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Calcineurin and skeletal muscle growth

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Recruitment determines the profile of fibre-type-specific genes expressed across the range of muscle fibres associated with slow, fast fatigue-resistant and fast fatiguable motor units. Downstream signalling pathways activated by neural signalling and mechanical load have been the focus of intensive research in past years. It is now known that Ca^{2+}-dependent calcineurin–nuclear factor of activated T cells and insulin-like growth factor 1 pathways and their downstream mediators contribute to these adaptive responses. These pathways regulate gene expression through muscle-specific (myocyte-enhancing factor 2, myoblast determination protein) and non-specific (nuclear factor of activated T cell 2, GATA-2) transcription factors. Transcriptional signals activated with increased contractile activity result in altered expression of fibre-type specific genes, including the myosin heavy chain isoforms and oxidative and glycolytic enzymes and a net change in muscle fibre-type composition. In contrast, transcriptional signals activated by increased load bearing result in hypertrophy or a growth response, a component of which involves satellite cell recruitment and fusion with existing adult myofibres. Calcineurin has been identified as a key mediator in the hypertrophic response, and the current challenge has been to determine the downstream target genes of this pathway. Exciting new data have emerged, showing that myostatin, a negative regulator of muscle growth, and utrophin, a cytoskeletal protein important in maintaining membrane integrity, are downstream targets of calcineurin signalling. Increased understanding of these mediators of muscle growth may provide strategies for the development of effective therapeutics to counter muscle weakness and muscular dystrophy.

Skeletal muscle hypertrophy: Myostatin: Nuclear factor of activated T cell and myocyte-enhancing factor 2 signalling

Although the contractile and metabolic phenotype of a muscle fibre is genetically pre-determined and modelled by developmental surges in thyroid hormone and other endocrine signals, it remains highly plastic in the adult mammal and can be adjusted to match the functional demands placed on it by the central nervous system. The diversity in muscle fibre expression and accumulation of contractile proteins in adult mammals appears to be, in large part, directed by neural activity. Depolarization of the sarcolemma in response to motor neuron firing triggers a release of Ca^{2+} from the sarcoplasmic reticulum and subsequent contraction of the muscle fibre. The amount and pattern of nerve-mediated depolarizations have a large influence on the expression and accumulation of contractile proteins within skeletal muscle fibres. It is well known that when muscles are denervated and then activated with patterns of electrical stimuli modelled after non-native nerve discharges they will convert to a phenotype consistent with the new neural activation profile. Thus, with appropriate stimuli, slow-twitch muscles can adapt to display a faster myosin heavy chain (Mhc) profile and fast-twitch muscles can switch over to a slower Mhc phenotype (Ausoni *et al*. 1990; Dunn & Michel, 1997, 1999). The fact that similar transformations in contractile proteins can be induced in cultured skeletal myotubes by modulating intracellular Ca^{2+} levels (Meissner *et al*. 2001) suggests the nerve may direct muscle-fibre phenotype via the fluctuations in Ca^{2+} that it evokes in these cells. Over the past few years some of the cellular and molecular factors that mediate these specific fibre responses to nerve-mediated depolarizations have been identified. Specifically, it has been shown that calcineurin (Cn), a Ca^{2+}-regulated phosphatase,

Abbreviations: CaM, calmodulin; Cn, calcineurin; CsA, cyclosporine A; IGF-1, insulin-like growth factor 1; MEF2, myocyte-enhancing factor 2; Mhc, myosin heavy chain; NFAT, nuclear factor of activated T cells.

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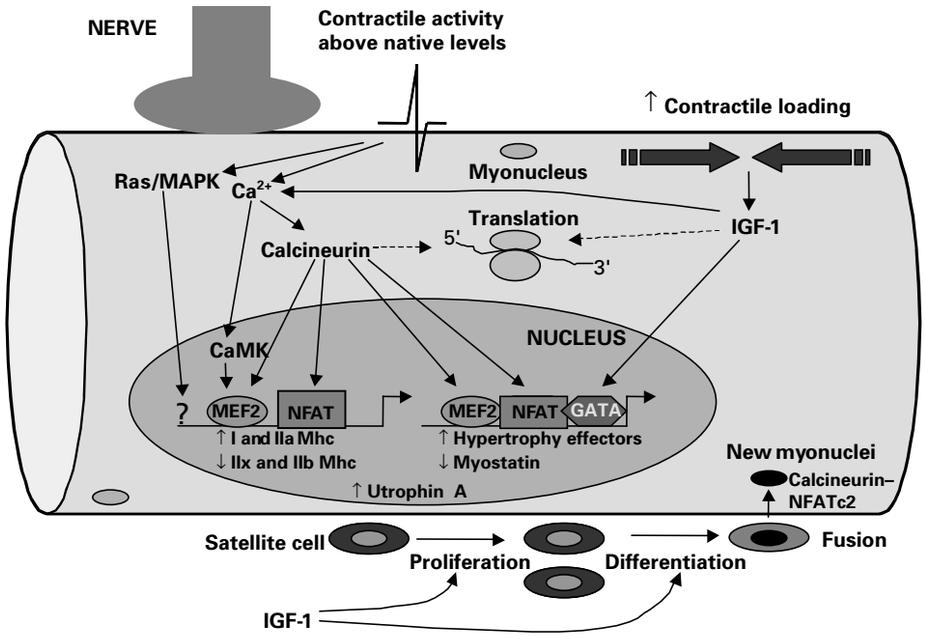

Fig. 1. Overview of muscle signalling mechanisms. Neural activation and mechanical loading of the muscle activate numerous intracellular signals that result in hypertrophic growth of the muscle. Neural recruitment results in sarcolemmal membrane depolarizations and subsequent increases in intracellular Ca^{2+} levels, with the resultant intracellular Ca^{2+} concentration dependent on the stimulation frequency. This increase in intracellular Ca^{2+} can activate a number of transcriptional pathways including the Ca^{2+}/calmodulin-dependent phosphatase calcineurin and Ca^{2+}/calmodulin-dependent kinase (CaMK) pathways. Calcineurin and its downstream transcription factor nuclear factor of activated T cells (NFAT) play an important role in the activation of type I and IIa myosin heavy chain (Mhc), oxidative enzyme and utrophin A genes as well as in the hypertrophy response through decreases in myostatin expression and altered expression of other unknown targets. Calcineurin also acts through myocyte-enhancing factor 2 (MEF2)-dependent transcription. In parallel with the calcineurin–NFAT and calcineurin–MEF2 signals, other intracellular signals are activated to effect growth. These signals are triggered by membrane depolarizations, contractile loading or the release of growth factors, such as insulin-like growth factor 1 (IGF-1), which activate hypertrophic responses through Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase and Akt/protein kinase B. One of the downstream effects of calcineurin and IGF-1 is activation of satellite cell proliferation and fusion with existing myofibres, which contributes, in part, to the resultant hypertrophy. ↑, ↓, Increase and decrease respectively; ⟵, established pathways; ----▸, possible pathways.

is a crucial modulator of skeletal muscle hypertrophy of all fibre types, fast or slow, *in vivo* (Dunn *et al*. 1999) and that Cn regulates fibre-type-specific genes (Chin *et al*. 1998; Dunn *et al*. 1999). In addition to the neural-evoked signals, increased muscle loading can also activate the release of various growth factors, including insulin-like growth factor 1 (IGF-1), which signals through both Cn-dependent and Cn-independent pathways (see Fig. 1) to induce muscle hypertrophy. The present paper will review both Cn-dependent and Cn-independent mechanisms of skeletal muscle growth and the downstream targets of Cn-dependent signalling.

Calcineurin regulation of skeletal muscle growth

The Ca^{2+}/calmodulin (CaM)-dependent phosphatase Cn (or protein phosphatase-2B) has recently come to the forefront as a molecular translator of low-amplitude sustained Ca^{2+} oscillations in skeletal muscle cells, as with other cell types (Bito *et al*. 1996; Dolmetsch *et al*. 1997; Olson & Williams, 2000*a*,*b*). Initial data implicating Cn in such a role have been provided in lymphocytes in which Cn signals to its target transcription factor, nuclear factor of activated T cells (NFAT), in response to low-amplitude prolonged-duration intracellular Ca^{2+} transients, such as those that occur in response to antigenic stimulation (Dolmetsch *et al*. 1997). Cn–NFAT has been shown to discriminate amongst different patterns of electrical stimuli in skeletal muscle cells (Liu *et al*. 2001). Activation of Cn in response to prolonged contractile work or during muscle fibre maturation is crucial to signalling the expression of slower more oxidative fibre-specific genes (Serrano *et al*. 2001) and skeletal muscle growth (Dunn *et al*. 1999; Mitchell *et al*. 2002). The *in vivo* administration of Cn inhibitors, cyclosporine A (CsA) or FK506, blocks muscle fibre hypertrophy in response to functional overload (Dunn *et al*. 1999) and hinders the recovery of muscle fibre size after unweighting-induced atrophy (Mitchell *et al*. 2002).

Additionally, administration of these Cn inhibitors induces slow-to-fast phenotype transformations in the normal weight-bearing or regenerating soleus (Chin *et al*. 1998; Bigard *et al*. 2000; Serrano *et al*. 2001) and prevents type IIb→IIx→IIa→I Mhc transitions in the functionally-overloaded plantaris (Dunn *et al*. 1999).

Cn-dependent signalling events are required for growth to occur in all (i.e. fast and slow) fibre types. This requirement was initially shown by studies in which the Cn inhibitors CsA or FK506 were administered to mice or rats at a dosage (25 mg/kg) that reduces Cn activity in skeletal muscles by ≥ 50%. This treatment prevents hypertrophy of all fibre types in the plantaris as well as fibre type transformations in response to functional overload (Dunn *et al*. 1999, 2000) without affecting animal health or ambulation (Dunn *et al*. 2002). A role for CaM-dependent signalling via Cn in the regulation of growth has also been identified. Overexpression of a binding peptide that inhibits CaM from signalling to target enzymes (CaM inhibitory peptide) mimics the effects of Cn inhibitors and blocks growth of overloaded muscle fibres that express this transgene (Dunn *et al*. 2000). This molecular-based evidence supports the pharmacological data and collectively provides convincing evidence for the role of Ca^{2+} and Cn–NFAT signalling in the hypertrophic response related to overload.

Controversy in the literature concerning the role of Cn-dependent signalling pathways in adaptive growth appears to be related to the dose dependency of CsA action (Dunn *et al*. 2002). CsA regimens using lower dosages (i.e. 5–10 mg/kg) and frequencies (once daily) of drug administration result in less-marked inhibition of Cn activity (i.e. ≤20%; Dunn *et al*. 2002; Fig. 2). These lower doses of CsA do not prevent muscle fibre hypertrophy in overloaded or regenerating rat skeletal muscles (Bodine *et al*. 2001; Serrano *et al*. 2001), although they are effective in inducing fibre phenotype transformations in the rat soleus (Chin *et al*. 1998; Bigard *et al*. 2000; Serrano *et al*. 2001). This CsA dose dependency has recently been confirmed in overloaded muscles (SE Dunn and RN Michel, unpublished results), showing clearly that 25 mg/kg twice daily effectively blocks hypertrophy of all plantaris fibre types whereas a dose of 10 mg/kg per d does not (see Fig. 2(a and b)). The fact that fibre hypertrophy is not prevented when Cn activity is only modestly compromised suggests that parallel signalling pathways co-regulate growth and can compensate to induce hypertrophy under these conditions. Consistent with this notion, protein levels of the growth effector IGF-1, as well as muscle regulatory genes and transcription factor proteins targeted by IGF-1 signalling such as myogenin (Adi *et al*. 2000) and GATA-2 (Musaro *et al*. 1999, 2001), are up regulated in a compensatory fashion in overloaded muscles when Cn activity is compromised with CsA (Dunn *et al*. 2000; Fig. 3). The requirement of accessory parallel signalling pathways for growth is further supported by the finding that overexpression of constitutively-active Cn in Tg mice does not induce skeletal muscle hypertrophy in normal weight-bearing animals (Dunn *et al*. 2000; Naya *et al*. 2000), nor does it potentiate fibre growth in response to overload (Dunn *et al*. 2000).

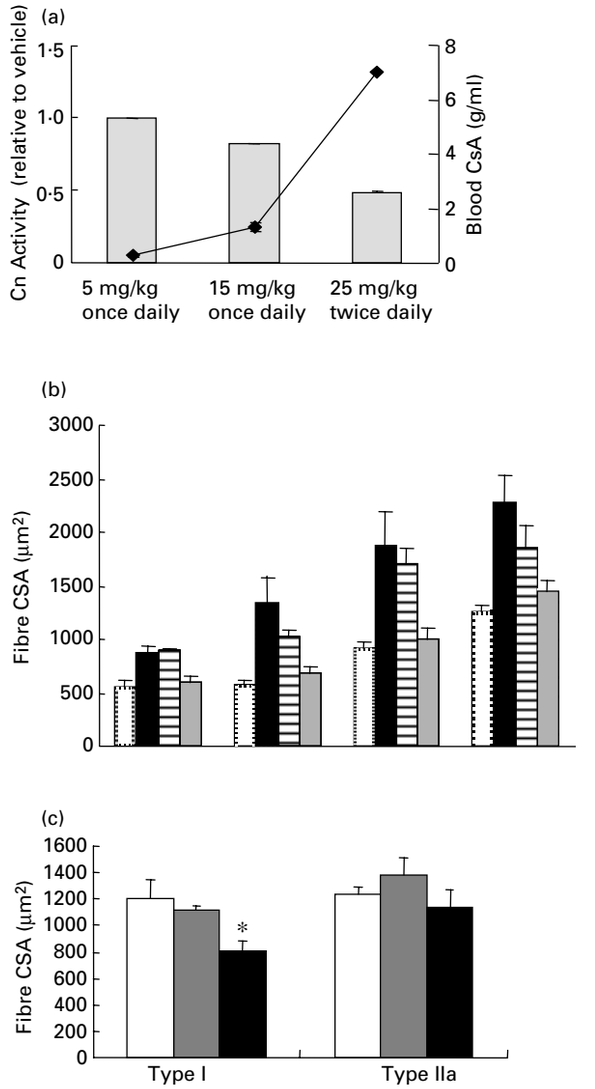

Fig. 2. Dose-dependent inhibition of compensatory overload and normal weight-bearing responses with calcineurin (Cn) and calmodulin inhibitors. (a) CD-1 mice were administered cyclosporin A (CsA) at 5 or 15 mg/kg per d or 25 mg/kg twice daily, resulting in dose-dependent increases in blood CsA level (◆–◆) and decreases in muscle Cn activity (□). (b) Overload hypertrophy of the plantaris muscle following removal of the soleus and gastrocnemius muscles (Ov) resulted in increased fibre cross-sectional area (CSA) of muscle fibres of all types (Ov–vehicle-treated (■) *v.* sham-operated–vehicle-treated (□)). This effect was inhibited by CsA treatment at 25 mg/kg twice daily (▩) but not at 10 mg/kg per d (▤). (c) Regenerating muscle fibre CSA in normal weight-bearing animals was reduced in type I but not in type IIa fibres from the 5229 line of transgenic mice (■) that overexpress a calmodulin inhibitory peptide (CaMBP) driven by the troponin I slow promoter, which limits expression to type I fibres. Wild-type mice (□) and transgenic mice from the 6444 line (▩), which have lower expression of the CaMBP transgene, had higher type I CSA. Values are means with their standard errors represented by vertical bars. Mean value was significantly lower than that for the other two groups: $^*P < 0{\cdot}05$.

Cn-dependent signalling pathways may also play a role in the maintenance of fibre size in specific muscles under normal weight-bearing conditions. Administration of CsA (25 mg/kg per d) orally to rats over 3 weeks causes plantaris and soleus muscles to lose some of their mass (Bigard *et al*. 2000). In contrast, administration of this same CsA dosage subcutaneously, or effective doses of FK506, does not affect the size of plantaris fibres in mice (Dunn *et al*. 1999, 2000). This discrepancy may relate to species-specific differences in the uptake, tissue distribution, clearance and *in vivo* effects of these drugs. Non-pharmacological evidence, however, suggests that Ca^{2+}/CaM-dependent signalling is required for the maintenance of fibre size. There is a marked decrease in the average size of regenerating soleus slow type I fibres in Tg mice overexpressing CaM inhibitory peptide, driven by the troponin I slow promoter (Dunn *et al*. 2000), under normal weight-bearing conditions. This effect is also dose-dependent, since a line of Tg mice (line 5229) with high levels of CaM inhibitory peptide expression display significantly ($P < 0{\cdot}05$) smaller regenerated calibre type I fibres compared with a Tg line (line 6444) with lower levels of CaM inhibitory peptide expression (see Fig. 2(c)). These data suggest that maintenance of growth is dose-dependently related to the level of Ca^{2+}/CaM-dependent signalling through Cn. Nonetheless, the effects of CsA and CaM inhibitory peptide on fibre size are subtle in the control or regenerating condition compared with the hypertrophic condition, emphasizing that Cn signalling pathways play a more substantive role in regulating fibre growth than in maintenance of size. In summary, Cn is necessary for skeletal muscle growth and appears to be the primary, although not the only, modulator of change in response to increased neural activation (Dunn *et al*. 1999, 2000, 2001).

Downstream mediators of calcineurin–nuclear factor of activated T cell signalling

Although Cn appears critical to mediating skeletal muscle hypertrophy, the downstream effector genes or targets in this process have yet to be clearly defined. Various downstream mediators of Cn-dependent signalling have been hypothesized (see Fig. 1) and include NFAT and myocyte-enhancing factor 2 (MEF2) proteins as well as GATA transcription factors. Similar to how these factors co-stimulate the transcriptional response of certain hypertrophic marker genes in the heart (Molkentin *et al*. 1998; Passier *et al*. 2000) and affect IGF-1 growth of skeletal myocytes *in vitro* (Musaro *et al*. 1999), these downstream transcription factors also appear to regulate skeletal muscle growth *in vivo*. GATA-2 expression is up regulated (Dunn *et al*. 2000) and NFAT more extensively dephosphorylated (Dunn *et al*. 2001) in hypertrophying skeletal muscles *in vivo*, suggesting that GATA–NFAT complexes may be at the nexus of neural activity-dependent and contractile load-dependent influences in skeletal muscles, via Cn and IGF-1 respectively. Despite the demonstrated importance of Cn–NFAT and Cn–MEF2 signalling events in regulating cardiac growth and the induction of the fetal programme of gene expression (i.e. atrial natriuretic factor (ANF), type I Mhc and α-skeletal actin) in cardiac hypertrophy (Rothermel *et al*. 2001), the growth-effector genes targeted by these transcription factors in both cardiac and skeletal muscle have not been identified. Findings that overexpression of constitutively-active Cn in cardiomyocytes is associated with the activation of c-Jun N-terminal kinase, extracellular signal-regulated kinase and protein kinase Cα and -φ raises the possibility that signalling of cardiac hypertrophy by Cn may occur through the production of an autocrine growth factor, such as IGF-1 (Molkentin, 2000). However, the links to cell proliferative factors (i.e. p21, cyclin-dependent kinase 2) that increase myocyte number or to translation initiation factors (i.e. eukaryotic initiation factor 2B, eukaryotic initiation factor 4E-binding protein 1) that increase protein synthesis have not been clearly established.

Recent converging lines of evidence also suggest that Cn may be sensitive to the loading state of the muscle and may cooperate with IGF-1 signalling pathways to effect growth. We found (SE Dunn and RN Michel, unpublished results) that there is an increase in the level of IGF-1 and the muscle regulatory factors myogenin and myf-5 at the mRNA level with overload and a further compensatory increase when Cn signalling is blocked with CsA (see Fig. 3).

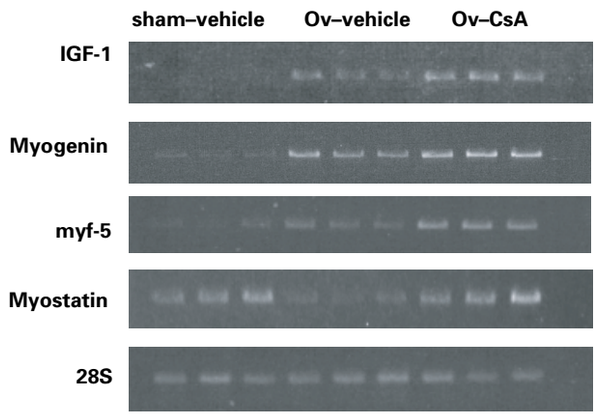

Fig. 3. Overload-induced up-regulation of IGF-1, myogenin, myf-5 and down-regulation of myostatin contributes to growth of plantaris muscle. Plantaris muscle from vehicle-treated sham-operated mice (sham–vehicle) and cyclosporin A (CsA)-treated (25 mg/kg twice daily) overloaded mice (OV–CsA) in which the soleus and gastrocnemius muscles were removed and analysed for expression of various growth-related factors. Total RNA was isolated from the distal portion of the plantaris muscle and 1 μg was reverse-transcribed to cDNA and amplified using primers specific for insulin-like growth factor 1 (IGF-1), the myogenic factors myogenin and myf-5, and myostatin. The 28S ribosomal RNA served as a loading control. Myostatin was amplified using primers that encoded sequences that are unique to the mouse sequence of this gene (accession number 042222) and not for its closely-related counterpart GDF-11. Transcript levels of IGF-1, myogenin and myf-5 were up regulated in response to overload, and were further increased in CsA-treated mice, suggesting that the IGF-1 dependent pathway for adaptation is up regulated when calcineurin-dependent growth is blocked. Myostatin, a negative regulator of growth, was down regulated in response to overload, and this response was countered by administration of CsA. These data indicate that myostatin may be a downstream target of calcineurin–nuclear factor of activated T cells signalling and suggest that hypertrophic growth requires counter-regulation of growth-inhibitory pathways.

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Similarly, studies (Dunn *et al*. 2000) have shown an up-regulation of GATA-2 with hypertrophy and a potentiated compensatory increase in this factor when Cn-mediated growth is blocked. These data strongly support parallel signalling via IGF-1-dependent and Cn-dependent pathways. Further support for a role of Cn signalling in response to increased load has come from studies showing Cn co-localization at the Z-line in cardiomyocytes, a region of the sarcomere that may play a role in the transduction of mechanical tension (Frey *et al*. 2000). Additionally, studies in *Dictyostelium* have shown that Cn is also activated by arachidonic acid (Kessen *et al*. 1999), a metabolite produced by muscles during stretch and mechanical loading (Vandenburgh *et al*. 1993). These data further support the notion that parallel pathways are required for adaptation in response to overload and suggest that compensatory increases in IGF-1, and possibly other signalling intermediates yet to be identified, signal via myogenin and myf-5 to increase myofibre growth. The IGF-1 pathways appear to be up regulated in a compensatory fashion when Cn-dependent signalling is inhibited.

Role of satellite cells in calcineurin-dependent and calcineurin-independent hypertrophy

Skeletal muscle fibre hypertrophy is characterized by an expansion of the size and number of myofibrils (Rosenblatt & Woods, 1992). This anabolic process is mediated by increases in muscle fibre transcriptional capacity and protein synthesis (Carson, 1997), and the activity-dependent regulated assembly of newly-translated proteins into sarcomeres (De Deyne, 2000; Torgan & Daniels, 2001). Increases in muscle fibre transcriptional capacity occur, in part, through the addition of satellite cell nuclei to growing fibres (Carson, 1997). Growth factors such as IGF-1, which are released from muscle fibres in response to increased contractile loading, cause satellite cells to proliferate, differentiate and fuse with pre-existing fibres, thus maintaining nucleus:cytoplasm of the fibre during growth (Adams & McCue, 1998). Recent findings suggest load-induced increases in protein synthesis are mediated in large part by the Akt–mammalian target of rapamycin pathway, in which mammalian target of rapamycin phosphorylates factors (i.e. 70 kDa ribosomal S6 protein kinase and eukaryotic initiation factor 4E-binding protein 1) involved in regulating the initiation factors of protein translation. Interference in either mammalian target of rapamycin signalling with rapamycin (Dunn *et al*. 1999; Bodine *et al*. 2001) or satellite cell activation via γ-irradiation of skeletal muscles (Rosenblatt & Parry, 1993; Phelan & Gonyea, 1997) severely hinders functional overload-induced hypertrophy, suggesting that both processes are critical to muscle growth.

While the downstream hypertrophic factors are not well understood, it is known that Cn is crucial to signalling at the onset of myogenesis (Abbott *et al*. 1998; Musaro *et al*. 1999; Semsarian *et al*. 1999; Delling *et al*. 2000; Friday *et al*. 2000). Thus, it is possible that Cn may affect muscle fibre hypertrophy via its stimulatory effects on satellite cell differentiation. Indeed, inhibition of Cn activity by CsA or the Cn-inhibitory peptide CAIN in cultured satellite cell lines or in primary cell culture before or coincident with growth factor withdrawal prevents their differentiation (Abbott *et al*. 1998; Musaro *et al*. 1999; Delling *et al*. 2000; Friday *et al*. 2000), whereas overexpression of constitutively-active Cn has the opposite effect and enhances this process (Delling *et al*. 2000; Friday *et al*. 2000). Cn may also regulate satellite cell fusion during muscle fibre hypertrophy via the NFATc2 transcription factor (Horsley *et al*. 2001). Primary cultures of myoblasts derived from NFATc2$^{-/-}$ mice, although displaying normal proliferation, differentiation and initial fusion into myotubes, fail to undergo subsequent fusion into larger more mature myotubes. This growth deficit is consistent with the phenotype of NFATc2$^{-/-}$ mice, in which muscle fibres are half the size and contain half the nuclei of their NFATc2$^{+/+}$ counterparts.

Whether satellite cell differentiation and fusion events during mature muscle fibre growth *in vivo* are Cn–NFAT-dependent and whether the role of Cn in regulating muscle growth is related to the activation of satellite cells has been tested. Using a compensatory overload model, as previously described (Dunn *et al*. 1999, 2000), we determined that the number of new fibres at 5 and 14 d of compensatory overload, as determined by bromodeoxyuridine incorporation and embryonic Mhc staining, is not altered by CsA treatment despite the fact that growth is blocked in these treated muscles (see Fig. 4). Thus, while satellite cell activation may contribute to the growth of fibres during overload, this pathway does not appear to be Cn dependent. Furthermore, although activation of satellite cells may well be important to such muscle adaptations during overload, Cn appears to affect fibre growth primarily by targeting processes that are intrinsic to existing adult myofibres (Dunn *et al*. 2001).

Insulin-like growth factor 1-induced hypertrophy: calcineurin-dependent and calcineurin-independent mechanisms

IGF-1 is a potent anabolic agent that can activate myocyte proliferation and myocyte differentiation, leading to muscle hypertrophy. These actions occur through different signalling pathways that include the Ras/Raf-1 pathway and mitogen-activated protein kinase pathways (proliferation), the phosphatidylinositol 3-kinase–Akt–mammalian target of rapamycin pathway (protein translation) and Ca^{2+}/Cn-dependent pathways (differentiation and cellular hypertrophy). Overexpression of IGF-1 induces myocyte hypertrophy of both C2C12 and L6 cells *in vitro* (Musaro *et al*. 1999; Semsarian *et al*. 1999) and transgenic mice *in vivo* (Musaro *et al*. 2001). The muscle hypertrophy observed *in vivo* is accompanied by an increase in tetanic force and is the result of an increase in myofibre diameter. Interestingly, mice with muscle-specific overexpression of IGF-1 show an attenuation of the age-related decrease in muscle size and regenerative capacity in response to injury; the latter is a result of an enhanced proliferative capacity (Musaro *et al*. 2001). IGF-1-induced hypertrophy *in vitro* is also the result of increased myocyte proliferation (i.e. increased myonuclei per myotube) as well as an increased rate of protein synthesis (Semsarian *et al*. 1999).

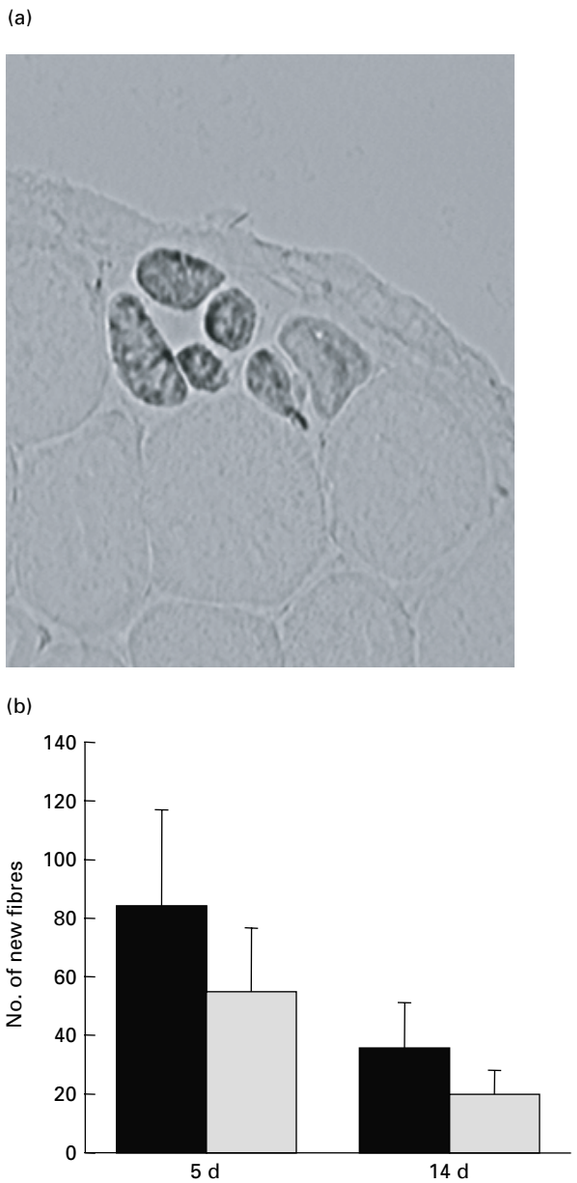

Fig. 4. Cyclosporin-dependent inhibition of muscle growth is not a result of inhibition of satellite cell differentiation. (a) Cross-sections of overloaded plantaris muscle from mice were stained for bromodeoxyuridine, a hallmark of satellite cell proliferation and fusion, or embryonic myosin heavy chain (Mhc), a marker of nascent muscle fibres. Embryonic Mhc-positive fibres are evident in the upper part of the fibre section. (b) The number of embryonic Mhc-positive fibres per muscle midbelly section were assessed in muscle from overloaded vehicle-treated (■) and overloaded CsA-treated (25 mg/kg twice daily; □) mice. Values are means with their standard errors represented by vertical bars. The number of new muscle fibres per midsection at 5 and 14 d of overload was not affected by CsA treatment, indicating that the prevention of overload hypertrophy by calcineurin inhibitors is not the result of a blocking of satellite cell activation leading to fibre hyperplasia.

At least part of the response to IGF-1 is a result of activation of the Cn–NFAT pathway (Musaro *et al*. 1999; Semsarian *et al*. 1999) via actions of IGF-1 on L-type Ca^{2+} channels, resulting in an increase in intracellular free Ca^{2+} concentration. Thus, increased IGF-1 would result in further increases in intracellular Ca^{2+} and activation of multiple Ca^{2+}-dependent signalling pathways. Inhibition of Cn–NFAT signalling with CsA blocks the IGF-1-induced increase in C2C12 proliferation, implicating this pathway in the regulation of the myocyte cell cycle. Cn–NFAT signalling also enhances myocyte fusion and thus differentiation into the more adult-like myotube state *in vitro* (Friday *et al*. 2000). Cn–NFAT signalling does not, however, appear to influence the size of myotubes once they have formed *in vitro*, since IGF-1 added after differentiation does not induce hypertrophy of fused C2C12 cells (Semsarian *et al*. 1999). This response may be different from the events that occur *in vivo*, since Cn–NFAT-mediated myofibre growth during muscle reloading is attenuated but not ablated in the absence of new myonuclei from satellite cells (Rosenblatt & Parry, 1993; Mitchell & Pavlath, 2001). Additionally, the *in vivo* response may involve counter-regulation of growth inhibitory pathways that may not be regulated in the same manner *in vitro*.

Novel downstream targets of calcineurin signalling: role of utrophin and myostatin in muscle adaptation

Activation of Cn-dependent signalling pathways is required for skeletal muscle hypertrophy (Dunn *et al*. 1999); however, the hypertrophic effector targets of this phosphatase have not yet been fully elucidated. Recent evidence identifies utrophin A and myostatin as possible downstream gene targets of Cn signalling in skeletal muscle. Utrophin is a cytoskeletal protein that forms part of the dystrosarcoglycan complex that plays an important role in maintaining membrane integrity, especially in the face of mechanical stress placed on the muscle during contractile activity. The lack of the utrophin homologue dystrophin results in muscular dystrophy and there is, therefore, therapeutic interest in the ability to up regulate utrophin expression. We have recently shown, in collaboration with Dr B Jasmin, that utrophin A is up regulated in a Cn-dependent manner, and that this gene is regulated by Cn-NFAT signalling in proportion to fibre oxidative capacity (Chakkalakal *et al*. 2003). Up-regulation of utrophin A in proportion to the level of fibre recruitment (i.e. type I ≥ IIa > IIx > IIb) may reflect an adaptation that allows the muscle to maintain membrane integrity in the face of increased mechanical strain related to higher levels of contractile activity.

Evidence is also reported here for the first time that Cn-dependent down-regulation of the growth inhibitory factor myostatin occurs during overload hypertrophy and implicates myostatin as a downstream effector of Cn–NFAT signalling (RN Michel and SE Dunn, unpublished results). Myostatin has recently come to the forefront as a negative regulator of muscle mass (McPherron *et al*. 1997). Mice null for myostatin display double the mass of their wild-type counterparts, which results from both increases in muscle fibre number and fibre size (McPherron *et al*. 1997). Moreover, overexpression of a dominant-negative form of this protein under the control of muscle creatine kinase promoter, which is expressed only in fully-differentiated

muscle cells, leads to fibre hypertrophy (by 35%), but does not affect fibre number (Zhu *et al*. 2000) This finding suggests that the effects of myostatin occur very early in development. This protein may play a role in modulating mass in response to changes in contractile loading, since its mRNA and protein levels are increased in the soleus after 10 d of unweighting, and this response is counteracted when muscles are periodically loaded (Wehling *et al*. 2000). If indeed the expression of myostatin is sensitive to loading, it would be expected that its expression would decrease in muscles when contractile loading is increased.

To test this hypothesis we investigated whether the expression of this growth factor is down regulated in mouse plantaris muscles subjected to functional overload. Moreover, to determine whether the expression of myostatin is regulated by Cn, the sensitivity of myostatin expression to CsA treatment was examined. The plantaris in each hindlimb of CD-1 mice was overloaded via surgical removal of the gastrocnemius and soleus, and then these mice and sham controls were injected with CsA (25 mg/kg) or FK506 (3 mg/kg) or vehicle twice daily for 5 d. mRNA levels of myostatin in these tissues were measured using RT–PCR (Dunn *et al*. 1999), with primers that encoded sequences that are unique to the mouse sequence of this gene but not for its closely-related counterpart, GDF-11. The transcript levels for myostatin decrease after overload and this response is countered by administration of CsA (see Fig. 3). These exciting new data suggest that activation of Cn in response to functional overload leads to down-regulation of the myostatin gene. This response may be of therapeutic value, since myostatin inhibition has been shown to play an important role not only in increasing muscle size but also in the prevention of obesity and type 2 diabetes (McPherron & Lee, 2002). Thus, Cn-mediated signalling may improve the muscle mass and counter muscle weakness and various myopathies, and, more importantly, may improve overall health in individuals with these metabolic diseases. Whether the improved metabolic state with myostatin inhibition is a direct effect of the abrogation of myostatin signalling or an indirect effect of increased muscle mass, and thus increased metabolically-active tissue, is not known. It is interesting to speculate that the Cn-dependent and myostatin signalling pathways may be counter-regulated during periods of growth and atrophy, and that therapeutic intervention of either one or both pathways would be beneficial for various metabolic disorders.

Calcineurin and the acquisition of a slower more-oxidative fibre phenotype

In addition to the role of Cn signalling in muscle hypertrophy, this phosphatase is known to play an important role in muscle fibre-type determination (Chin *et al*. 1998; Naya *et al*. 2000). Evidence to support this role was first provided by Chin *et al*. (1998), who have shown that increasing Cn activity in skeletal myocytes using a constitutively-active form of Cn induces the expression of troponin I slow and myoglobin promoters, but not the fast-muscle creatine kinase promoter. A role for Cn signalling *in vivo* has been confirmed by showing that administration of the Cn inhibitor CsA to rats induces type I→II fibre conversions in the soleus. The results of this study and a subsequent investigation (Wu *et al*. 2000) have provided the framework for the original model describing the Cn-dependent control of muscle fibre phenotype. According to this model, the sustained elevations in Ca^{2+} that occur in muscle fibres in response to the tonic firing of slow motor nerves activates Cn, which leads to the dephosphorylation and activation of MEF2 and NFAT proteins and the resultant transcription of slower-fibre-specific or oxidative genes (Chin *et al*. 1998; Wu *et al*. 2000). These elevations in Ca^{2+} also activate CaM kinases that coordinate with Cn to fully activate MEF2 (Wu *et al*. 2000). This model proposes that slow genes are selectively responsive to Cn signalling, because their transcriptional control regions contain NFAT and high-affinity MEF2-binding sites that are not present in the promoter regions of fast gene counterparts (i.e. troponin I fast; Chin *et al*. 1998; Wu *et al*. 2000).

The involvement of Cn in the signalling of a slow phenotype has since been corroborated by numerous *in vitro* (Delling *et al*. 2000; Swoap *et al*. 2000; Meissner *et al*. 2001; Torgan & Daniels, 2001) and *in vivo* studies (Bigard *et al*. 2000; Delling *et al*. 2000; Naya *et al*. 2000; Serrano *et al*. 2001). However, this hypothesis has been challenged by studies reporting the induction of fast Mhc (Torgan & Daniels, 2001) and certain fast- and muscle-specific promoters (Swoap *et al*. 2000; Allen *et al*. 2001) by activated Cn. Additionally, administration of CsA to mice, at a dose sufficient to inhibit Cn activity, not only blocks the accumulation of type I Mhc, but also prevents type IIb→IIx→IIa Mhc transitions in functionally-overloaded muscles (Dunn *et al*. 1999). Moreover, when more-potent Cn inhibitors (i.e. FK506 or CAIN) than CsA are used in rats, types I and IIa Mhc mRNA are both down regulated, and types IIx and IIb mRNA up regulated in regenerating and normal weight-bearing soleus muscles (Serrano *et al*. 2001). Further evidence supporting a role for Cn in mediating the expression of IIa Mhc is that stimulatory effects of the constitutively-active CnA transgene are much greater for IIa Mhc promoters (50- to 100-fold) than for IIx or IIb Mhc promoters (5- to 10-fold; Allen *et al*. 2001). Additionally, myoglobin, one of the first identified gene targets of Cn (Chin *et al*. 1998), is expressed in highly-oxidative type IIa fibres. Thus, Cn-dependent control of muscle fibre phenotype appears to be directed towards genes most expressed in highly-oxidative fibres and may involve coordinated regulation of genes related to the oxidative profile of a fibre. Interestingly, Cn has been shown to act synergistically with CaM kinase IV (although not normally expressed in muscle) to activate mitochondrial biogenesis and adoption of a slower-oxidative phenotype (Wu *et al*. 2002). The signals downstream of CaM kinases (primarily the transcriptional cofactor PPARγ co-activator 1α) appear sufficient to induce expression of genes related to oxidative metabolism and slower (types I and IIa) contractile elements (Lin *et al*. 2002; Wu *et al*. 2002). Thus, Cn-dependent control of fibre type may require coordination with other contractile activity-related or Ca^{2+}-related events or signalling pathways (i.e. CaM kinases) to induce the transcription of

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myofibrillar genes co-expressed in slower, more oxidative, fibres (types I or IIa fibre-associated). These signals are transduced via either MEF2 or NFAT, but only when muscle activity is above normal or at the upper range of that associated with relatively slower more-fatigue-resistant motor unit recruitment levels. As such, Cn signalling appears more a vehicle for adaptation towards a more-metabolically-efficient phenotype in response to increased muscle usage than a signalling agent for exclusive maintenance of slow type I fibre profiles in response to 'slow/chronic' patterns of nerve activation.

Summary

In summary, Cn-dependent pathways that signal through NFAT- and MEF2-dependent transcriptional intermediates play an important role in myofibre growth and adaptation to increased contractile activity. This adaptation may be achieved, in part, through regulation of specific downstream targets such as utrophin A and myostatin, and is supported by the parallel activation of Cn-independent IGF-1 and other Ca^{2+}-dependent pathways.

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