Dihydropyridine Receptor Gene Expression Is Regulated by Inhibitors of Myogenesis and Is Relatively Insensitive to Denervation

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Abstract

To evaluate developmental and physiological signals that may influence expression of the dihydropyridine-sensitive "slow" Ca^{2+} channel, we analyzed dihydropyridine receptor (DHPR) mRNA abundance in mouse skeletal muscle. Using synthetic oligonucleotide probes corresponding to the rabbit skeletal muscle DHPR, a 6.5 kb DHPR transcript was identified in postnatal skeletal muscle and differentiated C2 or BC_3H1 myocytes, but not cardiac muscle or brain. DHPR gene expression was reversibly suppressed by 0.4 nM transforming growth factor β-1 or by transfection with a mutant c-H-*ras* allele, nominal inhibitors of myogenesis that block the appearance of slow channels and DHPR. In contrast, both BC_3H1 and C2 myocytes containing the activated *ras* vector expressed the gene encoding the nicotinic acetylcholine receptor δ subunit, demonstrating that not all muscle-specific genes are extinguished by *ras*. Denervation stimulated DHPR gene expression less than 0.6-fold, despite 8-fold upregulation of δ-subunit mRNA and reciprocal effects on the skeletal and cardiac α-actin genes. Thus, DHPR gene induction is prevented by inhibitors of other muscle-specific genes, whereas, at most, relatively small changes in DHPR mRNA abundance occur during adaptation to denervation. (*J. Clin. Invest.* 1990. 85:781–789.)
calcium channels • differentiation • MyoD1 • oncogenes • skeletal muscle • transforming growth factor β-1

Introduction

The exact molecular mechanisms that might control the expression of calcium channels during muscle ontogeny and adaptation are presently not understood. For example, the density of dihydropyridine (DHP)[1]-sensitive Ca^{2+} channels is subject to homologous and heterologous regulation by DHPs and β-adrenergic ligands (1), may respond to membrane potential (2) or calcium itself (3), and might be altered in cardiomyopathies of animals (4) and man (5). Purification of the DHP receptor (DHPR) from transverse tubules of rabbit skeletal muscle (e.g., 6) and isolation of the corresponding cDNA (7, 8) have made Ca^{2+} channel gene expression accessible to molecular analysis, at least in skeletal muscle. The α_1-subunit protein contains the receptor site for both DHP derivatives and phenylalkylamines (6) and possesses structural similarity to voltage-gated Na^+ and K^+ channels (7, 9). The inference that the DHP-binding polypeptide might function as the voltage-sensing component of the proposed Ca^{2+} channel complex is strongly supported by the demonstration that DHPR cDNA restores both "slow" current and excitation-contraction coupling in cultured skeletal muscle cells from mice with the lethal mutation, muscular dysgenesis (10). A DHPR α_1 expression vector also is sufficient to produce voltage-gated, DHP-sensitive Ca^{2+} currents in murine L cells, which lack endogenous α_2, as well as α_1, subunits (11).

Studies to elucidate the molecular genetics of Ca^{2+} channel expression would be facilitated by model systems whose biochemical and biophysical differentiation can be precisely regulated. We have previously analyzed the DHP-sensitive Ca^{2+} channels formed in differentiating C2 and BC_3H1 muscle cells (12–14), whose slow kinetics of activation and DHP-binding properties correspond to those of skeletal muscle and are distinct from those found in neurons, smooth muscle, or cardiac muscle cells. Neither cell line expressed functional Ca^{2+} channels during proliferative growth, and the appearance of slow current was dependent on removal or depletion of serum growth factors (12, 14). Conversely, Ca^{2+} channel formation could be prevented by transforming growth factor β1 (TGFβ1), a peptide inhibitor of muscle differentiation (14–16). Equivalent results were produced by transfection with an activated allele of the H-*ras* proto-oncogene (12), encoding a guanine nucleotide binding protein that is postulated to mediate certain growth factor effects (12, 17–21). Like the induction of slow channels assayed by whole-cell clamp techniques, formation of DHP receptor sites was contingent on serum withdrawal, and was suppressed both by *ras* and by TGFβ1 (13).

These findings suggested that the ontogeny of Ca^{2+} channels during myogenesis may involve intracellular events similar to those that control formation of other muscle-specific gene products (20, 22). However, a number of issues were still unresolved. First, the synthesis of a functional ion channel might involve control at diverse levels. Subunit assembly, glycosylation, translational control, and alternative mRNA splicing each have been implicated as potential regulatory steps in ion channel formation (23–25), in addition to control at the level of gene transcription. Second, the inference remains open to question, that growth factors and oncogenes would neces-

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1. *Abbreviations used in this paper:* DHP, dihydropyridine; DHPR, dihydropyridine receptor; *gad,* glyceraldehyde-3-phosphate dehydrogenase; *mck,* muscle creatine kinase; nAchR δ, delta subunit of the nicotinic acetylcholine receptor; TGFβ1, type β-1 transforming growth factor.

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sarily suppress Ca^{2+} channel formation through a block to the induction of all muscle-specific genes. Finally, a variety of physiological signals including innervation and muscle activity stimulate dramatically the genes encoding other ion channels (e.g., 26–28), whereas the available physiological data suggest that denervation of mammalian skeletal muscle exerts at most a very modest effect on slow Ca^{2+} current (29; cf. 30) and asymmetric charge movement, the postulated gating current for Ca^{2+} release (31). Together, these observations indicate that DHPR gene expression might possess regulatory features both in common with and distinct from other ion channels in skeletal muscle.

As a first step toward understanding the molecular events that determine Ca^{2+} channel expression, we have investigated DHPR gene expression during myogenesis, both in vivo and in vitro. The present study was designed to ascertain whether DHPR gene expression in mouse skeletal muscle is tissue-specific and developmentally regulated. C2 and BC_3H1 muscle cells were analyzed to test the prediction that growth factors and oncogenes affect the expression of functional DHP-sensitive Ca^{2+} channels through a block to induction of the DHP receptor gene, and to establish whether all muscle-specific genes necessarily are suppressed by nominal inhibitors of the myogenic pathway. Finally, experiments were undertaken to examine the possible control of DHPR gene expression by denervation.

Methods

Northern blot hybridization. Total cellular RNA was size-fractionated by formaldehyde-agarose gel electrophoresis and transferred to nylon membranes (32–34). To identify putative DHP receptor transcripts, oligonucleotide probes were synthesized, complementary to nucleotides 1–60 and 4090–4131 of the DHP receptor cDNA isolated from transverse tubules of rabbit skeletal muscle (7). Hybridization of DHPR probes was performed at 42°C in 5× SSC/5× Denhardt's solution/5% $NaDodSO_4$/50% formamide/100 $\mu g \cdot ml^{-1}$ salmon sperm DNA; blots were washed twice in 3× SSPE/1% $NaDodSO_4$ at 50°C for 15 min, then in 1× SSPE/1% $NaDodSO_4$ at 50°C. The α-skeletal and α-cardiac actin oligonucleotide probes were complementary to specific 3′ untranslated portions of the corresponding cDNAs (35, 36). The δ subunit of the nicotinic acetylcholine receptor (nAchR δ) was analyzed using a 20-mer complementary to the 3′-untranslated nucleotides 1842–1861 of the murine sequence (37). Muscle creatine kinase (*mck*) mRNA was measured using the 0.88-kbp Pst I fragment of pHMCKIa (38). Expression of MyoD1, which becomes activated as an early event in the myogenic pathway and confers the muscle phenotype to certain non-muscle cells, was examined using nucleotides 580–639 of the *myc* homology domain (39). Oligonucleotide probes were end-labeled using T4 polynucleotide kinase, and cDNA restriction fragments were labeled by the random primer method (40). Blots were exposed to Kodak XAR-2 film at −70°C with intensifying screens and were quantitated by scanning densitometry.

Denervation. Denervation was performed on 11–12-wk-old C3H mice under general anesthesia with intramuscular ketamine (Parke-Davis Co., Morris Plains, NJ)/xylazine (Haver, Shawnee, KS)/acepromazine (Techamerica, Kansas City, MO). Segments of the sciatic nerve and proximal segments of the left tibial and peroneal nerves (1–2 mm) were excised at their bifurcation. Left hindlimb soleus and flexor digitorum longus muscles were collected at intervals after denervation, and the contralateral muscles served as controls.

Cell culture. Undifferentiated C2 cells (41) were maintained in Ham's medium F12 containing 20% fetal bovine serum (14). At 85% confluency, differentiation was induced in Dulbecco's modified Eagle's medium containing 2% horse serum. To induce differentiation of BC_3H1 muscle cells (42), cells were fed medium with fetal bovine serum reduced from 20% to 0.5% (12, 18, 43). All media were purchased from Gibco Laboratories (Grand Island, NY), and sera were obtained from Hyclone, (Logan, UT). Oncogene-transfected BC_3H1 cell lines (cotransfected with the neomycin phosphotransferase as a dominant selectable marker) were thawed from low-passage stocks of clonal isolates described previously (12, 18, 43) and were maintained in medium containing 400 $\mu g \cdot ml^{-1}$ G418. Clonal, *ras*-transfected C2 cells were a gift from Dr. Eric Olson (19). The homodimeric form of TGFβ1 isolated from porcine platelets was obtained from R&D Systems (Minneapolis, MN). (−)S Bay K 8644 (R5417), (+)R Bay K 8644 (R4407), and nifedipine were provided by Dr. Alexander Scriabine (Miles Laboratories, West Haven, CT).

Results

Dihydropyridine receptor gene expression is tissue-specific and developmentally regulated in vivo. To establish whether formation of the DHP-sensitive slow Ca^{2+} channel in skeletal muscle is regulated through control at a pretranslational level, DHPR mRNA abundance was analyzed by Northern hybridization, using synthetic oligonucleotides derived from the α_1 subunit of the rabbit skeletal muscle DHPR cDNA (7, 8). Our best results were obtained with the two oligonucleotides reported here, a 60-mer directed against the 5′-most portion of the coding sequence, and with a 42-mer corresponding to the S6 α-helix in domain 4. As shown in Fig. 1, using 15 μg of total cellular RNA per lane, a 6.5-kb putative DHPR α_1 transcript was identified in postnatal C3H mouse skeletal muscle, in agreement with the size of the transcript reported in rabbit muscle (7, 8). DHPR mRNA was detected even at 2 d, in agreement with the existence of functional slow channels in newborn mice (29), and its abundance increased 18-fold by 2 mo of age. Fast skeletal muscle (latissimus dorsi) expressed ~ 1.8-fold more DHP receptor mRNA than slow skeletal muscle (soleus), as might be anticipated on the basis of the greater T-tubular volume, slow current density, and DHP binding in fast muscle (44, 45). The probes were specific for skeletal muscle. Little or no binding was seen in mouse heart or brain, using either total RNA (Fig. 1) or polyadenylated RNA (Fig. 2). Equivalent results were obtained with either probe. This result agrees with other evidence that structural differences distinguish the DHP-sensitive Ca^{2+} channels in skeletal muscle from those in brain or ventricular muscle (47, 48), as suggested by the known disparities in channel kinetics and DHP affinity.

DHP receptor mRNA and protein are induced as late events during myogenic differentiation. Neither specific DHP binding sites (Fig. 3) nor DHPR mRNA (Fig. 4) was detected in proliferating C2 cells, which lack functional slow channels (14). Whereas expression of other muscle-specific genes (*mck*, α-cardiac actin, α-skeletal actin) was detected within 24 h or less of serum withdrawal, DHPR mRNA (like DHP receptor sites) first appeared after 2 d, in agreement with the absence of DHP-sensitive current at earlier timepoints; both "transient" Ca^{2+} channels and sodium channels also are induced in C2 cells one day or more before slow Ca^{2+} channels are found (14). DHPR mRNA was induced at least 10-fold after serum withdrawal. The abundance of DHPR mRNA 72 h after serum withdrawal was greater than in mouse skeletal muscle 2 d after birth, but less than that found in vivo at 7 or more days. At 2 and 3 d of serum withdrawal, Scatchard transformation of saturation analysis identified a single class of noninteracting

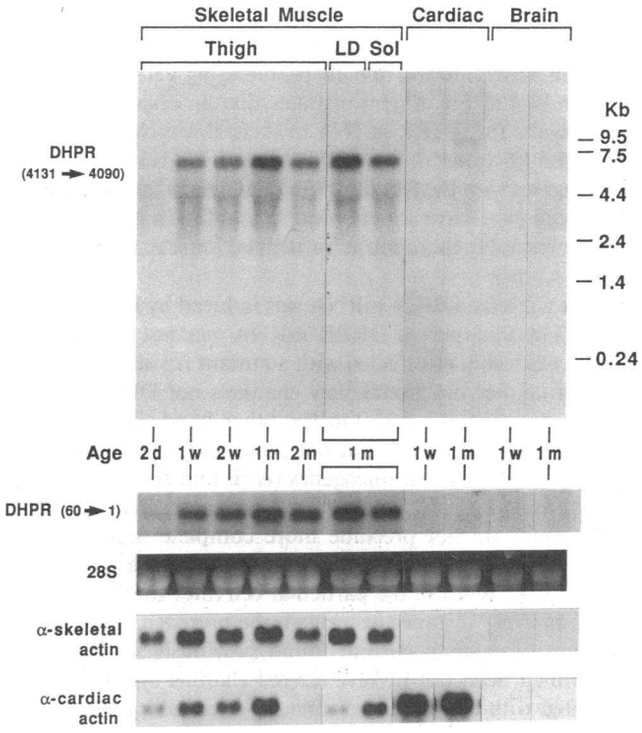

Figure 1. DHPR gene expression is tissue-specific and developmentally regulated in vivo. Total cellular RNA (15 $\mu g \cdot lane^{-1}$) was isolated from C3H mouse tissues at the ages shown, and was analyzed by Northern blot hybridization, using the probes indicated at the left of the figure. The specificity and developmental regulation of actin sequences utilized in the subsequent experiments is shown at the bottom of the figure. The specific activities and exposure times for autoradiography differed, and the hybridization signal intensities are not intended to represent the relative abundance of the respective transcripts. A nonspecific hybridization signal at 4.0 kb associated with the leading edge of 28S ribosomal RNA also was seen with oligonucleotides directed against other transcripts. 28S ribosomal RNA is shown for comparison. LD, latissimus dorsi; Sol, soleus.

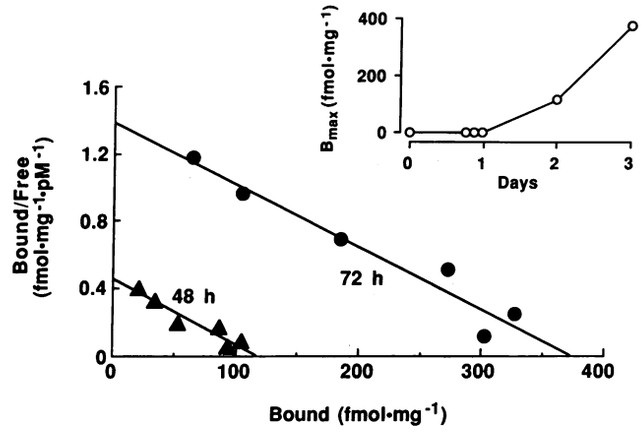

Figure 3. Development of [^{3}H]PN200-110 receptor sites in C2 muscle cells. C2 muscle cells were subjected to hypotonic lysis, homogenized, and analyzed for equilibrium binding of [^{3}H]PN200-110 as previously described (14). Scatchard transformation of saturation analysis is shown for C2 muscle cell membranes 48 and 72 h after mitogen withdrawal. Binding site density increased from 117 to 375 fmol · mg prot^{-1}, with little or no change in K_d (255 and 271 pM). (*Inset*) [^{3}H]PN200-110 binding site density in C2 muscle cells after mitogen withdrawal.

receptor sites with dissociation constants of 255 and 271 pM, and binding site densities of 117 and 375 fmol · mg prot^{-1}, respectively. These results are similar to the corresponding values for differentiated BC_3H1 cells (256 pM and 322 fmol · mg prot^{-1}; 13) and contrast with the disparity between ion channel sub-unit mRNA and binding site formation in other systems (23, 25).

TGFβ1 and an activated ras oncogene each prevent the formation of functional slow Ca^{2+} channels through a pretranslational mechanism. When TGFβ1 was added to C2 cells at 400 pM at the time of serum withdrawal, DHPR gene expres-

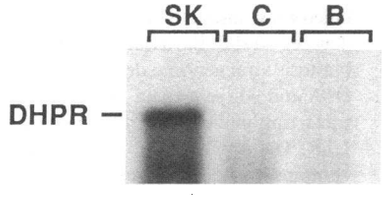

Figure 2. Distribution of the DHPR transcript. Polyadenylated RNA (ref. 46; 5 $\mu g \cdot lane^{-1}$) was analyzed by Northern blot hybridization. In this and the subsequent figures, the 42-mer complementary to nucleotides 4131–4090 was used as the DHPR hybridization probe. SK, skeletal muscle; C, cardiac muscle; B, brain.

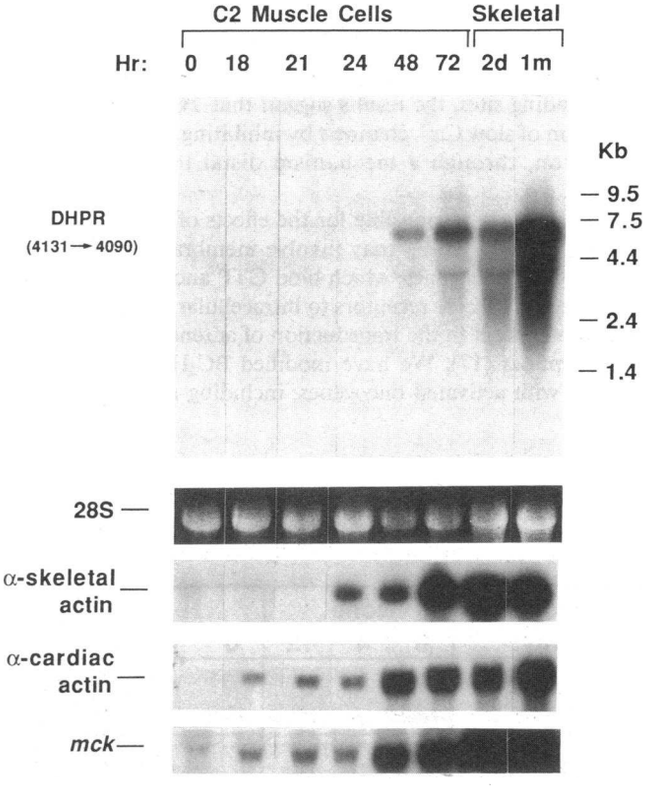

Figure 4. DHPR gene induction during myogenesis in vitro. Total cellular RNA isolated from C2 muscle cells after serum withdrawal for the intervals shown was analyzed by Northern blot hybridization as described in Methods and the legend to Fig. 1. Mouse skeletal muscle mRNA is shown for reference at the right of the figure. Comparable results were obtained with DHPR 42-mer used here and with the 5′ 60-mer. The exposures used for *mck* and the α-actins were selected to permit comparable hybridization signals in C2 muscle cells 72 h after mitogen withdrawal.

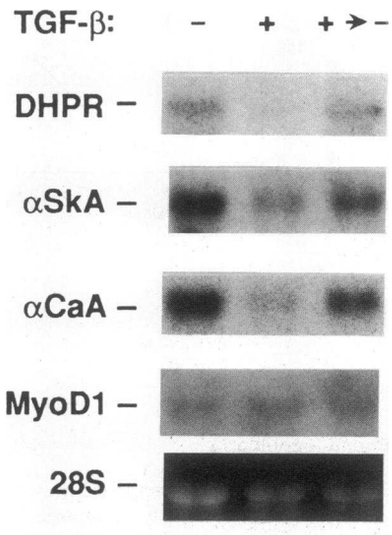

Figure 5. TGFβ1 prevents the formation of functional slow channels by suppressing DHPR gene expression. C2 muscle cells were subjected to mitogen withdrawal on day 0 in the presence of (−) vehicle or (+) 400 pM TGFβ1 for 4 d. TGFβ1 was removed from a portion of the cultures on day 2 (+ → −).

sion was reversibly suppressed by ~ 80% (Fig. 5). This finding concurs with the known ability of TGFβ1 to inhibit the induction of muscle-specific genes (15, 16), as illustrated for the α-actins. In contrast, TGFβ1 did not suppress the myogenic determination gene, MyoD1, whose expression had been reported to be constitutive within the myogenic lineage (39); recently, TGFβ1 has been shown to inhibit MyoD1 gene expression, at 10-fold higher concentrations than in the present study (49). Taken together with the previous evidence that TGFβ1 blocks the appearance of functional slow channels and DHP binding sites, the results suggest that TGFβ1 affects the formation of slow Ca^{2+} channels by inhibiting α_1 subunit gene expression, through a mechanism distal to the action of MyoD1.

The pathway responsible for the effects of peptide growth factors including TGFβ may involve membrane proteins encoded by *ras* oncogenes, which bind GTP and are believed to couple growth factor receptors to intracellular effectors, much as G-proteins act in the transduction of adrenergic agonists or neuropeptides (17). We have modified BC_3H1 cells by gene transfer with activated oncogenes, including *ras* (12, 18, 43). We showed that *ras* selectively prevents the appearance of channels that require serum withdrawal, but has no effect on the K^+ channels found in BC_3H1 cells, which are expressed equally in differentiated and in proliferating cells (12). The Northern blot in Fig. 6 demonstrates that an oligonucleotide probe specific for DHPR mRNA in skeletal muscle also identified a 6.5-kb transcript in differentiated BC_3H1 cells. This finding agrees with the fact that slow channels in the nonfusing BC_3H1 cell line have kinetic and ligand-binding properties that best resemble those found in skeletal muscle, not smooth muscle (12–14).

As in C2 cells, DHPR mRNA was induced by serum withdrawal (Fig. 6), whereas DHPR mRNA was not detected in BC_3H1 cells stably transfected with a mutant *ras* allele (Fig. 6), in which neither functional slow channels nor DHP binding sites are detected (12, 13). On the other hand, DHPR gene expression was decreased, but not prevented, in cell lines expressing other activated oncogenes (*erb*B and *myc*), in which the accumulation of Ca^{2+} channels is merely delayed (12). These results do not preclude more complete inhibition of DHPR at higher copy number or levels of oncogene expression than those achieved in the particular cell lines studied. Nonetheless, in every instance examined, the presence, absence, or reduction of calcium current found previously in these cell lines (which need not involve altered channel synthesis) was associated with the presence, absence, or reduction of DHPR mRNA, respectively. Thus, the control of Ca^{2+} current density in transfected BC_3H1 cells occurs in large part at the level of DHPR gene induction.

Notwithstanding the absence of α-bungarotoxin binding sites in the *ras*-transfected cells (18), in contrast *ras* did not abolish the induction of nAchR δ, whose potential regulation by transfected oncogenes has not previously been examined. nAchR δ mRNA was upregulated 10-fold in *ras*-transfected BC_3H1 cells after mitogen withdrawal, and the level of expression was roughly equivalent to that attained in control cells (Fig. 6). Since BC_3H1 muscle cells differentiate through a mechanism independent of MyoD1 (39, 50), to exclude the possibility that induction of nAchR δ in the presence of a transforming *ras* gene was merely due to an anomaly in their myogenic program, DHPR and nAchR δ transcript accumu-

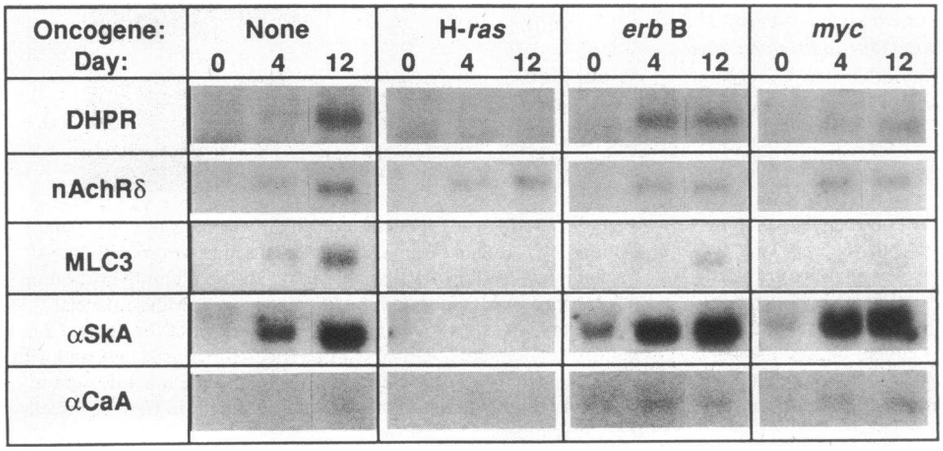

Figure 6. An activated *ras* oncogene prevents DHPR gene induction through a mechanism that is permissive for expression of nAchR δ. RNA was isolated and analyzed following mitogen withdrawal for the intervals shown. The construction and characterization of clonal BC_3H1 cell lines, stably cotransfected with pSV2neo and oncogene expression vectors, have been detailed previously (13, 19, 44). *Erb*B denotes v-*erb*B, the avian erythroblastosis viral genome deleted in the *erb*A and *gag* sequences, under the transcriptional control of the viral LTR. The *myc* expression vector contains murine c-*myc* exons 2 and 3 downstream from the SV40 promoter, and H-*ras* indicates the Val^{12} allele of human c-H-*ras*-1, under the control of its own promoter.

lation also was investigated in C2 cells containing the activated *ras* allele, which similarly fail to bind α-bungarotoxin (19). In concordance with the results shown for BC_3H1 cells, nAchR δ mRNA was readily detected in *ras*-transfected C2 cells following serum withdrawal, whereas the DHPR transcript was suppressed (Fig. 7). Thus, the inhibition of DHPR gene expression by *ras* does not merely involve a block to the myogenic pathway.

DHP receptor gene expression is relatively insensitive to denervation. To test the prediction, suggested by the existing biophysical data, that muscle activity also might exert differing effects on DHPR vs. nAchR gene expression, we analyzed RNA from fast and slow skeletal muscle 15 d after denervation, using the contralateral muscles as control (Fig. 8). No more than a 0.35- to 0.52-fold increase in DHPR gene expression was detected. These results contrast with the upregulation of mRNA encoding nAchR δ (up to eightfold). Qualitatively similar results were obtained at earlier time points, as well. Moreover, in agreement with a recent study (51), denervated soleus muscle also exhibited reciprocal effects on the two sarcomeric actin genes: decreased expression of α-skeletal actin mRNA, concomitant with increased abundance of the α-cardiac actin transcript (up to 4.5-fold) associated with embryonic and neonatal skeletal muscle. Thus, changes in DHPR gene expression after denervation were ~ 10-fold less than in the other muscle-specific genes which were tested.

To test the related hypothesis that stimulatory and inhibitory DHPs might themselves influence DHPR gene expression, we subjected C2 cells to serum withdrawal in the presence of vehicle alone, the optically pure DHP agonist (−)S Bay K 8644 (R5417), or the antagonist, nifedipine, at submaximal concentrations (1 μM). At 1 μM, the amplitude of "slow" current elicited by voltage pulses to 0 mV was increased fivefold by (−)S Bay K 8644 and decreased 60% by nifedipine (not shown). Under the conditions tested, nifedipine stimulated DHPR gene expression no more than 0.5-fold, at 2 d (Fig. 9). Unlike denervation, chronic exposure to nifedipine did not produce a large increase in nAchR δ mRNA levels. In the presence of (−)S Bay K 8644, little or no change in DHPR gene expression was measured; comparable results were obtained using the antagonist stereoisomer, (+R) Bay K 8644 (R4407; not shown). Thus, concentrations of DHPs sufficient to alter slow Ca^{2+} current up to fivefold did not exert potent effects on DHPR gene expression, in good agreement with the limited degree of homologous regulation shown by DHP binding in other excitable cells (1).

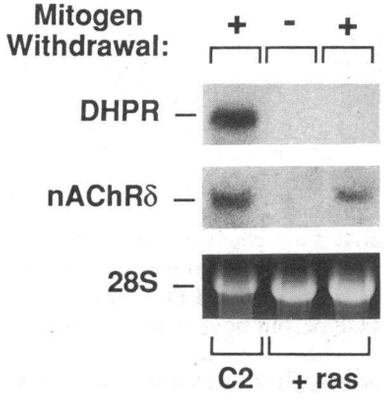

Figure 7. DHPR gene expression is extinguished in C2 muscle cells bearing the activated *ras* allele, whereas nAChRδ mRNA is induced after serum withdrawal. C2 cells stably transfected with the same H-*ras* construct as in Fig. 6 were differentiated for 4 d; the C2 cells included as a positive control were differentiated for 7 d and are not intended for direct comparison.

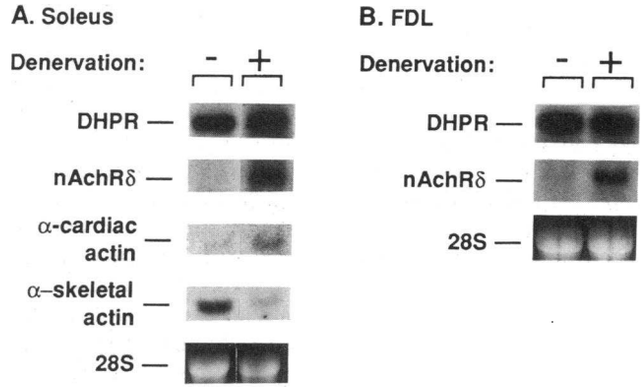

Figure 8. DHPR gene expression in the soleus and flexor digitorum longus after denervation. (+) Denervated and (−) contralateral control muscles were analyzed 15 d after nerve transection.

Discussion

DHPR gene expression during myogenesis in vivo. Our experimental findings suggest the conclusion that DHPR gene expression in mouse skeletal muscle is both tissue-specific and developmentally regulated in vivo. Previous studies have shown that the proportional volume of muscle fiber occupied by the T-tubular system increases about fivefold in mouse skeletal muscle, during the first few weeks after birth (45), as does the density of functional slow Ca^{2+} channels (29). Both slow current (52) and DHP binding sites (53) also increase after birth in rat skeletal muscle. In contrast, DHP-insensitive, T-type Ca^{2+} channels become undetectable within three to four weeks of birth (29, 52). The results favor the interpretation that quantitative differences in slow Ca^{2+} channel expression during myogenesis involve, at least in part, control at a pretranslational level. The absence of a hybridization signal in ventricular muscle and brain agrees with known disparities

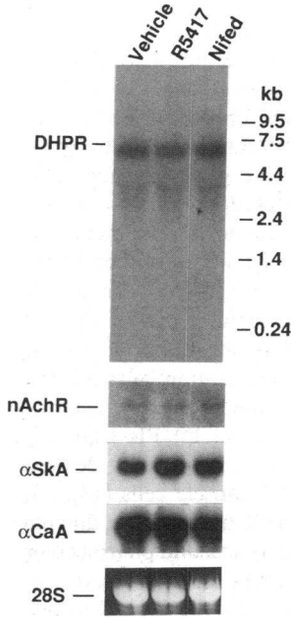

Figure 9. DHPR gene expression in C2 muscle cells subjected to mitogen withdrawal in the presence of vehicle or the DHPs shown (1 μM). Similar results were obtained at 20 μM.

between the DHP-sensitive slow channel in skeletal muscle and classical L-type channels in other excitable tissues, and agrees with recent confirmation that the DHP receptor in other cell types may possess a differing, though related, primary structure (48).

DHPR mRNA was identified in both fast (latissimus dorsi) and slow (soleus) muscle. Despite differences in the voltage-dependence of both asymmetric charge movement and the inactivation of excitation-contraction coupling (reviewed in reference 54), this finding is not unexpected, as slow Ca^{2+} channels in fast and slow muscle display similar kinetics and DHP sensitivity (44). The apparent abundance of DHPR mRNA in fast versus slow muscle varied no more than two-fold, in agreement with their relative T-tubular volume (45), slow current amplitude, asymmetric charge movement, and density of DHP binding sites (44). More precise analysis of these and other small fluctuations in transcript abundance can best be performed by quantitative nuclease protection, using the autologous murine DHPR α_1 subunit sequence as the hybridization probe.

DHPR gene expression during myogenesis in vitro. In agreement with the conclusion that the increase in slow current density after birth could be accounted for by increased DHPR mRNA abundance, in cultured muscle cells we found that the induction of slow Ca^{2+} channels by serum withdrawal (and their suppression by TGFβ1 or activated *ras* genes) all involve control at a pretranslational level. Both DHPR mRNA and functional DHP binding sites appeared during the interval from 24 to 48 h after serum withdrawal, and a single class of the binding sites existed, with an affinity typical of DHP receptors in adult skeletal muscle. Therefore, even if a second step were rate-limiting for the binding site to become functional, the latency between message induction and expression of the mature receptor must be less than 24 h (cf. 25). It is unknown whether ion movement through slow Ca^{2+} channels is responsible, even in part, for the effects of extracellular Ca^{2+} on muscle development (55). Although the appearance of Ca^{2+} currents is one of the earliest events during myogenesis in certain primitive marine chordates (56), in C2 muscle cells, the genes encoding α-cardiac actin, α-skeletal actin, and *mck* each were induced before slow channels could be detected by Northern blot, ligand binding, or even whole-cell clamp analysis. These experimental findings do not exclude a possible role for DHP-sensitive channels at densities too low to be detected by the three procedures used, or bear on the likelihood that DHP-sensitive channels might participate in the activity-dependent modulation of muscle-specific genes at later stages (57). In agreement with this interpretation, dysgenic muscle lacking slow channels also is reported to form sarcomeres (58) and express creatine kinase (59), though at diminished levels.

Despite the theoretical possibility that growth factors might inhibit the opening probability for slow channels, through protein kinase C or other transduction pathways (e.g., 60), the results shown here support the interpretation, instead, that TGFβ1 prevents the appearance of functional slow channels (and DHP binding sites) at the level of DHPR transcript abundance. Furthermore, TGFβ1 does not acutely decrease the amplitude of slow current in C2 muscle cells (14). The presence of the skeletal muscle DHPR transcript in differentiated BC_3H1 cells concurs with biophysical and pharmacological evidence that BC_3H1 cells express DHP-sensitive Ca^{2+} channels that are distinct from the L-type high-threshold channels of smooth muscle, cardiac muscle and neurons, and are identical in most respects to those found in C2 cells or skeletal muscle transverse tubules. That BC_3H1 cells have the properties of skeletal muscle cells (but are defective for terminal differentiation) is substantiated by their expression of sarcomeric muscle contractile protein genes (61), as well as the muscle determination gene, myogenin (50). Conversely, the absence of functional slow Ca^{2+} channels in BC_3H1 cells stably transfected with an activated *ras* gene can, like the action of extracellular TGFβ, be accounted for by a block to accumulation of the DHPR transcript. These findings demonstrate that a guanine nucleotide-binding protein can alter calcium current by regulating DHPR gene expression, in addition to the direct effects of other G proteins on Ca^{2+} channel gating (e.g., 60). Our investigations are consistent with recent evidence that *ras* can suppress the function of a regulatory DNA motif shared by several unrelated muscle-specific genes (21).

An unanticipated finding in the present study was the induction of nAchR δ gene expression in *ras*-transfected BC_3H1 and C2 cells after serum withdrawal, which is noteworthy in several respects. First, the presence of nAchR δ mRNA indicates that activation of c-H-*ras* does not preclude the appearance of all muscle differentiation products, as might be inferred from previous results (12, 18, 19, 62, 63). A mechanistic basis for the disparate effects of *ras* on multiple muscle-specific genes would be conjectural at present. However, studies with a reversibly inducible *ras* expression vector indicate that impairment of differentiation is titratable (62), suggesting that the threshold for inhibition by *ras* might vary systematically between genes. Second, analogous dosage effects might conceivably contribute to the more complete inhibition of Ca^{2+} channel formation by *ras* than by other oncogenes (cf. 43, 64), and the possibility therefore exists that properties differing, for example, between *ras*- and *erb*B-transfected cells are not inherent to these genes. However, although equivalency of dosage has not been yet established for v-*erb*B, the *ras*-transfected BC_3H1 cells contain only a single copy of the activated allele, under the control of its own promoter, and c-H-*ras* mRNA abundance is increased no more than threefold (18). By contrast, the SV40-driven c-*myc* gene produced *myc* mRNA levels that were 20- to 40-fold greater than those in control cells (43). Other studies demonstrating "complete" inhibition of myogenesis by v-*erb*B (65), v-*myc* (65, 66) or activated c-*myc* genes (64) have each retained at least 2% fetal bovine serum in the media used to induce differentiation, a concentration four times greater than that used here.

Furthermore, the presence of nAchR δ mRNA in both *ras*-transfected BC_3H1 and C2 cells, which fail to express α-bungarotoxin binding sites (18, 19), demonstrates that suppression of an ion channel need not involve each subunit mRNA. Activated *ras* genes also fail to suppress the nAchR α gene in transfected C2 cells (67). Thus, the highly regulated pattern of DHPR α_1 subunit gene expression neither is a foregone conclusion, nor can be predicted on the basis of measurements of current density and ligand binding. Finally, *ras* and other activated oncogenes produce contrasting effects in cell lineages whose DHP-sensitive Ca^{2+} channels differ from the slow channels in skeletal muscle. For example, *ras* leads to induction of calcium channels in neuronal PC12 cells (67a), and selectively suppresses T-type, but not L-type, channels in NIH 3T3 fibroblasts (68).

Conversely, the absence of DHPR mRNA despite contin-

ued MyoD1 gene expression in C2 cells treated with TGFβ1 also merits comment. Though MyoD1 itself recently was shown to be repressed by TGFβ1 (49), as well as by *ras* (63, 67), suggesting that the peptide might act on the myogenic pathway by inhibiting this muscle regulatory gene, this was achieved at tenfold higher concentrations than those in the present study, may have been further potentiated by the inclusion of 5% horse serum, and was demonstrated only in myoblasts derived from a pluripotent cell line, 10T1/2, by exposure to 5-azacytidine. Moreover, the fact that exogenous MyoD1 genes fail to overcome the block to differentiation created by TGFβ (49) or by transient transfection with *ras* (67; cf. 63) concurs with our finding that repression of DHPR by TGFβ1 can coexist with expression of the endogenous MyoD1 gene. MyoD1 is a nuclear phosphoprotein (69), suggesting the likelihood that posttranslational events may confer the ability to transactivate muscle-specific genes.

DHPR gene expression and physiological adaptation. In addition to regulated expression during muscle ontogeny, certain ion channels (and other differentiation products) also are subjected to quantitative or qualitative control in postnatal muscle fibers by physiological signals such as denervation, cross-innervation, activity and manipulation of ion flux (27–29, 57). The possibility has been suggested that cellular oncogenes might serve as "third messengers" that couple membrane currents to adaptive changes in gene expression in electrically excitable cells (70). However, in agreement with the finding that denervation fails to increase asymmetric charge movement (31) or Ca^{2+} current density (29; cf. 30), we observed that denervation exerts relatively little effect on DHPR gene expression, under conditions that markedly alter genes encoding other ion channels in muscle. In principle, DHP ligands might also be expected to influence DHPR expression, yet neither an agonist nor an antagonist altered DHPR gene expression in C2 cells by more than 60%. These changes were one-fifth or less as great as those reported for Na^+ channel mRNA following denervation (26) or inhibition of Na^+ current (28) in rat skeletal muscle. Possible impediments to resolving this issue in culture include disparities between acute effects versus chronic exposure to DHPs, consequences of resting potential (71), steady-state inactivation, and residual current or charge movement. Alternative tests of the role of membrane activity may be instructive (57).

The observations in the present report suggest that both the developmental regulation and tissue-specificity of Ca^{2+} channel expression are achieved in part at the level of DHPR transcript availability. In contrast, DHPR gene expression was relatively insensitive to physiological events, such as denervation and channel antagonists, which produce diverse alterations in other membrane or contractile proteins in skeletal muscle. This distinction may be a more general phenomenon, as shown by studies of cardiac muscle. Though adaptation to a hemodynamic load downregulates cardiac expression of the Ca^{2+} ATPase (72), evokes the fetal isoforms of sarcomeric proteins (73, 74), and reinduces ventricular expression of atrial natriuretic factor (73, 74), in contrast, DHP binding (per gram of ventricle or milligram protein) does not change (75). Thus, the density of Ca^{2+} channels is unlikely to account for the increased Ca^{2+} current observed in ventricular myocytes of hypertensive rats (76). The largest changes in gene expression produced by denervation each involve transitions toward embryonic isoforms (tetrodotoxin insensitive Na^+ channels [77], α-cardiac actin [51], and acetylcholine receptors with embryonic channel properties, in which the γ sub-unit is re-expressed [78]), whereas only a single class of DHP receptor sites (13, 53) and DHP-sensitive Ca^{2+} channels (12, 14, 29, 52) appears to exist during mammalian skeletal muscle development. Taken together with other evidence that Ca^{2+} channel density varies little in mammalian skeletal muscle once terminal differentiation has occurred, the present study fosters speculation that the existence of diverse pathways that alter Ca^{2+} channel opening may obviate, in part, the need for channel number to be highly regulated by denervation or other physiologic interventions.

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