Insulin-like Growth Factor-1 Increases Skeletal Muscle Dihydropyridine Receptor α_{1S} Transcriptional Activity by Acting on the cAMP-response Element-binding Protein Element of the Promoter Region*

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Previous work from our laboratory has shown that insulin-like growth factor 1 (IGF-1) increases the expression of the skeletal muscle dihydropyridine receptor (DHPR) α_{1S} subunit by regulating DHPR α_{1S} nuclear transcription. In this study, we investigated the mechanism by which IGF-1 enhances expression of the DHPR α_{1S} gene. To this end, the promoter region of the mouse DHPR α_{1S} gene was recently cloned and sequenced and various promoter deletion-luciferase reporter constructs were used. These constructs were transfected into C2C12 cells and IGF-1 effects were measured by recording luciferase activity. IGF-1 significantly enhanced DHPR α_{1S} transcription in those constructs carrying cAMP-response element-binding protein (CREB) binding site but not in CREB core binding site mutants. Gel mobility shift assay using a double stranded oligonucleotide for the CREB site in the promoter region, and competition experiments with excess unlabeled or mutated promoter oligonucleotide, and unlabeled consensus CREB oligonucleotide demonstrated that IGF-1 induces CREB binding to the DHPR α_{1S} promoter. IGF-1-mediated enhancement in charge movement was prevented by incubating the cells with antisense but not with sense oligonucleotides against CREB. These results support the conclusion that IGF-1 regulates DHPR α_{1S} transcription in muscle cells by acting on the CREB element of the promoter.

In this study we investigated the mechanisms by which insulin-like growth factor 1 (IGF-1) regulates the expression of the skeletal muscle L-type Ca^{2+} channel or dihydropyridine-sensitive receptor DHPR α_{1S}. IGF-1 is a peptide structurally related to proinsulin and has a primary role in promoting skeletal muscle differentiation and growth (1). We have shown that IGF-1 regulates the ion permeation function of the dihydropyridine (DHP)-sensitive L-type Ca^{2+} channel in skeletal muscle (2, 3). DHPR and ryanodine receptor and sarcoendoplasmic reticulum Ca^{2+} content are directly involved in regulating the amplitude of the muscle fiber Ca^{2+} influx (see Ref. 4). Prior studies from our laboratory have shown that the age-related decrease in the number of DHPR and ryanodine receptor 1 isoforms can be prevented by overexpression of IGF-1 in skeletal muscle (5). We have also shown that IGF-1 enhances skeletal muscle charge movement, [\(^{3}H\)PN200–110 binding sites, and DHPR α_{1S} message expression in single muscle fibers from adult rats (6). Whether IGF-1 regulates DHPR α_{1S} expression by acting on specific consensus sequences of the DHPR α_{1S} 5′-flanking region is not known. To address this issue, a combination of molecular and electrophysiological techniques was used in the present study.

The DHPR α_{1S} (known also as Ca_{v}1.1 α_{1S}, or CaCh1) is encoded in the human chromosome 1q31–32 by the CACNA1S gene and expressed exclusively in skeletal muscle (7, 8). The DHPR consists of five subunits (α_{1}, α_{2}, β, γ, and δ), α_{1} being the subunit that senses changes in membrane voltage, forms the Ca^{2+} conduction pore, binds to dihydropyridines, and interacts with the sarcoplasmic reticulum Ca^{2+} release channel or ryanodine receptor 1 isoform to release Ca^{2+} from the organelle into the myoplasm in response to membrane depolarization (9–11). The DHPR is located at the infoldings of the sarcolemma, named T-tubule, and plays a critical role in excitation-contraction coupling (4). Because of the pivotal role of the DHPR α_{1S} subunit in excitation-contraction coupling, its expression is of crucial importance for skeletal muscle contraction. DHPR α_{1S} cDNA restores excitation-contraction coupling in dysgenic mice (12, 13). DHPR α_{1S} subunit expression is subject to regulation by a series of factors, including aging (5, 14), development (15), calcium (16), trophic factors (14, 17, 18), activity (19), and muscle denervation (20–22). Recently, we have demonstrated that IGF-1 and age regulate DHPR α_{1S} gene transcription in murine skeletal muscle (23). All these factors result in changes in DHPR α_{1S} subunit abundance. Despite the diverse modulation of channel expression, the molecular mechanisms underlying this process are not known. The recent characterization of the DHPR α_{1S} 5′-flanking region allows for a better understanding of channel transcription and abundance (24). In that study, we have identified the consensus sequence for three transcription factors involved in the regulation of the DHPR α_{1S} expression in muscle cells. Deletion experiments in the core of the consensus sequence for these
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transcription factors and antisense procedures support that GATA-2, CREB, and SOX-5 play a significant role in the DHPR α1S transcription and DHPR α1S subunit functional expression in differentiated skeletal muscle cells. Whether these transcription factors mediate IGF-1-induced DHPR α1S expression enhancement is not known.

EXPERIMENTAL PROCEDURES

Cell Culture—The mouse C2C12 muscle cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA), cultured in standard conditions, and maintained in growth medium (Dulbecco’s modified Eagle’s medium, supplemented with 20% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin). Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin was used as the differentiation medium.

Charge Movements Recordings—For charge movement recordings C2C12 cells were plated on glass coverslips and mounted in a small flow-through Lucite chamber positioned on a microscope stage. Myotubes were continuously perfused with the external solution (see below) using a push-pull syringe pump (WPI, Saratoga, FL). Cells were voltage-clamped in the whole cell configuration of the patch clamp (25) using an Axopatch-200B amplifier (Axon Instruments, Foster City, CA). Micropipettes were pulled from borosilicate glasses (Boralex) using a Flaming-Brown micropipette puller (PM7, Sutter Instrument Co., Novato, CA) to obtain electrode resistance ranging from 2 to 4 MΩ. The composition of the internal solution (pipette) was (mM): 140 Cs-aspartate, 5 Mg-aspartate, 10 Cs-EGTA, 10 HEPEs, pH adjusted to 7.4 with CsOH. The high concentration of Mg2+ in the pipette solution helped to maintain the preparation stable for longer time. The external solution contained (mM): 145 tetraethylammonium hydroxide-Br, 2 CaCl2, 0.5 Cd2+, 0.3 La3+, 10 HEPEs, and 0.001-0.003 tetrodotoxin (26). Solution pH was adjusted to 7.4 with CsOH. This solution was used for forming gigaseals. For charge movements recording, calcium current was blocked with a solution containing (mM): 145 tetraethylammonium hydroxide-Br, 2 CaCl2, 0.5 Cd2+, 0.3 La3+, 10 HEPEs, and 0.001-0.003 tetrodotoxin (26). The integral of the charge movement (see below) was used for the statistical analysis.

Whole cell currents were acquired and filtered at 5 kHz with pClamp 6.04 software (Axon Instruments). A Digidata 1200 interface (Axon Instruments) was used for A-D conversion. Membrane current during a voltage pulse, P, was initially corrected by analog subtraction of linear components. The remaining linear components were digitized on-line using hyperpolarizing control pulses of one-quarter test pulse amplitude (–P/4 procedure) (20). The four control pulses were applied before the test pulse. Charge movements were evoked by 25 ms depolarizing voltage steps from the holding potential (–80 mV) to command potentials ranging from 70 to 70 mV. Intramembrane charge movements were calculated from myoblast and myotubes of C2C12 cells was extracted as described (23). Briefly, the cells were collected and washed in Tris-buffered saline. The cell pellets were resuspended in cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) by gentle pipetting. After swelling on ice for 15 min, 10% Nonidet P-40 solution was added and vortexed for 10 s. After centrifugation, the nuclear pellets were suspended in cold buffer C (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and rocked at 4°C for 15 min. The supernatants were frozen in aliquots at –70°C. Protein concentration was determined with Coomassie Plus Protein Reagent (Pierce Chemical Co.).

Nuclear Protein Extraction and Gel Mobility Shift Assay—Nuclear protein extracts were purified from skeletal muscle and C2C12 cell were prepared by reverse transcriptase-PCR, cloned into pCRII vector, and linearized with BamHI (New England Biolabs Inc., Beverly, MA). DHPR α1S probe (140 bp plus 82 bp vector fragment) was labeled with [32P]UTP by in vitro transcription. The probe for 28S RNA consists of 115 bp plus the 67-bp vector fragment. Both the pTRI-RNA-28S antisense control template and the RNA century marker template set (Ambion Inc.) were also labeled by the in vitro transcription method using different dilutions of [32P]UTP.

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Data are expressed as mean ± S.E. with the number of observations (n).

RESULTS
We investigated the effects of IGF-1 on DHPR α1 subunit protein, DHPR α1S mRNA, and the role of IGF-1 in the basal expression of the DHPR α1 subunit, to identify the element(s) involved in the IGF-1-mediated regulation of DHPR α1S expression. We also defined the transcription factor(s) critical for IGF-1 effects on DHPR α1S promoter-luciferase fusion plasmid, and examined the functional effects of antisense oligonucleotides for this transcription factor on charge movement.

IGF-1 Increases DHPR α1S Expression in Muscle Cells—We have previously demonstrated that IGF-1 enhances DHPR α1S protein expression (6) by increasing nuclear transcriptional activity in skeletal muscle (31). To study the specific regulatory elements involved in IGF-1-mediated enhancement of DHPR α1S, we examined first the effects of IGF-1 on the levels of expression of the channel subunit in C2C12 cells. To this end, a combination of immunoprecipitation and Western blot was used to improve the signal of the DHPR α1 subunit. Repeated determinations (n = 4) revealed that the DHPR α1 subunit expresses in myotubes and that IGF-1 increases the expression of DHPR α1 subunit protein. The IGF-1 concentration (20 ng/ml) and the exposure time (3 days in differentiation medium) used in the present work have been found optimal to enhance DHPR α1S expression in rat skeletal muscle primary culture (6). Fig. 1 shows a band at 210 kDa corresponding to the DHPR α1 subunit in C2C12 myotubes. The size of the band was determined using molecular weight markers as explained under “Experimental Procedures.” The size of the DHPR α1 subunit band is similar to that reported previously (32–34). This band is enhanced in myotubes treated with IGF-1 but not in cells treated with IGF-1 plus the IGF-1R tyrosine kinase inhibitor I-OME-AG538 (25 μM). It is also apparent that the inhibitor by itself does not modify the expression of the DHPR α1 subunit (Fig. 1). For these experiments, 500 μg of total proteins extracted from C2C12 cells lysates were used for immunoprecipitation with goat anti-DHPR α1 antibody. The precipitates were analyzed in SDS-PAGE gels and detected by Western blot as described above (“Experimental Procedures”). To explain the increase in DHPR α1 protein we examined whether IGF-1 enhances DHPR α1S mRNA in C2C12 cells.

IGF-1 Enhances DHPR α1S Gene Expression—Repeated RPA analysis revealed that a 140-bp protected fragment corresponding to DHPR α1S mRNA was present in total RNA samples from C2C12 myotubes (Fig. 2). Molecular weight was determined using radiolabeled RNA markers loaded on the same gel as described above. RPA was performed using specific cRNA probes for mouse DHPR α1S and 28 S rRNA (see “Experimental Procedures”). DHPR α1S expression in C2C12 myotubes was recorded in four experiments in which 25 μg of RNA from proliferating and differentiated cells corresponding to the same cell passage were analyzed. These results together with data on mouse skeletal muscle (24) support the concept that differentiation provides the conditions for DHPR α1S expression. Fig. 2 also shows that IGF-1 significantly increased DHPR α1S expression, a phenomenon that can be prevented by I-OME-AG538. No effects of this agent by itself were recorded by DHPR α1S expression (Fig. 2).

IGF-1 Enhances the Expression of DHPR α1S Promoter Deletion Constructs in Muscle Cells—To analyze the DHPR α1S 5'-flanking region elements that may play a role in controlling IGF-1-dependent enhancement of DHPR α1S gene transcription, a gene chimera was created by cloning the 5'-flanking sequence and a portion of exon 1 of the DHPR α1 subunit (−1076 to +129) upstream of a luciferase reporter gene. Chimera deletion constructs were made starting at the 5’-end of the chimera and progressing in the 3’ direction. Full and deletion constructs were subcloned into pGL3/basic plasmid and transfected into C2C12 cells. The pGL3/basic vector containing the luciferase reporter gene and lacking the DHPR α1S DNA was used as a control. Fig. 3 illustrates the full-length 5'-flanking region (−1076/+128)-luciferase reporter gene and 3 deletion constructs. The most distal 5’-end nucleotide from the transcription start site identifies each construct.

The expression of Luc/P-1076 has been shown to be dependent on cell differentiation and subtype (24). In our prior study (24) we have shown that the maximum luciferase activity was recorded at day 5 in differentiation medium in transfected C2C12 cells with the Luc/P-1076 construct. In the present work, we selected day 3 for luciferase activity recording because the C2C12 cells exposed to IGF-1 started to detach from the bottom of the chamber thereafter. Fig. 3 shows the relative luciferase activity recorded in the four 5'-flanking promoter-luciferase gene constructs transfected simultaneously into different groups of C2C12 cells. The relative luciferase activity was normalized to the Luc/P-1076 construct luminescence signal. Luciferase activity was much greater for Luc/P-1076 than for any of the other three constructs (p < 0.001) (n = 5). Enhancer and repressor elements in the 1.2 kb of the 5'-flanking region of the DHPR α1S have been discussed previously (24). Fig. 3 also shows that IGF-1 increases significantly the
luciferase activity at concentrations equal or greater than 50 μM I-OMe-AG538 or IGF-1 plus I-OMe-AG538 and control was 1.55, relative to the luminescence signal of the Luc/P-1076 construct (100%). The ratio between cells treated with I-OMe-AG538 or IGF-1 plus I-OMe-AG538 and control was 1.55, 1.53, and 1.46, respectively.

**Fig. 2.** IGF-1 increases DHPR α1S expression detected by ribonuclease protection assay. Total RNA (25 μg) extracted from control (untreated), and treated with I-OMe-AG538 alone or IGF-1 plus I-OMe-AG538 C2C12 myotubes, were assessed separately by RPA using specific cRNA probes for mouse DHPR α1S (140 bp plus 82 bp corresponding to the vector fragment). Results were normalized to the levels of 28 S rRNA (115 plus 67 bp of the vector). The ratio between cells treated with I-OMe-AG538 or IGF-1 plus I-OMe-AG538 and control was 1.55, 1.53, and 1.46, respectively.

**Fig. 3.** IGF-1 enhances the expression of DHPR α1S promoter deletion constructs expressed in C2C12 cells. The 5'-flanking region was progressively deleted from the 5'-end and fused to the pGL3 basic vector. The deletion constructs are numbered relative to the transcription start site. The relative luciferase activity was normalized to the luminescence signal of the Luc/P-1076 construct (100%). +, indicates the statistically significant difference compared with myotubes treated with IGF-1 for each deletion construct. The results presented are the mean ± S.E.

Luc/P-1076
Luc/P-1076/CREB-deleted construct
Luc/P-1076/CREB-deleted construct plus I-OMe-AG538

**Fig. 4.** Blockade of IGF-1 effects on CREB-mediated DHPR α1S expression. A. transient expression of the full promoter-luciferase construct (Luc/P-1076) in C2C12 cells treated with a combination of IGF-1 and the inhibitor I-OMe-AG538 (10–100 μM). The firefly luciferase activity was normalized to co-transfected pRL-TK renilla values. *, indicates statistically significant differences compared with myotubes treated with IGF-1 plus inhibitor (10–25 μM). B. deletion of the CREB binding site in the full-length Luc/P-1076 construct prevents the effects of IGF-1 and I-OMe-AG538 on DHPR α1S expression. Asterisks indicate the statistically significant differences with control cells transfected with Luc/P-1076 constructs treated and untreated with IGF-1.

**Role of CREB in DHPR α1S Transcription**—To determine the role of specific transcription factors in DHPR α1S transcription, deletion constructs of the Luc/P-146 construct were produced (Fig. 5A). Luc/P-146 is the shortest construct that exhibits reliable and reproducible luciferase signal and is significantly enhanced by IGF-1. Therefore, this is the construct that was used for mutational analysis. Fig. 5B shows the relative luciferase activity for three different constructs consisting of 4-bp deletions at the center of CREB, GATA-2, and MZF1 binding site sequences, respectively. Deletions in the binding sequence for CREB and GATA-2 led to significant reductions in luciferase activity in untreated cells (p < 0.01) (n = 5), underscoring the importance of these factors in DHPR α1S transcription (24). The Luc/P-146 GATA-Mut construct showed a significant potentiation of the luciferase activity in the group of cells treated with IGF-1 for 3 days; however, this effect was not recorded in cells transfected with the Luc/P-146 CREB Mut. These results support the concept that CREB binding to its consensus sequence in the DHPR α1S promoter region is needed for IGF-1 regulation. The relative luciferase activity in the cells transfected with the four chimeric constructs and that even the shortest construct (Luc/P-146) tested exhibits IGF-1 potentiation.

**Blockade of IGF-1 Effects on DHPR α1S Expression**—Recombinant IGF-1 has been used in the experiments described above, however, C2C12 cells can synthesize and secrete IGF-1 and express IGF-1R (35). Therefore, we examined whether endogenous, in addition to exogenous, recombinant IGF-1 modulate the expression of DHPR α1S. To this end, untreated and IGF-1-treated cells expressing the full promoter-luciferase construct (Luc/P-1076) were examined for the expression of DHPR α1S. To this end, untreated and IGF-1-treated cells expressing the full promoter-luciferase construct (Luc/P-1076) were examined for the expression of DHPR α1S in the presence or absence of 10–100 μM I-OMe-AG538 (36). I-OMe-AG538 has been selected among several tyrosine kinase inhibitors because of its reported action on IGF-1R kinase (36) and to its significantly less pronounced inhibition of C2C12 cell differentiation. Fig. 4A shows that I-OMe-AG538 decreases luciferase activity at concentrations equal or greater than 50 μM (double asterisks), whereas recombinant IGF-1 significantly enhances this signal in the absence of I-OMe-AG538 (single asterisk). The IGF-1-mediated enhancement of DHPR α1S expression was blocked by the inhibitor in the whole range of concentrations tested (10–100 μM) (n = 5). These experiments indicate that 25 μM, the I-OMe-AG538 concentration used in this work, blocks exogenous IGF-1 effects but does not alter the basal DHPR α1S expression. To investigate whether CREB is the only IGF-1 regulatory element, we tested the effect of IGF-1 on luciferase activity in myotubes transfected with the Luc/P-1076/CREB-deleted construct (Fig. 4B). Luciferase activity was significantly different in control (Luc/P-1076), the CREB-deleted construct, and the CREB-deleted construct plus 25 μM I-OMe-AG538. IGF-1 enhanced luciferase activity only in the full control construct but not in the CREB-deleted construct in the presence or absence of the inhibitor (Fig. 4B). These results support the concept that CREB mediates IGF-1 regulatory effects on DHPR α1S gene expression.
IGF-1 in the IGF-1-mediated potentiation of Luc/P-146 MZF-1-Mut. These results do not support a role for IGF-1 enhanced the luciferase activity in cells transfected with No explanation for this effect is obvious at the present time.

conclusion that IGF-1 modulates AG538 alone or in combination with IGF-1 give support to the expression. Control experiments using the inhibitor I-OMe-DHPR

ment that GATA is important for expression (24).

The low level of activity of Luc/P-146 GATA-Mut could suggest that GATA is important for DHPR α1S expression. To further assess this issue, the effects of sense and antisense for GATA-2, in the presence and absence of IGF-1, were examined in cells transfected with Luc/P-146 MZF-1-Mut. These results do not support a role for MZF-1 in the IGF-1-mediated potentiation of DHPR α1S gene expression. Control experiments using the inhibitor I-OMe-AG538 alone or in combination with IGF-1 give support to the conclusion that IGF-1 modulates DHPR α1S expression by acting on the CREB element of the DHPR α1S promoter region.

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Nuclear Proteins Involved in IGF-1-mediated Enhancement of DHPR α1S Gene Transcription.—To determine the key elements involved in IGF-1-mediated enhancement of the DHPR α1S gene transcription, we performed gel shift assays as an alternative approach to the deletion constructs described above. This procedure allows us to identify the nuclear proteins involved in IGF-1 effects on the DHPR α1S gene. Nuclear proteins from IGF-1-treated myotubes were extracted as described (23). To further support that CREB is binding to the promoter, a double stranded oligonucleotide for the putative CREB site in the promoter was used as the probe for the assay (D8). As additional tests of binding specificity, the effects of competition with 50-fold excess CREB, unlabelled consensus CREB oligonucleotide, or mutated oligonucleotide (D8mut) have been studied. Fig. 7A shows the DNA-protein complexes resolved on 10% native polyacrylamide gel electrophoresis and visualized in x-ray film. The a and b bands disappear in the presence of 50-fold excess of unlabeled CREB or 50-fold excess of D8 oligonucleotide, but not in the presence of 50-fold excess of mutant D8 (D8mut). We repeated the experiment four times with consistent results. The band corresponding to the free probe is missing in Fig. 7 because the gel was run for 90 min to better separate the a and b bands. These experiments provide further evidence for the role of CREB in IGF-1-mediated enhancement of the DHPR α1S gene transcription. As a further control Fig. 7B shows a gel mobility assay for nuclear extracts (5 μg) from IGF-1-treated and untreated (control) myotubes.

Effects of Antisense Oligonucleotide for CREB on Charge Movement.—As a functional expression of the DHPR α1 subunit we recorded charge movement in differentiated myotubes after 36–48 h incubation in 1 μM sense or antisense oligonucleotides for CREB plus IGF-1 and the results were compared with recordings in control cells not incubated in sense or antisense. Although the DHPR α1 subunit accounts for only 70% of the total nonlinear capacity of the membrane (27), we preferred the recording of charge movement to calcium current because of the direct relationship between the integral of charge movement and the levels of channel expression in the sarcolemma (37). Also, the amplitude of the L-type calcium current varies in response to channel regulation and percentage of silent channels (2, 38). As the charge movement reported here is similar to that recorded previously with the patch clamp (39) but higher than that recorded with other techniques (20, 40, 41), we studied both linear capacitive transients for the voltage steps from −80 to −90 mV, and the unsubtracted current traces for the
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In the present study, we identified CREB as the transcription factor involved in IGF-1 regulation of the DHPR $\alpha_{1S}$ promoter-luciferase fusion plasmids. Gel shift competition assays and experiments using the IGF-1R inhibitor I-OMe-AG538 demonstrated that IGF-1 enhanced the levels of phosphorylated CREB. Patch clamp recording showed that antisense oligonucleotide against CREB decreases charge movement and prevents the effect of IGF-1 on DHPR $\alpha_{1S}$ expression in C2C12 myotubes.

**IGF-1 Increases DHPR $\alpha_{1S}$ Expression in Muscle Cells**—A series of experiments included in the present study demonstrate that IGF-1 increases the expression of the DHPR $\alpha_1$ subunit protein as a result of increasing gene transcription in C2C12 cells. These results are consistent with previous studies performed in mouse skeletal muscle and rat muscle primary culture that demonstrate that IGF-1 increases DHPR $\alpha_{1S}$ nuclear transcription (6, 31). We investigated whether endogenous or recombinant IGF-1 has an effect on DHPR $\alpha_{1S}$ gene expression. To this end, we measured the effects of the IGF-1R tyrosine kinase inhibitor I-OMe-AG538 (36) on luciferase activity in transfected myotubes with various DHPR $\alpha_{1S}$ promoter-luciferase fusion constructs. This recently synthesized compound inhibits IGF-1R autophosphorylation and the activation of the downstream targets protein kinase B and Erk2 (36). IC$_{50}$ concentration (25 $\mu$M) (Fig. 4) (36) was used for these experiments. Using this concentration of I-OMe-AG538, no effects on luciferase activity or cell differentiation were observed. This is in contrast with marked effects of the protein kinase inhibitor genistein on cell differentiation (data not shown). A more specific effect of I-OMe-AG538 on the IGF-1R could explain the differential effects on cell differentiation. These experiments support a role for IGF-1 on DHPR $\alpha_{1S}$ expression. Whether muscle differentiation and modulation of DHPR $\alpha_{1S}$ expression share common signaling pathways cannot be addressed at the present time.

**CREB Mediates IGF-1-dependent Enhancement of DHPR $\alpha_{1S}$ Gene Transcription**—We have recently reported the cloning of the mouse L-type/DHPR $\alpha_{1S}$ subunit gene 5' flanking sequence and the specific sequences necessary for basal transcription and control of the DHPR $\alpha_{1S}$ expression. Deletion analysis of the 5' flanking region in the DHPR-luciferase fusion gene indicates that cis-acting regulatory elements in the proximal 146 bp appear to be essential for skeletal muscle cell-specific expression of the DHPR $\alpha_{1S}$ subunit gene. Transfection of the deletion construct Luc/P-146 in C2C12 cells resulted in significant luciferase activity. CREB and GATA-2 consensus sequences in the 146 bp upstream of the transcription start site are critical for DHPR $\alpha_{1S}$ expression (24). In the present study we investigated the role of CREB, GATA, and MZF-1 as potential mediators of IGF-1-dependent enhancement of DHPR $\alpha_{1S}$ transcription. The study of the consensus sequence for these transcription factors is based on their expression in the first 146 bp upstream of the transcription start site. We have found that mutation in the consensus sequence for CREB and GATA significantly decreases luciferase activity. These results are consistent with a recent report from our laboratory (24). Significant potentiation of the DHPR $\alpha_{1S}$ expression was induced by IGF-1 on the cells transfected with the GATA mutant but not with CREB. Ablation of the myeloid zinc finger consensus MZF-1 binding sequences did not significantly modify luciferase activity (24). These results support the concept that CREB
mediates IGF-1 potentiation of DHPR \( \alpha_{1S} \) transcription. Several consensus sequences have been described in the DHPR \( \alpha_{1S} \) promoter region spanning from nucleotides −146 to −1076, SOX-5 being one of them (24). We have reported previously that SOX-5 regulates DHPR \( \alpha_{1S} \) transcription. The role of SOX-5 in DHPR \( \alpha_{1S} \) transcription was not analyzed in the present work because Luc−146, a construct lacking a consensus binding sequence for SOX-5, exhibits potentiation of the DHPR \( \alpha_{1S} \) transcription evoked by IGF-1. Ongoing studies are exploring the role of specific signaling pathways linking IGF-1R activation and CRE binding to the DHPR \( \alpha_{1S} \) promoter region. Phosphorylation at a single Ser133 residue greatly enhances the activity of CREB bound to the response element CRE. Therefore, phosphorylated CREB is the active form that regulates gene transcription. Whether CREB is phosphorylated by protein kinase A, C, or any other calcium-dependent protein kinase in response to IGF-1R activation is not currently known.

CREB-mediated IGF-1 Potentiation of Charge Movement—Charge movements are currents arising on movement of charge molecules dwelling in the membrane (42). The integral of the recordings are directly related to the number of moving charged molecules (37). The maximum control charge movement reported here is similar to that reported by us previously (24) but 1.35- and 2.3-fold higher than that reported by other groups (40, 43), using microelectrode techniques. Although several voltage-gated channels contribute to charge movement, these recordings represent mainly the activity of the DHPR \( \alpha_1 \) subunit (27). We have performed high affinity radioligand binding assays in muscle cells treated with IGF-1 to determine whether the effect on charge movement results from an increase in DHPR \( \alpha_1 \) subunits or on the remaining 30% of the charge not attributable to the L-type Ca\(^{2+}\) channel (18). In that publication (18), we reported a significant increase in binding sites in the cells treated with the same concentration of IGF-1 used in the present study.

In the present work we have recorded a significant effect of antisense oligonucleotides for CREB on charge movements as an indication of down-regulation of the number of DHPR \( \alpha_1 \) subunits expressed in the sarcolemma. We have also reported that IGF-1 does not enhance charge movement in the presence of CREB-antisense. These results, together with the data on DHPR \( \alpha_1 \) subunit expression and DHPR \( \alpha_{1S} \) mRNA, support the concept that IGF-1 enhances the charge movement arising from DHPR \( \alpha_1 \) subunits. Whether antisense oligonucleotides for CREB result in decreased expression of other skeletal mus-
cle voltage-gated ion channels is not known. This possibility cannot be ruled out at the present time.

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Insulin-like Growth Factor-1 Increases Skeletal Muscle Dihydropyridine Receptor α1S Transcriptional Activity by Acting on the cAMP-response Element-binding Protein Element of the Promoter Region

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Insulin-like growth factor-1 increases skeletal muscle dihydropyridine receptor \( \alpha_{\text{IS}} \) transcriptional activity by acting on cAMP-response element-binding protein element of the promoter region.

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Page 50541, Fig. 8C: The numbers on the right are inverted. They should read 30, 10, \(-10\), and \(-30\) from top to bottom. The correct figure is shown below.

**Fig. 8.** Effects of antisense oligonucleotide for CREB on charge movement. Charge movement recorded in differentiated myotubes incubated in 1 \( \mu \)M sense or antisense oligonucleotides for CREB plus IGF-1. Results are compared with records in control cells not incubated in sense or antisense oligonucleotides. A, \( Q_m \)-membrane voltage relationship for cells treated with 20 ng/ml IGF-1 alone (filled circles), sense (triangles), or antisense (squares) for CREB plus IGF-1 and control (open circles) C2C12 cells. Data points were fitted to a Boltzmann equation (see text, Equation 1). Charge movement recordings were in the \(-30\) to 30 mV range for control (B), IGF-1 plus CREB-antisense (C), IGF-1 (D), and IGF-1 plus CREB sense-treated cells (E). The best fitting parameters for \( Q_{\text{max}}, V_{\text{Q1/2}}, \) and \( K \) recorded in the four experimental groups are included in Table I. Dotted lines indicate the baseline.

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