forest University School of Medicine (WFUSM). Mice were killed by cervical dislocation. Animal handling and procedures were approved by the Animal Care and Use Committee of WFUSM.

Microsome preparation

Isolation of the t-tubule membrane was performed using a modified version of the protocol by (Knudson *et al.* 1989). Briefly, whole muscles were dissected and pulverized in liquid nitrogen and then homogenized in ice-cold Buffer A (20 mM sodium pyrophosphate, 20 mM sodium phosphate monobasic, 1 mM $MgCl_2$, 0.5 mM EDTA, 303 mM sucrose with complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) using a handheld tissue tearor. Homogenate was centrifuged at 7,000 g for 20 min at 4°C, and the pellet discarded. Supernatant was filtered through four layers of cheesecloth and centrifuged at 100,000 g for 90 min at 4° C in a Beckman Type Ti.70i rotor. The pellet was rinsed with ice cold PBS and resuspended with a glass homogenizer in fresh Digitonin buffer (1% digitonin (w/v), 185 mM KCl, 1.5 mM $CaCl_2$, 10 mM HEPES pH 7.4 with complete protease inhibitor cocktail). Samples were left on ice for 1h and then vortexed. Protein concentration was measured using bicinchoninic protein assay using BSA digitonin standards.

Antibodies

Primary antibodies used for immunoblots were monoclonal $VD2_1$ to $Ca_V\beta_{1a}$ (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), monoclonal IIF7 to $Ca_V1.1$; a generous gift from Dr. Kevin P. Campbell of the University of Iowa, and actin (Chemicon International, Temecula, CA, USA). NA931V goat anti-mouse (Amersham Health, Little Chalfont, Buckinghamshire, UK) was used as a secondary antibody.

Western blots

For $Ca_V\beta_{1a}$ subunit, microsomes were mixed with equal volume β-mercaptoethanol buffer and boiled for 5 minutes. For $Ca_V1.1$, microsomes were mixed with equal volume double strength Urea buffer and incubated at room temperature for 30 min (Murray & Ohlendieck 1998).SDS-PAGE was conducted using a 4.5 % stacking gel with a 10% resolving gel in a Mini-Protean gel system (BioRad Laboratories, Hemel-Hemptstead, Herts., UK). Gels were transferred to PVDF membranes (Amersham Health, Little Chalfont, Buckinghamshire, UK) overnight at 4° C. Blots were blocked in 5% non-fat dry milk with 0.1% Tween in TBS for $Ca_V\beta_{1a}$ and PBS for all other antibodies. Primary antibody concentrations were as follows: 1:1000 ($Ca_V\beta_{1a}$), 1:5000 ($Ca_V1.1$) and 1:250,000 for actin. Secondary antibodies were used at a 1:5000 dilution. Band intensity was measured using Kodak Gel Doc imaging system.

Quantitative Real Time RT-PCR

Total RNA was isolated using TriReagent according to the manufacturers protocol (Molecular Research Center, Cincinnati, OH), treated with DNAse I (New England Biolabs, Ipswich, MA) and reverse transcribed to cDNA using random hexamers and reverse transcriptase (Promega, Madison, WN) according to manufacturers protocol. Following reverse transcription, cDNA expression was assessed by quantitative real time RT-PCR (Taqman Gene Expression Master Mix kit, Applied Biosystems, Foster City, CA, USA) on a Stratagene MX3000P. Primers and probes for $Ca_V1.1$ were purchased from Applied Biosystems.

Muscle Electroporation

Intramuscular plasmid injection and electroporation were performed according to Schertzer *et al.* (2006) and DiFranco *et al.* (2006). Briefly, FDB or TA muscles were injected with 30 Al of 0.5 U/Al hyaluronidase and injected 1 hr later with 20 μg $Ca_V\beta_{1a}$–YFP or 20 μg $Ca_V\beta_{1a}$ siRNA equal volume saline solution. A pair of platinum plate electrodes was placed under the skin on adjacent sides of the muscle. Eight, 150 V, 20-ms square-wave pulses of 1-Hz frequency were generated using a Grass stimulator (Grass S48; W. Warwick, RI, USA) and delivered to the muscle. The polarity was then reversed and a further 8 pulses were delivered to the muscle. For sham electroporations, the same protocol was followed with a saline-only injection.

siRNA sequences

C2C12 cells were transfected with five siRNA sequences (Open Biosystems, Huntsville, AL, USA) using FuGENE 6 (Roche Diagnostics, Indianapolis, IN, USA). The source of shRNA has accession # NM_031173. The following five siRNA sequences were used:

Seq. 69048:
CCGGCCAGTGGTAATGAAATGACTACTCGAGTAGTCATTTCATTACCACTGG
TTTTTG

Seq. 69049:
CCGGCCCAGCAAACACATCATCATTCTCGAGAATGATGATGTGTTTGCTGGG
TTTTTG

Seq. 69050:
CCGGCGAGGGAAGTCTCAATCCAAACTCGAGTTTGGATTGAGACTTCCCTC
GTTTTTG

Seq. 69051:
CCGGCCTCGGATACAACATCCAACACTCGAGTGTTGGATGTTGTATCCGAG
GTTTTTG

Seq. 69052:
CCGGGCTCAGGAGAAATCTCAGCTTCTCGAGAAGCTGAGATTTCTCCTGAG
CTTTTTG

Charge movement recordings

Enzymatically dissociated flexor digitorum brevis (FDB) fibers were transferred to a small, flow-through Lucite chamber positioned on a microscope stage. Fibers were continuously perfused with the external solution using a push-pull syringe pump (WPI). Only fibers exhibiting a clean surface and no contracture were used for electrophysiological recordings. Muscle fibers were voltage-clamped using an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981; Wang *et al.* 1999). Patch pipettes were pulled from borosilicate glass (Boralex, WPI, Sarasota, FL, USA) using a Flaming Brown micropipette puller (P97, Sutter Instrument Co., Novato, CA, USA) and then fire-polished to obtain electrode resistances ranging from 450 to 650 kΩ. In the cell-attached configuration, the seal resistance was in the range of 1–4.5 GΩ, and in the whole-cell configuration, values ranged between 75 and 120 MΩ (Wang *et al.* 1999). The pipette was filled with the following solution: 140 mM Cs-aspartate, 5 mM Mg-$aspartate_2$, 20 mM Cs_2EGTA (ethylene glycol-bis(aminoethyl ether)-N,N,N’N’-tetraacetic acid), and 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), and pH was adjusted to 7.4 with CsOH (Adams *et al.*, 1990; Wang *et al.*, 1999). The external solution contained: 150 mM TEA(tetraethylammonium hydroxide)-CH_3SO_3, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Na-HEPES, 0.05 mM BTS(N-benzyl-p-toluenesulfonamide), and 0.001 mM

tetrodotoxin (Delbono 1992; Delbono *et al.* 1997). Solution pH was adjusted to 7.4 with CsOH. All the experiments were conducted at room temperature (21–22°C).

Confocal microscopy

FDB fibers fluorescence was analyzed using a Radiance 2100 confocal microscope (Bio-Rad/Zeiss, Thornwood, NY, USA). YFP fluorescence was detected at 488nm. Confocal microscope fluorescence acquisition parameters were maintained constant across recordings are described above.

Single Intact Muscle Fiber Contraction

At time of sacrifice, FDB muscles were carefully dissected and pinned into a Petri dish lined with Sylgard (Dow Corning, Auburn, MI, USA) in a Ca^{2+}-containing physiological solution (see below). All contraction experiments were carried out at room temperature (21–22°C). Single intact fiber dissection followed procedures previously published (Lannergren & Westerblad 1987; Gonzalez *et al.* 2000). Following dissection, tendons of single intact fibers were placed in custom-made micro-clips, and these clips were connected to a force transducer and a micropositioner for length control. Fibers were adjusted to optimum length (L_O) by using single twitches, elicited by 0.5-ms square wave pulses at 10 V. Once at L_O, fibers were stimulated with 350-ms trains of pulses, using frequencies varying from 50 to 100 Hz. The stimulation frequency that elicited maximum force was used for the remainder of the experiment (Payne *et al.* 2004).

Statistical analysis

All data are presented as means ± SE. Data were analyzed with *Student* t-test or one-way repeated measures ANOVA, with Tukey's multiple comparisons test applied *post hoc* when appropriate. An alpha value of $P < 0.05$ was considered significant.

ACKNOWLEDGMENTS

The present study was supported by grants to Osvaldo Delbono from the National Institutes of Health/National Institute on Aging (AG13934, AG033385, and AG15820), the Muscular Dystrophy Association (MDA #33149), and the Wake Forest University Claude D. Pepper Older Americans Independence Center (P30-AG21332). The authors would like to thank Dr. Kurt G. Beam (University of Colorado Health Sciences, Department of Physiology and Biophysics) for providing the $Ca_V\beta_{1a}$–YFP plasmid, Dr. Franz Hofmann (University of Saarland, Pharmacology and Toxicology) for the initial cDNA sequence of $Ca_V\beta_{1a}$, and Dr. Hang Shi and Dr. Juan Codina for their expert assistance with the qRT-PCR experiments.

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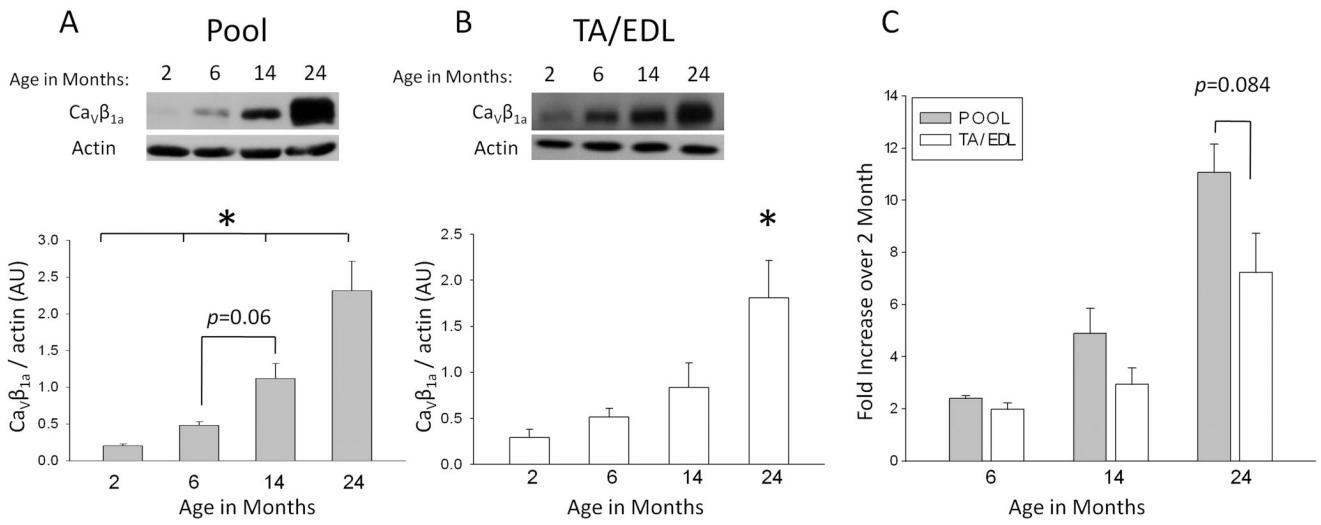

Fig. 1. Relative $Ca_V\beta_{1a}$ expression across the lifespan of FVB mice

(A) Representative immunoblot of $Ca_V\beta_{1a}$ (52 kDa) expression in P100 fractions collected from gluteus and hamstring muscles (pool) at 2, 6, 14, and 24 months of age (24 months represents the very old age of this strain). Below is the mean $Ca_V\beta_{1a}$ band intensity for each age group (n=4), normalized to the intensity of the corresponding actin band. Each age group reached a statistically significant difference from all of the others, with the exception of 6 and 14 month age groups. Data represent mean ± SE. *$P \leq 0.01$ (one-way ANOVA). (B) The same as A, except representative of TA and EDL muscle groups (n=4). The 24 month age group reached significant difference from all other ages. *$P < 0.01$(one-way ANOVA). (C) Mean fold increase of pool and TA/EDL samples by age. Values represent normalized sample values from each age group divided by the corresponding 2 month normalized value.

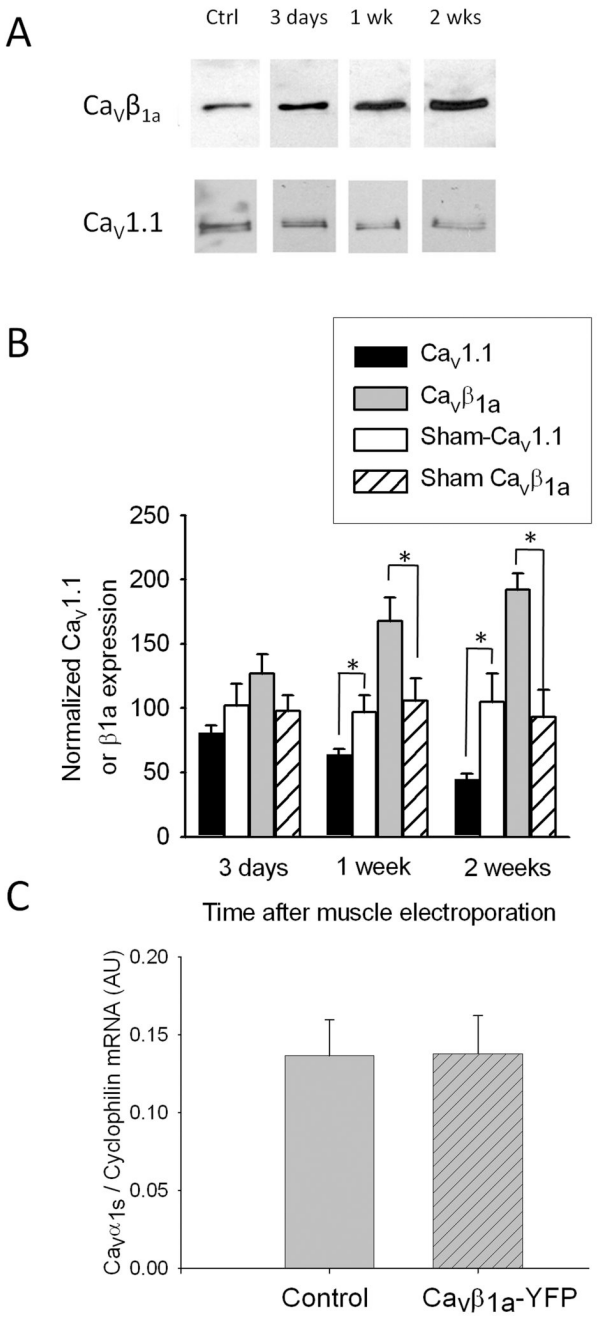

Fig. 2. $Ca_V1.1$ and $Ca_V\beta_{1a}$ expression in TA muscles following *in vivo* electroporation of a $Ca_V\beta_{1a}$-YFP plasmid

(A) Representative immunoblots from control muscle (lane 1) and from muscles harvested 3 days (lane 2), 1 week (lane 3), and 2 weeks (lane 4) post-electroporation. (B) $Ca_V1.1$ and endogenous $Ca_V\beta_{1a}$ protein expression in muscle following $Ca_V\beta_{1a}$-YFP electroporation (n=4 for 3 days and 2 weeks, n=3 at 1 week), normalized to control (non-electroporated) muscle. Sham electroporation (n=4 at each time point) produced no significant difference in relative $Ca_V1.1$ or $Ca_V\beta_{1a}$ expression. Data represent mean ± SE. *P <0.001 (one-way ANOVA). (C) $Ca_V1.1$ mRNA levels measured using qRT-PCR in control (non electroporated, n=3) and $Ca_V\beta_{1a}$-YFP electroporated (2 weeks, n=4) animals.

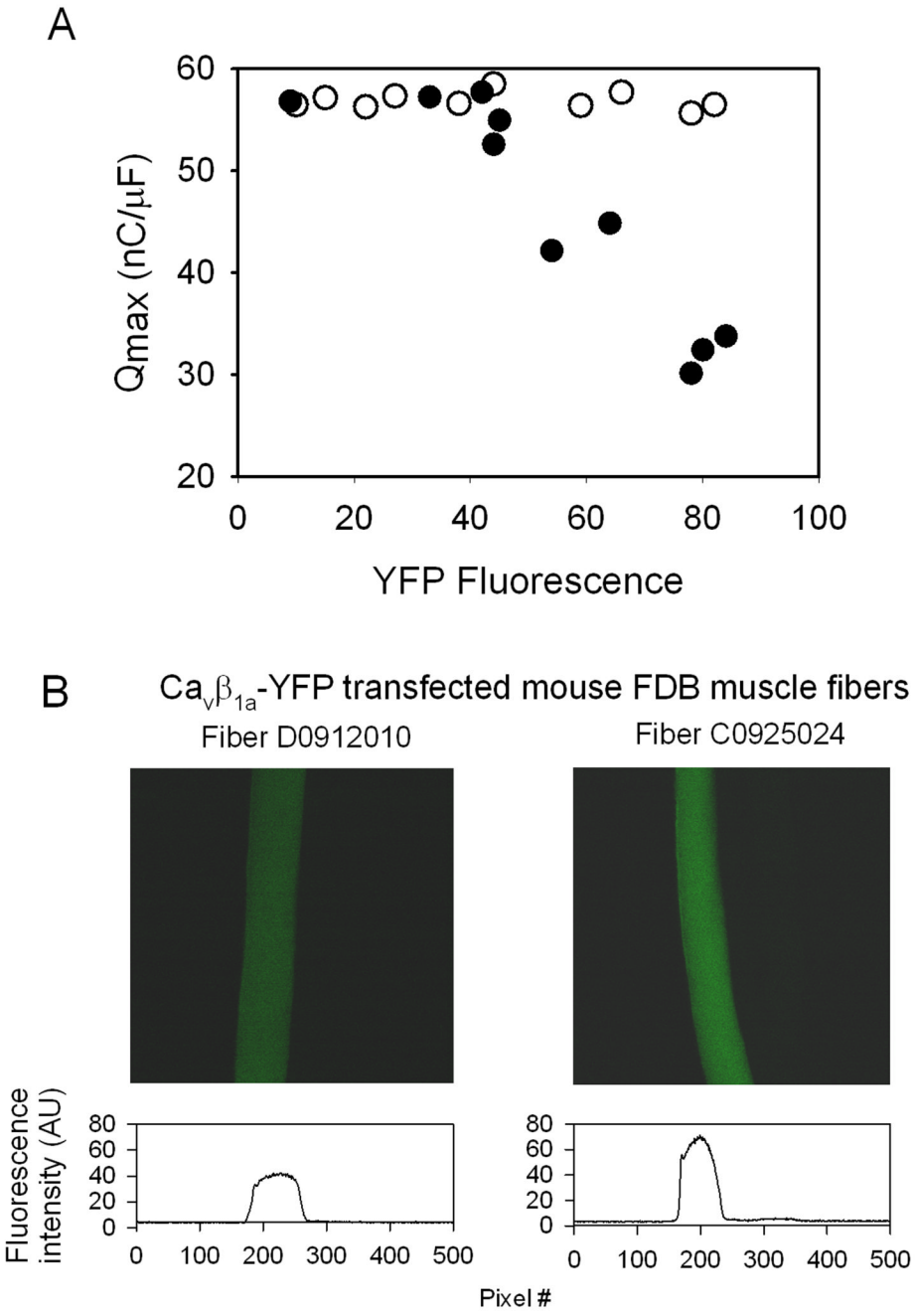

Fig. 3. $Ca_V1.1$ charge movement (Q) and YFP ($Ca_V\beta_{1a}$ -YFP) fluorescence (F) in electroporated FDB fibers

(A) Q_{max}-F relationship of single fibers electroporated with $Ca_V\beta_{1a}$ –YFP (●)(n=10) or GFP (○)(n=10). Detection of F above ~40 AU correlates with decreased charge movement (r^2=0.91). (B) Top - two representative fibers from A. Fiber D0912 exhibits F=42 AU, Q=52.6 nC/µF. Fiber C0925 exhibits F=71.3 AU, Q=26.6 nC/µF. (Also see Fig. 4 for more detail on these two fibers). Fluorescence acquisition parameters on confocal microscope: 20x objective, zoom = 3.5x, Iris = 3.8mm, Gain = 80, box size = 512×512 pixels (172.6×172.6 µm), pixel dwell time 37.76 µs. Excitation wavelength = 488 nm, Emission = 528 nm. Bottom – Illustration of the fluorescence intensity across the fibers.

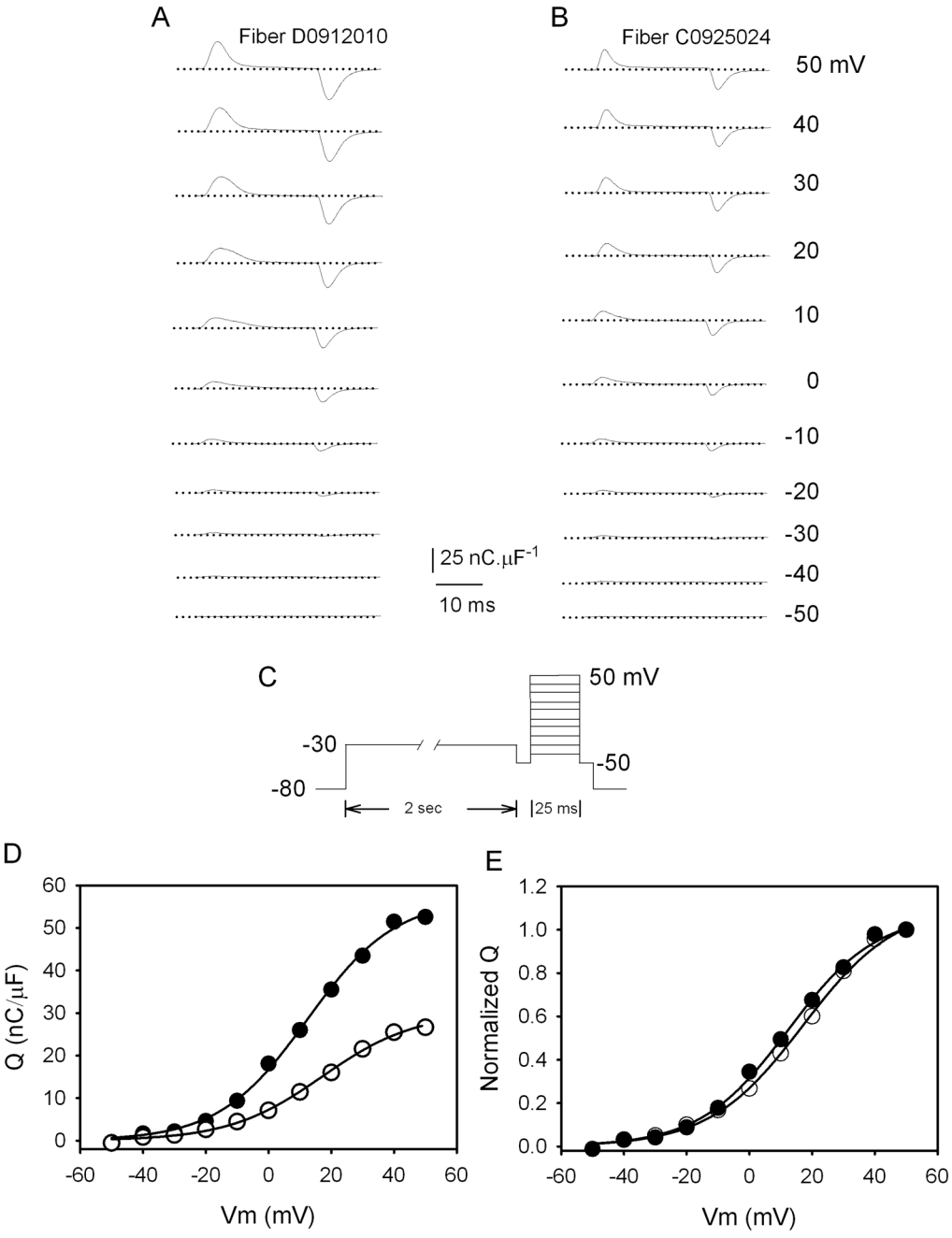

Fig. 4. Charge movement from FDB fibers expressing $Ca_V\beta_{1a}$-YFP

(A – B) Charge movement recordings from fiber D0912 (A), representing a fiber expressing low levels of $Ca_V\beta_{1a}$ -YFP (indicated by F intensity in Figure 3), and from fiber C0925 (B), representing a fiber expressing high levels of $Ca_V\beta_{1a}$-YFP (indicated by F intensity in Figure 3). (C) The voltage step protocol. The holding potential was −80 mV. A 2-sec pre-pulse at −30 mV was followed by a 15-ms repolarization to −50 mV, followed by a 25-ms test pulse of varying membrane voltage and repolarization to −50 mV. (D) Absolute Q-V relationship of fibers D0912 (●) and C0925 (○). Note that the fiber which expresses higher levels of $Ca_V\beta_{1a}$-YFP displays lower charge movement. (E) Relative Q-V relationship of the same fibers in D. Q values were normalized to Q_{max} for each fiber. Overexpression of $Ca_V\beta_{1a}$ -YFP does not

shift the Q-V curve in the voltage axis or modify the steepness of the curve. Data points were fitted to a Boltzmann equation of the form: $Q = Q_{max}/ [1+ \exp(V_{1/2} - V_m)/k]$, where Q_{max} is the maximal charge; $V_{1/2}$ is the charge half-activation potential; V_m is the membrane potential; and k is the steepness of the curve. Q_{max}, $V_{1/2}$, and K values were: 56.9 nC/μF, 12.3 mV and 3.37 for fiber D0912, and 30.2 nC/μF, 17.1 mV, and 14.9 for fiber C0925.

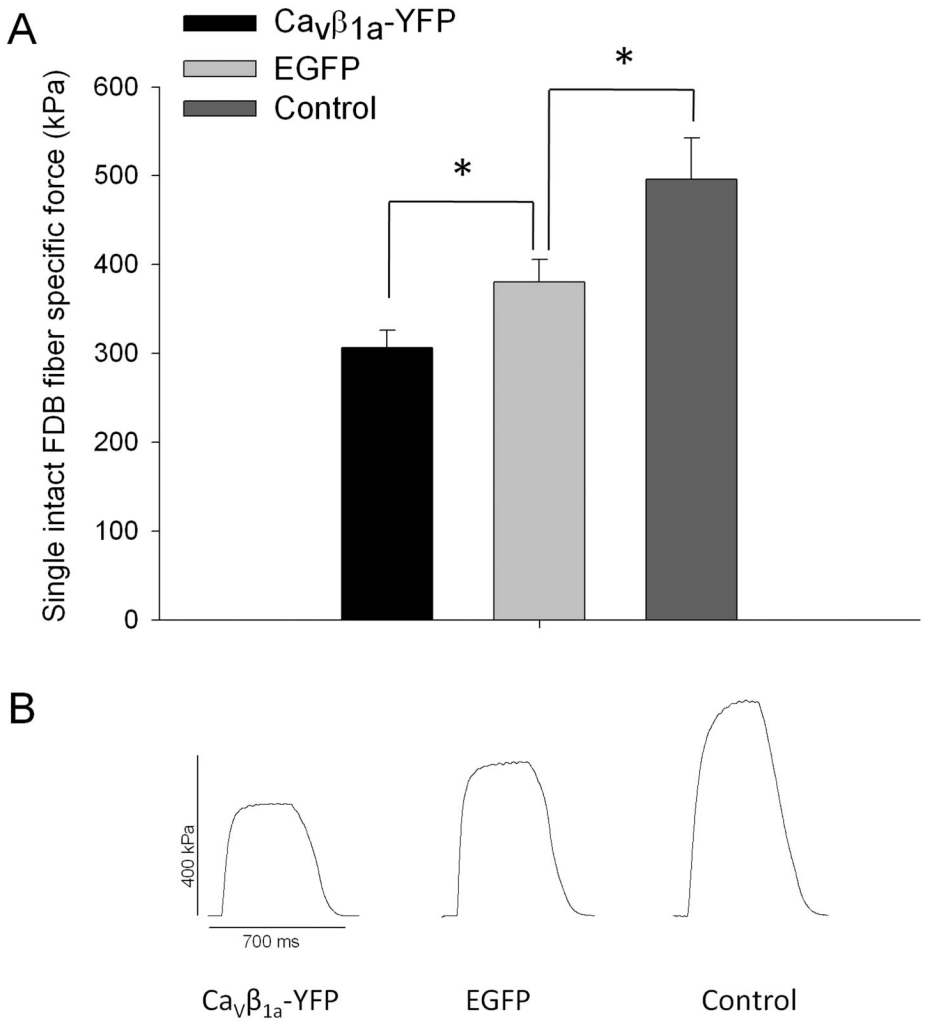

Fig. 5. Specific force of single intact FDB fibers from young mice electroporated and expressing Ca$_V$β$_{1a}$-YFP or GFP

(A) Specific force (kPa) of single intact FDB fibers from Ca$_V$β$_{1a}$-YFP (n=7), GFP (n=7) and non-electroporated controls (n=3). Ca$_V$β$_{1a}$-YFP expressing fibers display significantly lower specific force compared to GFP expressing fibers. Data represent mean ± SE. *P<0.05 (one-way ANOVA) (B) Representative tetanic contraction traces from fibers expressing Ca$_V$β$_{1a}$–YFP, GFP, and control groups.

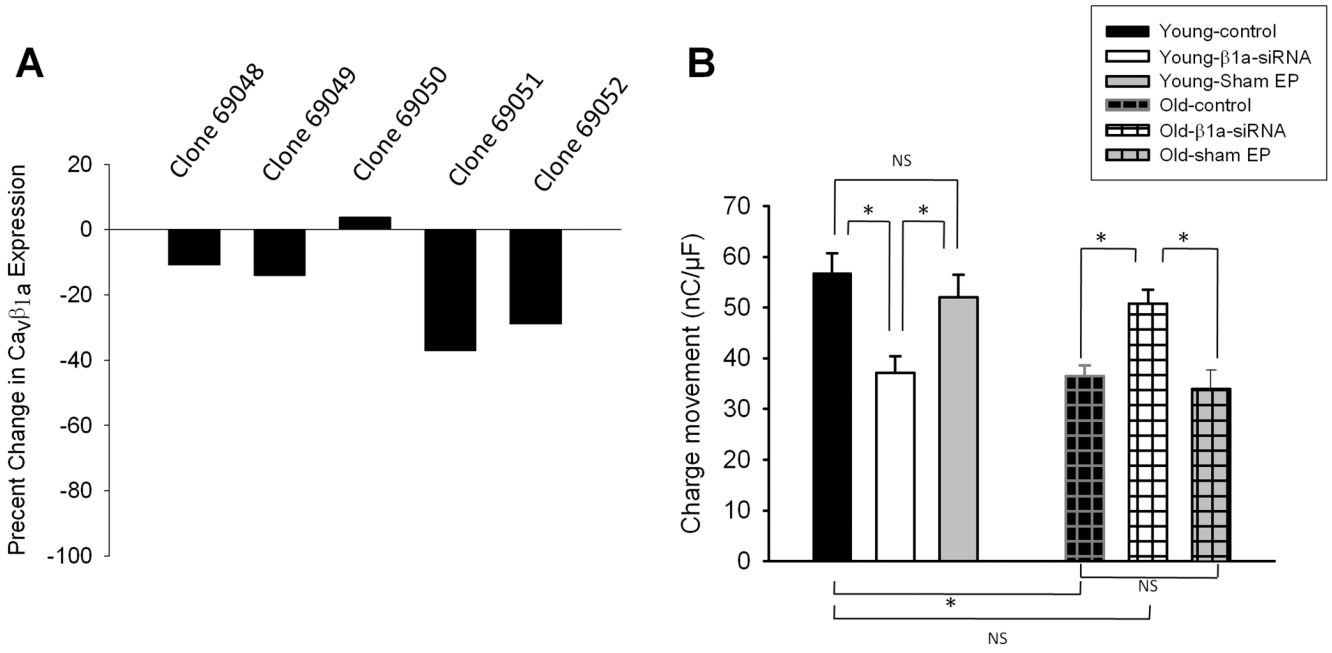

Fig. 6. siRNA restoration of charge movement *in vivo*

(A) Effect of five different siRNA sequences on $Ca_V\beta_{1a}$ expression in C2C12 myotubes. Data are presented as percent change in immunoblot band intensity versus control. Clones 69051 and 69052 cause the greatest decline in endogenous $Ca_V\beta_{1a}$ expression (37% and 28% decline, respectively). (B) Charge movement from FDB muscle fibers 7–11 days post-electroporation. FDB muscles were electroporated with 20 μg of siRNA sequence 69051 and 10 μg of siRNA sequence 69052. Fibers were dissociated, patch-clamped, and subjected to the same protocol as in Figure 4. The number of fibers examined for young control, sham GFP-electroporated and $Ca_V\beta_{1a}$ siRNA electroporated was 13, 10, and 17, respectively, and for old control, $Ca_V\beta_{1a}$ siRNA and sham electroporated was 21, 16 and 11, respectively. Data represent mean ± SE. *P < 0.05 (one-way ANOVA).