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Ca^{2+} Calmodulin Kinase and Calcineurin Mediate IGF-1-induced Skeletal Muscle Dihydropyridine Receptor α_{1S} Transcription

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Abstract. The skeletal muscle L-type Ca^{2+} channel or dihydropyridine(DHP)-sensitive receptor is a key molecule involved in membrane voltage-sensing, sarcoplasmic reticulum Ca^{2+} release, and muscle contraction. Previous work from our laboratory has shown that the insulin-like growth factor-1 (IGF-1) increases skeletal muscle L-type Ca^{2+} channel or dihydropyridine-sensitive receptor $DHPR\alpha_{1S}$ transcriptional activity by acting on the cyclic AMP response element binding protein (CREB) element of the promoter region; however, the cellular signaling mediating this process is not known. In this study, we investigated the signaling pathway whereby IGF-1 enhances the expression of $DHPR\alpha_{1S}$ in C2C12 myotubes, using a molecular, pharmacological and electrophysiological approach. We found that inhibition of the Ca^{2+}/Calmodulin (CaM)-dependent protein kinase or calcineurin, influenced IGF-1-induced increase in $DHPR\alpha_{1S}$ expression, as detected by recording the luminescence of the $DHPR\alpha_{1S}$ promoter–luciferase fusion construct and by immunoblot analysis of the DHPR α1 subunit. IGF-1 significantly increased CaM kinase and calcineurin activity and the cellular levels of phosphorylated CREB in a time-dependent manner. The role of CaM kinase and calcineurin in $DHPR\alpha_{1S}$ expression was confirmed by functional recording of the effects of the inhibition of the kinase and phosphatase on IGF-1-mediated enhancement of charge movement. These results support the conclusion that IGF-1 controls CREB phosphorylation by activating a phosphorylation and dephosphorylation cascade, which ultimately modulates the $DHPR\alpha_{1S}$ gene transcription.

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Key words: Skeletal muscle — Dihydropyridine receptor — IGF-1 — CREB — CaM Kinase — Calcineurin — Calcium channels — Gene transcription

Introduction

The characterization of the cellular signaling that mediates the action of trophic factors on the expression of voltage-gated ionic channels in excitable cells is functionally relevant due to the effects of these factors in cell proliferation, differentiation, maintenance, and repair, among other functions. Also, some of these functions are evoked by activation of voltage-sensitive ion channels (*see* below). In the present study, we investigated the signaling pathway whereby IGF-1 enhances the expression of the skeletal muscle L-type Ca^{2+} channel or dihydropyridine(DHP)-sensitive receptor $DHPR\alpha_{1S}$. IGF-1 has a primary role in neuromuscular trophism in adult and senescent mammals (Barton-Davis et al., 1998; Rabinovsky et al., 2003; Renganathan, Messi & Delbono, 1998; Rabinovsky et al., 2003). We have demonstrated that IGF-1 regulates the expression and function of the DHPR, a key molecule involved in excitation-contraction coupling. IGF-1 regulates ion permeation through DHPR in skeletal muscle (Delbono, Renganathan & Messi, 1997; Renganathan, Sonntag & Delbono, 1997c) and its voltage-sensing property (Wang, Messi & Delbono, 2002). Prior studies from our laboratory have shown that the age-related decrease in the number of DHPR and RyR1 can be prevented by transgenic overexpression of IGF-1 in skeletal muscle (Renganathan, Messi & Delbono, 1997a). We have shown that IGF-1 also enhances skeletal muscle charge movement, the number of DHP binding sites and $DHPR\alpha_{1S}$ message in single fibers from adult rats (Wang et al., 1999b). More

recently, we have demonstrated that IGF-1 regulates $DHPR\alpha_{1S}$ expression by acting on the CREB element of the $DHPR\alpha_{1S}$ 5′-flanking region (Zheng, Wang & Delbono, 2002a).

The DHPR α_1 subunit 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 RyR1, to release Ca^{2+} from the organelle into the myoplasm in response to membrane depolarization (Block et al., 1988; Meissner, 1994; Schneider, 1994). Due to the central role of the DHPR α_1 subunit in excitation-contraction coupling, its expression is of crucial importance for skeletal muscle contraction. $DHPR\alpha_{1S}$ cDNA restores excitation-contraction coupling in dysgenic mice (Tanabe et al., 1988, 1990). DHPRα_{1S}-subunit expression is subject to regulation by trophic factors (Renganathan et al., 1997b, 1998; Wang, Messi & Delbono, 1999a), activity (Saborido et al., 1995) and muscle denervation (Delbono, 1992; Delbono & Stefani, 1993; Pereon et al., 1997). Recently, we have demonstrated that IGF-1 and age regulate $DHPR\alpha_{1S}$ transcription in murine skeletal muscle (Zheng, Messi & Delbono, 2001). These factors result in changes in DHPRα_{1S}-subunit expression. Despite the diverse modulation of channel expression, the molecular mechanisms underlying this process are not known. The characterization of the $DHPR\alpha_{1S}$ 5′-flanking region allowed for a characterization of channel transcription and abundance (Zheng, Wang & Delbono, 2002b). We have also shown that IGF-I significantly enhanced $DHPR\alpha_{1S}$ transcription by acting on the CREB element of the promoter (Zheng et al., 2002a). To characterize the signaling pathway linking IGF-1 activation and $DHPR\alpha_{1S}$ expression, we used a combined molecular, pharmacological and electrophysiological approach. The results reported here support the conclusion that CaM kinase and calcineurin regulate $DHPR\alpha_{1S}$ gene transcription by modulating CREB phosphorylation.

Materials and Methods

Cell Culture

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

Construction of $DHPR\alpha_{1S}$ Promoter—luciferase fusion plasmid

A partial fragment of the DHPRα_{1S} promoter (P-146) (Zheng et al., 2002b) (GenBank accession number for the 5′ flanking region is AF343753) was fused with the luciferase reporter gene as explained previously (Zheng et al., 2002b). The deletion construct was generated by PCR cloning, using specific primers. The correct orientation of the construct and sequence was confirmed by DNA sequencing (ABI Prism Cycle Sequencing, Perkin Elmer Co, Norwalk, CT). The Luc/P-146 construct was transfected into C2C12 cells following described procedures (Zheng et al., 2002a; Zheng et al., 2002b).

Transfection of C2C12 with $DHPR\alpha_{1S}$ Promoter—Luciferase Fusion Plasmid and Dual Luciferase Assay

All of the plasmids used in the present work were purified with QIAfilter (Qiagen, Valencia, CA). Cells were plated at a density of 2×10^4 cells, grown in growth medium on 35 mm dishes till reaching 70% confluence. For cell transfection, 1 μg of each plasmid and 200 ng of the control vector pRL-TK (Promega) were mixed with 2 μl FUGENE6 (Roche, Indianapolis, IN) for 20 min at room temperature and added to the medium directly. Cells were induced to differentiate by changing to differentiation medium and cultured for 3 days. Cell lysis was prepared using a passive lysis buffer for the dual Luciferase reporter assay (Promega, Madison, WI). Luciferase and renilla activity were measured using a luminometer (Turner 20E, Sunnyvale, CA) and expressed as arbitrary units. Values for the luciferase assay were normalized to renilla luciferase activity to minimize differences in transfection efficiency for each experiment.

Immunoprecipitation and Western Blot

For immunoprecipitation and Western blot analysis, C2C12 cells were washed with PBS, treated with 1% digitonin buffer (1% digitonin, 185 mM KCl, 1.5 mM $CaCl_2$, 10 mM HEPES pH 7.4) on ice and centrifuged at $10,000 \times g$ for 10 min at 4°C. Protein concentration was measured using BCA Protein Assay (Pierce). The lysate (500 μg total cellular protein) was precleared by adding 0.5 μg of the appropriate control IgG (normal goat IgG) together with 20 μl of resuspended volume of the appropriate agarose conjugate (Protein G-Agarose). After centrifugation at $1,000 \times g$, the supernatant was transferred to a fresh tube on ice. Goat anti-DHPRα_{1S} N-19 primary antibody (1 μg) (Santa Cruz Biotechnology, Santa Cruz, CA) was added and incubated. Only goat IgG was added to the control tube. The resuspended volume of the Protein G-Agarose (20 μl) was added to each tube and incubated at 4°C on a rotating device overnight. After centrifugation ($1,000 \times g$), the pellets were washed with PBS and resuspended in 20 μl of electrophoresis sample buffer. The samples were boiled for 2–3 min and analyzed in 10% SDS-PAGE gel. Rainbow molecular weight markers (Amersham Pharmacia Biotech, Piscataway, NJ) were loaded. Proteins were transferred from the gel to nitrocellulose membrane. Nonspecific binding was blocked by incubating the nitrocellulose membrane in 5% milk Tris buffer saline Tween (TBST) for 30–60 minutes at room temperature. Incubation in the primary antibody (1:100 diluted in blot buffer) was done for 1 h at room temperature and washed three times for 5 min each with TBS, 0.05% Tween-20. The membrane was incubated with anti-Goat IgG conjugated with horseradish peroxidase, washed, and finally incubated in ECL Reagent (Pierce) and visualized in X-ray films.

For CREB and phospho-CREB detection, the blots were blocked using a solution containing 20 mM Tris (pH 7.6), 140 mM NaCl, 1% nonfat powdered milk, 1% BSA (bovine serum albumin) and 0.1% Tween 20, for 1 h, and then probed with either anti-CREB or anti-phospho-CREB antibody (Cell Signaling Technology, Beverly, MA) in a solution of 20 mM Tris HCl (pH 7.6), 140 mM NaCl, 1% bovine serum albumin, 0.1% Tween 20, for 2 h

(Gangolli et al., 2000). The blots were then washed and incubated with horseradish peroxidase-conjugated secondary antibody and visualized using the Amersham ECL system. Autoradiograms were scanned and analyzed with KODAK-1D Image Analysis Software (Eastman Kodak Company, Rochester, NY). Phospho-CREB levels were quantified and normalized to total CREB levels.

CaM Kinase Activity

CaM kinase activity was measured using a CaM kinase assay kit (Upstate Biotechnology, Lake Placid, NY). The specific substrate peptide for CaMK II was KKALRRQETVDAL (autocamtide II). Desalted samples (10 μl) were combined with substrate solution (30 μl) containing 10 μg autocamtide II or peptide γ, 1 μM protein kinase A inhibitor peptide, 1 μM protein kinase C (PKC) inhibitor peptide, 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT and 1 mM $CaCl_2$ (assay dilution buffer; ADB). For the control reactions, 10 μl of the desalted sample were combined with 30 μl ADB without peptide γ, or autocamtide II. After this, 10 μl [γ-^{32}P] ATP (1 μCi) (ICN) diluted in ADB, 500 μM ATP and 75 mM $MgCl_2$ was added to each sample and incubated at 30°C for 10 min. After incubation, a 25 μl aliquot was spotted onto p81 phosphocellulose papers, which were placed in a glass scintillation vial containing 15 ml of 0.75% phosphoric acid. After 30 min mixing at room temperature, the washing reagent was decanted. Each paper disk was then mixed with 10 ml of 0.75% phosphoric acid, followed by 10 ml acetone, for 10 min, at room temperature. Each reagent was decanted after each washing. Finally, 10 ml of scintillant Ecoscint H (National Diagnostics, Atlanta, GA) was added and the radioactivity remaining on each binding paper counted in a liquid scintillation counter. Nonspecific binding of [γ-^{32}P] ATP to the binding paper without substrates (peptide γ, autocamtide II) was subtracted from each sample, including the substrate.

Calcineurin Activity

Muscle calcineurin activity was measured using the serine/threonine phosphatase assay system (Promega). The procedures followed the manufacturer's protocol with some modifications. In brief, cell lysates in the kinase assay buffer above were desalted using Sephadex G25 spin columns to remove the excess of phosphate. Aliquots of desalted samples were added to the assay buffer containing (in mM): 100 NaCl, 50 Tris, 6 $MgCl_2$, 0.5 $CaCl_2$, 0.5 mM DTT, and 0.025% NP-40 containing Ser/Thr phosphopeptide RRA(pT)VA, as a substrate for calcineurin, and okadaic acid (Calbiochem, San Diego, CA). The control groups were treated with EGTA assay buffer containing (in mM): 10 EGTA, 100 NaCl, 50 Tris, 6 $MgCl_2$, 0.5 $CaCl_2$, 0.5 DTT, 0.025% NP-40. Samples were incubated at 30°C for 10 min, after which 50 μl of molybdate dye/additive mixture were added to stop the reaction. The optical density at 630 nm (OD_{630}) was determined for each sample. Calcineurin-specific phosphatase activity was determined by subtracting the OD_{630} of samples with assay buffer from the OD_{630} of samples in the presence of EGTA. The amount of free phosphate was determined by using a phosphate standard curve, which was performed in parallel with the samples. Total protein content of each group was determined in triplicate, using the BCA protein assay method. Calcineurin activity was normalized to total protein levels.

Chemicals

The following compounds: FPT Inhibitor III or {(E,E)-[2-Oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic acid, (2,2-dimethyl-1-oxopropoxy)methyl ester, Na}; PD98059 (2′-amino-3′-methoxyflavone), SB 203580-Sulfone or [4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1H-imidazole], ZM 336372 or {N-[5-(3-dimethylaminobenzamido)-2-methylphenyl]-4-hydroxybenzamide}, bisindolylmaleimide I or {2-[l-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide}, KT5823, KN-62 {1-[N,O-*bis*-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine}, H-89 {N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl)}, cyclosporine A, and a-Cyano-(3-methoxy,4-hydroxi,5-iodo)cinnamoyl-(3′,4′-dihydroxyphenyl)ketone or tyrphostin I-OMe-AG 538 were purchased from Calbiochem, and Wortmannin from Alomone Labs (Jerusalem, Israel). FPT III and cyclosporine A were dissolved in distilled water and ethanol, respectively, whereas the remaining drugs were dissolved in DMSO. Vehicles were used at a final dilution of 1:1000 or higher in the culture dishes.

Charge-Movement 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 (Hamill et al., 1981) using an Axopatch-200B amplifier (Axon Instruments, Union City, CA). Micropipettes were pulled from borosilicate glasses (Boralex) using a Flaming Brown micropipette puller (P97, Sutter Instrument, Novato, CA) to obtain electrode resistances ranging from 2–4 MΩ. The composition of the internal solution (pipette) was (mM): 140 Cs-aspartate; 5 Mg-aspartate$_2$, 10 Cs_2EGTA, 10 HEPES; pH was adjusted to 7.4 with CsOH. The high concentration of Mg^{2+} in the pipette solution helped to maintain the preparation stable for a longer time. The external solution contained (mM): 145 TEA (tetraethylammonium hydroxide)-Br, 10 $CaCl_2$, 10 HEPES and 0.001 tetrodotoxin (Beam & Knudson, 1988). Solution pH was adjusted to 7.4 with CsOH. This solution was used for forming gigaohm seals. For charge-movement recording, calcium current was blocked with a solution containing (mM): 145 TEA-Br, 2 $CaCl_2$, 0.5 Cd^{2+}, 0.3 La^{3+}, 10 HEPES and 0.001-0.003 tetrodotoxin (Adams et al., 1990). The maximum 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 digitally subtracted on-line using hyperpolarizing control pulses of one-quarter test pulse amplitude ($-P/4$ procedure) (Delbono, 1992). 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 as the integral of the current in response to depolarizing pulses (charge on, Q_{on}) and were expressed per membrane capacitance (coulombs per farad). The complete blockade of the inward calcium current was verified by the Q_{on}-Q_{off} linear relationship. Membrane capacitance was calculated as the integral of the transient current in response to a brief hyperpolarizing pulse from −80 mV (holding potential) to −90 mV.

Statistical Analysis

Data have been analyzed using Student's *t*-test or analysis of variance (ANOVA). A value of $P < 0.05$ (*) was considered

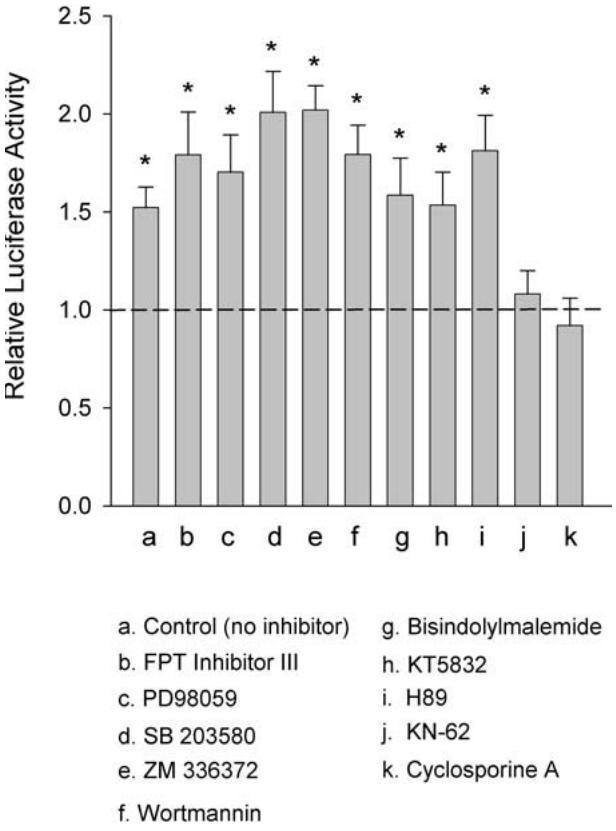

Fig. 1. Effects of protein kinase inhibitors on luciferase activity measured in C2C12 myotubes transfected with the Luc/P-146 fusion construct. Relative luciferase activity measured in transfected myotubes and expressed as the ratio between cells incubated in IGF-1 plus inhibitor and cells treated with inhibitor alone (*b–k*). The first column (*a*) is the ratio between IGF-1-treated and non-treated cells. The statistical analysis compares IGF-1-treated and non-treated cells (*a*) or cells treated with IGF-1 plus inhibitor and inhibitor alone (*b–k*). Asterisks indicate the level of statistical significance ($P < 0.05$). Experiments labeled *a–k* correspond to incubation in different inhibitors, as indicated. Data are expressed as mean ± SEM and the number of observations was 10–15 per group.

significant. Data are expressed as mean ± SEM with the number of observations (*n*).

Results

CaM Kinase and Calcineurin Inhibition Prevent IGF-1-mediated Potentiation of $DHPR\alpha_{1S}$ Gene Transcription in Muscle Cells

Our laboratory has reported that the IGF-1R tyrosine kinase inhibitor I-OMe-AG538 (Blum, Gazit & Levitzki, 2000) prevents IGF-1-mediated increase in $DHPR\alpha_{1S}$ transcription in muscle cells (Zheng et al., 2002a). We have also shown that the enhancement of Ca^{2+} influx through the muscle DHPR α1 subunit is mediated by PKC activation (Delbono et al., 1997). However, the signaling linking the activation of IGF-1R and the increase in $DHPR\alpha_{1S}$ gene transcription is currently unknown. To identify the key signaling pathways involved in IGF-1–induced $DHPR\alpha_{1S}$ gene transcription, we tested the effects of inhibitors of cell signaling cascades on luciferase activity measured in C2C12 myotubes transfected with the Luc/P-146 fusion construct (Fig. 1). We have reported previously that CREB mediates the IGF-1 effect on $DHPR\alpha_{1S}$ transcription (Zheng et al., 2002a). Although the P-146 construct exhibits 25% of the activity depicted by P-756, it contains a consensus sequence for CREB and has been shown to respond to IGF-1 stimulation with an increase in luminescence (Zheng et al., 2002a; Zheng et al., 2002b).

For these experiments, C2C12 cells incubated for three days in differentiation medium were exposed to compounds that inhibit key signaling molecules in the cell (*see* below) (Fig. 1). These inhibitors were renewed daily to compensate for possible degradation. IGF-1 was added 30 min after the addition of the inhibitor to the culture medium, and luminiscence was recorded three days afterward. 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 $DHPR\alpha_{1S}$ expression in rat skeletal muscle primary culture (Wang et al., 1999b). The concentration of various enzyme inhibitors used in the present study corresponds to the IC-50 values reported in the literature (*see* below). Results are expressed as the ratio between the luciferase activity recorded in cells incubated in 20 ng/ml IGF-1 plus inhibitor and cells treated with inhibitor alone (bars *b–k*). The first column compares IGF-1-treated and non-treated cells (bar *a*). The statistical analysis compares IGF-1-treated and non-treated (*a*) or IGF-1 plus inhibitor-treated and inhibitor-alone-treated (*b–k*) cells. We performed control experiments to test the effect of the vehicle used to dilute the reagents on $DHPR\alpha_{1S}$ expression. The vehicles were used at a final dilution of 1:1000 or higher in the culture medium (*see* Methods). No significant differences were found between cells treated or non-treated with the vehicle alone. The three experimental cell groups, control (non-treated), treated with a kinase or phosphatase inhibitor, and treated with their vehicle alone, were studied in the same batch of C2C12 myotubes.

Fig. 1 shows that IGF-1 increases $DHPR\alpha_{1S}$ expression by myotubes by about 50% compared to control (non-treated) cells, confirming published data (Zheng, Wang & Delbono, 2001; Zheng et al., 2002b). Despite the influence of PKC activation on skeletal muscle DHPR α1 subunit function (Delbono et al., 1997), the inhibition of this enzyme with 100 nM bisindolylmalemide (Ku, Cheng & Wang, 1997) did not prevent the IGF-1-induced enhancement of $DHPR\alpha_{1S}$ transcription. Similarly, the inhibition of

PKA or PKG induced by 1 μM H89 (Nojima, Kimura & Kimura, 1994) or 2 μM KT5832 (Grider, 1993), respectively, did not prevent IGF-1 effects on gene transcription (Fig. 1). It has been shown that IGF-1 induces phosphatidylinositol (1,4,5)-trisphosphate (PI-3) kinase activation in muscle cells (Chakravarthy et al., 2000; Rommel et al., 2001), however, the use of the inhibitor Wortmannin (50 nM) (Cross et al., 1995), did not prevent the effect of IGF-1 on $DHPR\alpha_{1S}$ transcription. Although MAP kinase activation mediates some of the cellular effects of IGF-1 in muscle cells (Chakravarthy et al., 2000), PD98059 at a concentration known to inhibit the phosphorylation of ERK1/2 in the classical ERK1/2 signaling pathway (20 μM) (Alessi et al., 1995) did not prevent the IGF-1 induced enhancement of $DHPR\alpha_{1S}$ transcription (Fig. 1). CaM kinase plays multiple roles in the cell (Soderling, 1999) and particularly in cardiac excitation-contraction coupling (Wu, Colbran & Anderson, 2001); however, its role in $DHPR\alpha_{1S}$ expression has not been tested. In the present study, we investigate the involvement of CaM kinase in $DHPR\alpha_{1S}$ expression using the kinase inhibitor KN-62 (Minami, Inoue & Hidaka, 1994). This inhibitor has been reported to be highly selective for CaM kinase (Davies et al., 2000). The concentration of the CaM kinase inhibitor used in this study (10 μM) is within the range reported in previous publications (Brozinick et al., 1999; Vazquez, de Boland & Boland, 2000; Wolfe et al., 2002). Fig. 1 shows that KN-62 inhibits IGF-1-mediated potentiation of $DHPR\alpha_{1S}$ transcription. Calcineurin, a serine/threonine protein phosphatase, plays multiple roles in skeletal muscle (Friday et al., 2003; Glass, 2003), however, its role in modulating DHPR gene expression in not known. Here, we found that cyclosporine A, a calcineurin inhibitor, at a concentration of 5 μM, precludes IGF-1-mediated potentiation of $DHPR\alpha_{1S}$ transcription. The cyclosporine A concentration was used at a concentration reported in previous publications (Musaro et al., 1999; Gooch et al., 2001; Rommel et al., 2001). Based on the results included in Fig. 1, it seems that the inhibition of calcineurin or CaM kinase has a similar effect on $DHPR\alpha_{1S}$ transcription.

To determine whether IGF-1 increases DHPR α1 subunit protein in C2C12 myotubes, immunoprecipitation and immunoblot studies were performed in cells treated with IGF-1, KN-62, or both. Figure 2*A* shows a band at 210 kDa, corresponding to the DHPR α1 subunit. The size of this band was determined using molecular weight markers and is similar to that reported previously (De Jongh, Merrick & Catterall, 1989; Morton & Froehner, 1989; Rotman, Murphy & Catterall, 1995). This band is enhanced in myotubes treated with IGF-1, a phenomenon not recorded in myotubes treated with IGF-1 plus 10 μM KN-62. It is also apparent that KN-62 alone decreased the expression of the $DHPR\alpha_{1S}$, which may be secondary to inhibition of basal CaM kinase activity (*see* below). For these experiments, 500 μg total proteins extracted from cell lysates were used. Figure 2*B* shows the values of DHPR α1 subunit expression normalized to control (non-treated cells) for IGF-1, KN-62, and KN-62 plus IGF-1-treated cells in 5 immunoblots from 5 different pools of myotubes. It appears that IGF-1 significantly enhances the expression of the DHPR α1 subunit, a phenomenon that is prevented by KN-62.

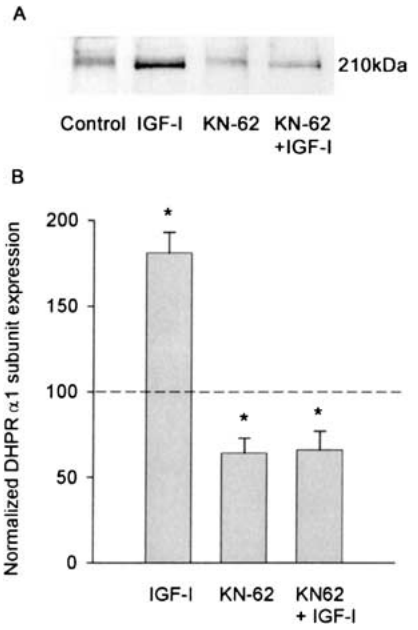

Fig. 2. Immunoblot analysis of DHPR α1 subunit expression. (*A*) Western blot analysis of C2C12 myotubes treated with 20 ng/ml IGF-1, 10 μM KN-62 or KN-2 plus IGF-1. The band at 210 kDa corresponds to the DHPR αl subunit. The size of the band was determined using molecular weight markers as explained in Materials and Methods. For these experiments, 500 μg total proteins extracted from myotube lysates were used. (*B*) Quantification of the bands measured in 5 experiments from 5 different pools of C2C12. Bars represent the magnitude of IGF-1, KN-62, or KN62 plus IGF-1 effects normalized to control (non-treated) cells. The asterisk indicates statistically significant difference between treated and untreated cells. Values are expressed as mean ± SEM.

To determine the time course of IGF-1 effect on CaM kinase activity we analyzed 7 experiments corresponding to 7 different pools of myotubes. Figure 3 shows a significant increase in enzyme activity 5 min after adding IGF-1 to the culture medium. This effect remained statistically significant at 10 and 30 min after the application of the growth factor.

IGF-1 Induces CREB Phosphorylation in Muscle Cells

In the following group of experiments we investigated whether IGF-1 induces CREB phosphorylation in

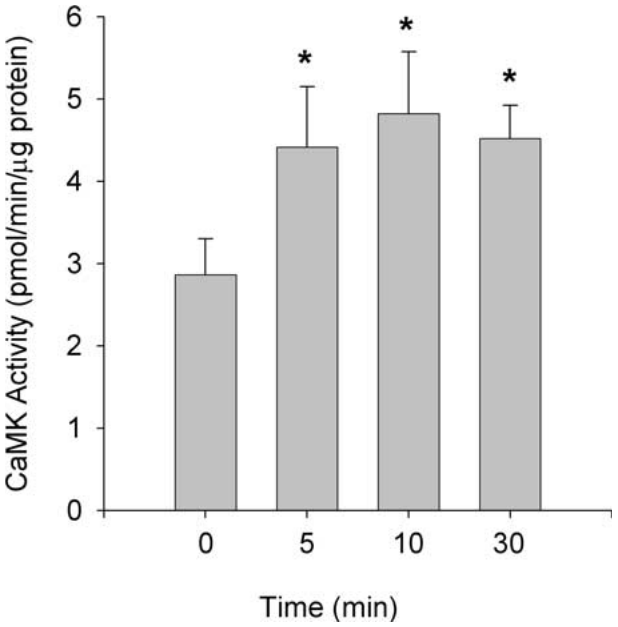

Fig. 3. Time course of CaM kinase activity in C2C12 myotubes treated with IGF-1. CaM kinase activity recorded in control (no IGF-1 treatment) and IGF-1-treated C2C12 myotubes for 5, 10, and 30min. Asterisks indicate statistical significance ($P < 0.05$). Data are expressed as mean ± SEM and the number of observations was 7 different cell groups.

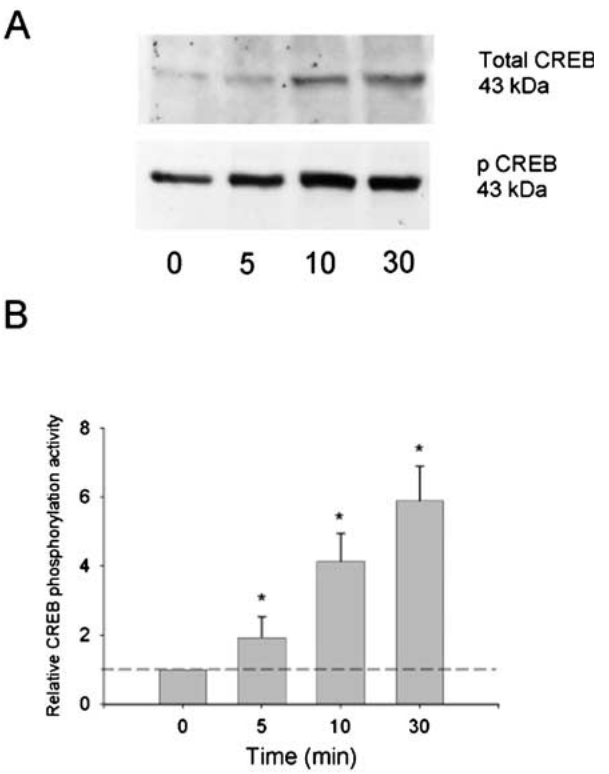

Fig. 4. IGF-1 induces CREB phosphorylation in C2C12 myotubes. Phospho- and total-CREB were measured by Western blot using specific antibodies. (*A*) CREB phosphorylation was measured in cultured myotubes incubated in IGF-1 for 0, 5, 10, and 30 min. (*B*) Mean of five experiments showing IGF-1-mediated enhancement of CREB phosphorylation in C2C12 myotubes. The dashed line represents the reference activity of myotubes not treated with IGF-1. Asterisks indicate statistical significance ($P < 0.05$).

C2C12 myotubes. To this end, we used specific antibodies against phospho- and total-CREB for Western blot experiments. To quantify the magnitude of the IGF-1 effect on CREB phosphorylation, and measure the time course of this process with a similar time frame as that used for CaM kinase activity (*see* Fig. 3), CREB phosphorylation was measured in cultured myotubes incubated in IGF-1 for 0, 5, 10, and 30 min (Fig. 4*A*). It is apparent that IGF-1 evokes a time-dependent increase in myotube phospho-CREB. Figure 4*B* shows that phospho-CREB (pCREB) formation in C2C12 myotubes increased steadily during this period. The bars represent the mean of five experiments. The comparison of these results with those on CaM kinase activity shows that the activity of this enzyme increases significantly between 5 and 30 min of exposure to IGF-1, which is associated with a sustained formation of phospho-CREB.

Inhibition of CaM Kinase Prevents Phospho-CREB Formation

It has been shown that the inhibition of CaM kinase prevents the effect of IGF-1 on $DHPR\alpha_{1S}$ transcription (Fig. 1) and DHPR α1 protein expression (Fig. 2). We have also found a temporal relationship between CaM kinase activation and CREB phosphorylation (Figs. 3 and 4). In the following group of experiments we explored the effects of CaM kinase inhibition on phospho-CREB levels in C2C12 myotubes. Figure 5*A* shows that the CaM kinase inhibitor KN-62 prevents the increase in CREB phosphorylation in response to IGF-1 and that this compound decreases by itself phospho-CREB formation, consistent with the effect on $DHPR\alpha_{1S}$ expression shown in Fig. 2. Similar results were obtained in 6 other experiments. Figure 5*B* shows the quantification of these experiments. IGF-1 enhances significantly pCREB formation. Incubation in 10 μM KN-62 results in a significant decrease in the detected levels of pCREB, probably associated with inhibition of basal CaM kinase activity. The sequential application of KN-62 and IGF-1 also resulted in a significant decrease in pCREB formation. This effect can probably be explained by the same mechanism. These results support a causative relationship between CaM kinase activation and CREB phosphorylation and consequently between CaM kinase activation and $DHPR\alpha_{1S}$ transcription.

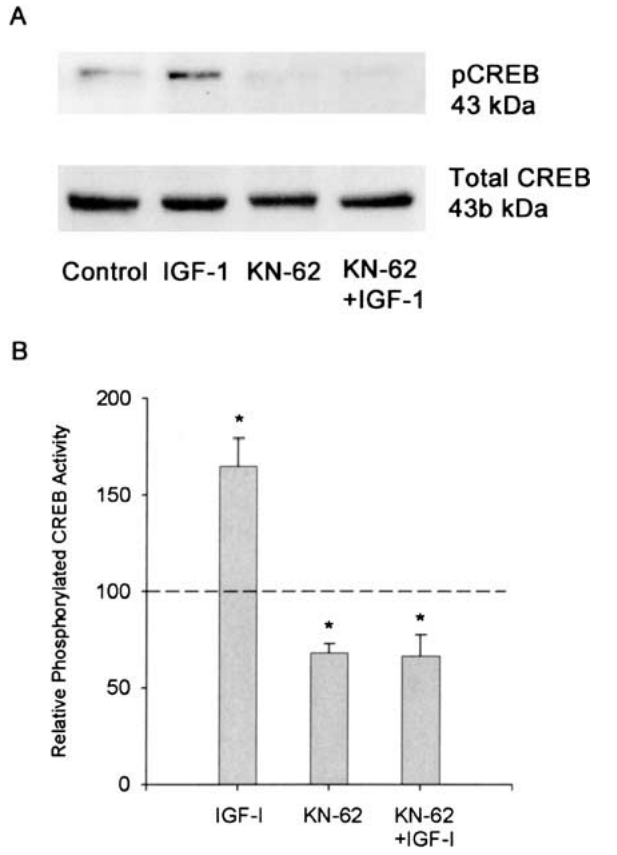

Fig. 5. Effects of IGF-1 and KN-62 on phosphorylated and total CREB in C2C12 myotubes. (*A*) Representative immunoblot of six experiments showing phosphorylated and total CREB in myotubes treated with 20 ng/ml IGF-1, 10 μM KN-62, or KN-62 plus IGF-1. The bands for pCREB and total CREB correspond to 43 kDa. The size of the bands was determined using molecular weight markers (*see* Methods). (*B*) Phosphorylated CREB normalized to control experiments recorded in the absence of IGF-1. Bars represent mean ± SEM for 6 immunoblots corresponding to 6 separate cell batches.

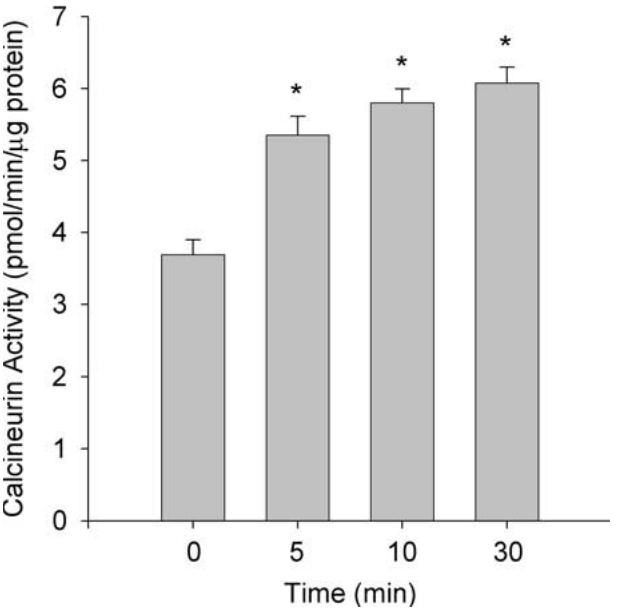

Fig. 6. Time course of calcineurin activity in C2C12 myotubes treated with IGF-1. Calcineurin activity recorded in control (no IGF-1 treatment, zero time) and treated C2C12 myotubes for 5, 10, and 30 min with 20 ng/ml IGF-1. Asterisks indicate statistical significance ($P < 0.05$) compared with values at zero time. Data are expressed as mean ± SEM and the number of observations was 7 different cell groups.

Calcineurin activity and its role in phospho-CREB formation

The following experiments investigated the involvement of calcineurin in $DHPR\alpha_{1S}$ transcription. Figure 6 shows the time-dependent changes in calcineurin activity in myotubes exposed to IGF-1. A significant increase in calcineurin activity was recorded at 5, 10 and 30 min after adding IGF-1 to the culture medium. These results indicate that IGF-1 modulates the enzyme activity. To determine whether increases in calcineurin activity have an impact on CREB phosphorylation, we analyzed the effects of cyclosporine alone or combined with IGF-1 on phospho-CREB formation by immunoblot. Figure 7*A* shows representative immunoblots for phospho- and total-CREB. It appears that IGF-1 and/or cyclosporine enhance phospho-CREB formation. Figure 7*B* plots the quantification of 6 experiments. IGF-1, cyclosporine, and IGF-1 plus cyclosporine significantly increased phospho-CREB formation ($P < 0.05$). However, no significant differences among these three groups were detected ($P > 0.05$). These results are consistent with those included in Fig. 1, showing that the effect of IGF plus cyclosporine is not significantly different from that in cyclosporine alone. Our interpretation of these results is that inhibition of CREB dephosphorylation increases $DHPR\alpha_{1S}$ transcription. The addition of IGF-1 to cells preincubated in cyclosporine does not further increase $DHPR\alpha_{1S}$ transcription, probably due to the high levels of CREB phosphorylation induced by calcineurin inhibition. Therefore, the ratio between the effect of IGF-1 plus cyclosporine and the inhibitor alone does not display an enhancing effect on $DHPR\alpha_{1S}$ transcription. In summary, these results suggest that CaM kinase and calcineurin mediate IGF-1-induced enhancement of $DHPR\alpha_{1S}$ transcription.

Effects of CaM Kinase and Calcineurin Blockade on Charge Movement

To investigate the functional effects of CaM kinase and calcineurin blockade we recorded charge movement in differentiated myotubes as a functional expression of the DHPR α1 subunit. Charge movement was recorded after 36–48 h incubation in 20 ng/ml IGF-1 plus 10 μM KN-62 and compared to recordings

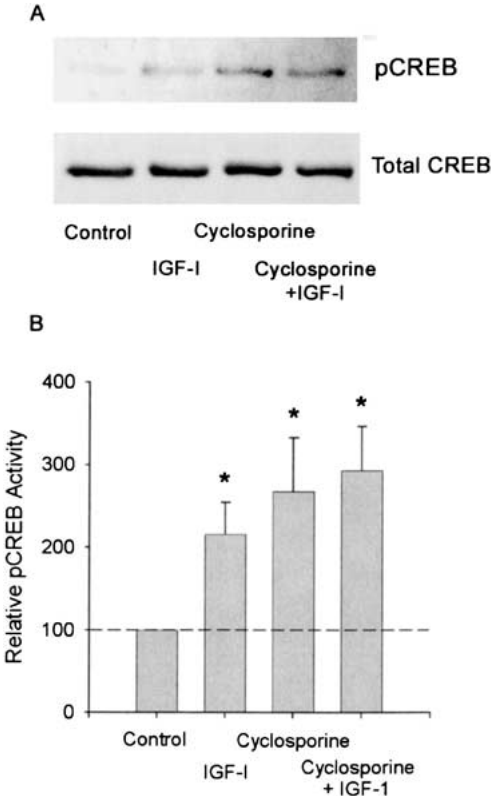

Fig. 7. Effects of IGF-1 and cyclosporine on phosphorylated and total CREB in C2C12 myotubes. (*A*) Representative immunoblots using specific antibodies for phospho- and total-CREB in control (no treatment), 20 ng/ml IGF-1, 10 μM cyclosporine, and cyclosporine plus IGF-1. (*B*) Relative phospho-CREB activity. Bars represent mean ± SEM of 6 experiments. The dashed line represents the reference activity of control (not treated) myotubes.

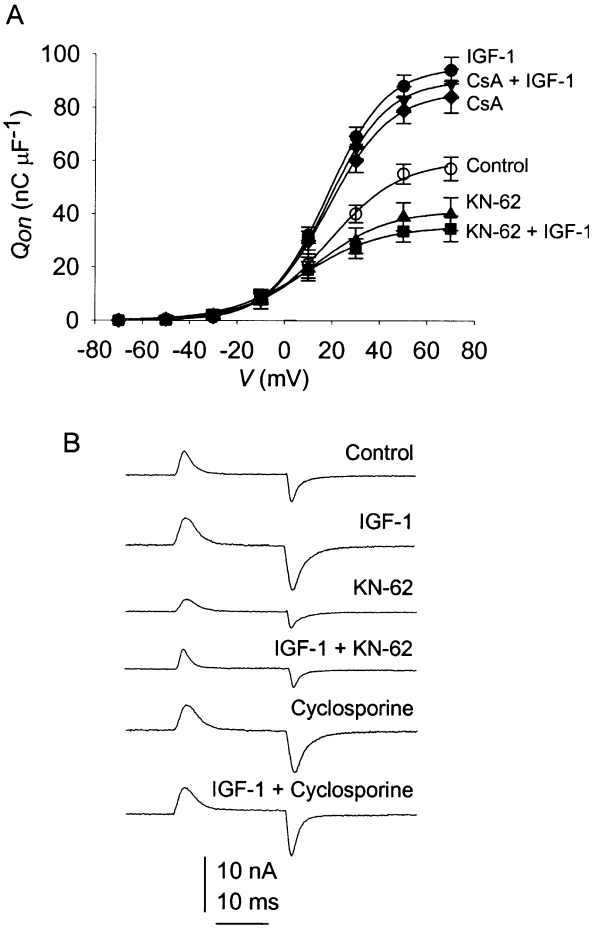

Fig. 8. Effects of the CaM kinase inhibitor K-62 on charge movement. (*A*) Q_{on}–voltage relationship for C2C12 cells treated with IGF-1 and/or KN-62 and/or cyclosporine (CsA) and control (non-treated) myotubes. Data points were fitted to a Boltzmann equation (*see* Eq. 1). The best-fitting parameters for Q_{max}, $V_{Q1/2}$ and K recorded in C2C12 cells for the six experimental groups are included in Table 1. (*B*) Illustration of representative charge movement recordings at 50 mV, corresponding to the experimental groups included in (*A*).

in C2C12 myotubes incubated in IGF-1, KN-62, or control cells (no treatment). Although DHPR α1 subunits account for 70% of the total non-linear capacity of the membrane (Adams et al., 1990), we preferred the recording of charge movement to calcium current due to the direct relationship between the integral of charge movement and the levels of channel expression in the sarcolemma (*see* below). Also, the amplitude of the L-type Ca^{2+} current varies in response to channel regulation and percentage of silent channels (Fleig & Penner, 1995; Delbono et al., 1997). As the charge movement reported here is similar to that recorded previously with the patch clamp (Wang et al., 2002; Zheng et al., 2002a) but higher than that recorded with other techniques (Hollingworth & Marshall, 1981; Dulhunty & Gage, 1983; Delbono, 1992), we studied both, linear capacitive transients for the voltage steps from −80 to −90 mV and the unsubtracted current traces for the test and control steps. No current in the subpulses was detected in either control or test fibers (n = 3–4 fibers per group), confirming prior observations (Zheng et al., 2002a). Differences in the recording technique and time after current blockade could account for the variability in the reported maximum charge movement. IGF-1 did not exert any effects per se on muscle fiber capacitance, as reported recently (Zheng et al., 2002a).

Electrophysiological recordings were performed after two days of incubation in IGF-1 and/or KN-62. The CaM kinase inhibitor was added 30 min before IGF-1 to the culture medium. Charge movement was normalized to membrane capacitance to control for variations in myotube size. Recordings were carried out on the same batches of myotubes that were exposed to cyclosporine A and KN-62 and used for the measurements described above. Figure 8*A* shows the Q_{on}-voltage relationship for the C2C12 cells treated with IGF-1 and/or KN-62 and control (non-treated) myotubes. The effects of IGF-1 alone are consistent with previous reports from our laboratory (Zheng

Table 1. Best-fitting parameters describing the voltage dependence of charge movement in C2C12 muscle cells

Experimental groups	*n*	Best-fitting parameters		
		Q_{max} (nC μF^{-1})	$V_{Q1/2}$ (mV)	*K*
Control	34	57 ± 4.3	18 ± 2.7	14 ± 1.8
IGF-1	28	84 ± 3.7*	19 ± 1.8	15 ± 2.3
KN-62	26	36 ± 2.7*	20 ± 1.7	16 ± 2.2
IGF-1 + KN-62	25	39 ± 3.4*	21 ± 1.9	13 ± 3.2
Cyclosporine A	20	80 ± 3.4*	17 ± 2.0	15 ± 1.9
Cyclosporine A + IGF-1	22	82 ± 3.6*	19 ± 2.2	16 ± 2.4

Experimental data were fit to the Boltzmann equation described in the text. Q_{max}: maximum charge movement; $V_{Q1/2}$: charge movement half activation potential; *K*: steepness of the curve. IGF-1: 20 ng/ml. KN-62: 10 μM. Cyclosporine: 10 μM. Asterisks indicate statistically significant difference to control (non-treated) cells ($P < 0.05$); *n*, number of cells.

et al., 2002b). For the analysis of the voltage dependence of the charge, data points were fitted to a Boltzmann equation of the form:

$$Q_{on} = Q_{max}/(1 + \exp[V_{Q1/2} - V_m]/K), \quad (1)$$

where Q_{max} is the maximum charge, V_m is the membrane potential, $V_{Q1/2}$ is the charge movement half-activation potential, and *K* is the steepness of the curve. The best fitting parameters for Q_{max}, $V_{Q1/2}$ and *K* recorded in cells for the four experimental groups are included in Table 1. Figure 8*B* illustrates the charge movement recorded at 50 mV. It is apparent that IGF-1 significantly enhances charge movement and that KN-62 per se decreases significantly the maximum charge movement. It also appears that KN-62 inhibits the IGF-1 effect on charge movement.

The charge movement (Q_{on})-current relationship was also studied in myotubes exposed to cyclosporine and/or IGF-1 (Fig. 8*A*). Cyclosporine alone or the combination of IGF-1 plus cyclosporine enhanced charge movement (Fig. 8*B*). Charge movement recorded in cells incubated in IGF-1, cyclosporine A, or IGF-1 plus cyclosporine A, were not significantly different. These results are consistent with the expression of the DHPR in cells transfected with the Luc/P-146 fusion construct (Fig. 1) and CREB phosphorylation in cells exposed to cyclosporine A in the presence or absence of IGF-1 (Fig. 7).

Discussion

In the present work we have explored the molecular signaling linking IGF-1R activation and $DHPR\alpha_{1S}$ gene transcription and DHPRα1 subunit expression. Based on our results we propose a model in which CaM kinase and calcineurin are involved in IGF-1-induced $DHPR\alpha_{1S}$ expression. IGF-1R activation triggers CaM kinase and calcineurin activity to regulate CREB through a phosphorylation and dephosphorylation mechanism. Inhibition of CaM kinase in the presence of IGF-1 allows for a predominance of CREB dephosphorylation and inhibition of $DHPR\alpha_{1S}$ transcription, while the inhibition of calcineurin triggers CREB phosphorylation in response to inhibition of dephosphorylation plus CaM kinase activity, which results in $DHPR\alpha_{1S}$ transcription enhancement.

CaM Kinase and Inhibition of Calcineurin Increase $DHPR\alpha_{1S}$ Transcriptional Activity

Previous work from our laboratory has shown that IGF-1-mediated enhancement of $DHPR\alpha_{1S}$ expression requires the activation of the IGF-1R-tyrosine kinase, as demonstrated by inhibition of this process by the IGF-1R-tyrosine kinase inhibitor I-OMe-AG 538 (Blum et al., 2000; Zheng et al., 2002a). Subsequently, a signaling cascade should be triggered to phosphorylate the transcription factor CREB, which is involved in $DHPR\alpha_{1S}$ transcription (Zheng et al., 2002a). A number of signaling pathways converge on CREB (Kornhauser et al., 2002; Lonze & Ginty, 2002) and dynamic multiphosphorylation pathways activate and modulate gene expression (Deisseroth & Tsien, 2002). Enzyme inhibitors have been used to determine the major cell signaling pathways involved in $DHPR\alpha_{1S}$ gene transcription. KN-62 acting as a CaM kinase inhibitor inhibited IGF-1-induced $DHPR\alpha_{1S}$ transcription. Multiple roles for calcineurin have been described, particularly in muscle differentiation and hypertrophy (Friday et al., 2003; Glass, 2003), however, its role in $DHPR\alpha_{1S}$ transcription and excitation-contraction coupling is not known. Cyclosporine A, a calcineurin inhibitor, inhibited CREB dephosphorylation. These results support a role for CaM kinase and calcineurin in skeletal muscle IGF-1 signaling. Although KN-62 and cyclosporine A are widely used compounds to explore the involvement of Ca^{2+}-Calmodulin kinase and calcineurin in cell signaling, the possibility that side effects unrelated to kinase or phosphatase inhibition might be responsible for some of the results

reported here, cannot be ruled out. None of the other compounds, at the concentration tested in this work, prevented the effect of IGF-1 on $DHPR\alpha_{1S}$ transcription, including MAP-kinase, IP-3 kinase, PKC, PKA, and PKG inhibitors. Inhibitors at the IC50 concentrations used in this study have been found effective in inhibiting specific cellular kinase-mediated reactions (*data not shown*). The participation of these signaling pathways in IGF-1-mediated $DHPR\alpha_{1S}$ transcription cannot be ruled out completely at the present time, based on potential secondary effects of the antagonist used in this work.

Role of CaM Kinase and Calcineurin in CREB-mediated Transcription

We have shown that IGF-1 elicits elevations in CaM kinase in C2C12 myotubes. To determine whether IGF-1-induced increases in CaM kinase result in CREB phosphorylation, we measured pCREB in myotubes treated with IGF-1 and/or KN-62. The IGF-1-induced increase in pCREB was prevented by the CaM kinase inhibitor KN-62. Whether CaM kinase II or IV are involved in IGF-1-$DHPR\alpha_{1S}$ signaling is not known at the present time. CaM kinase IV, which has been involved in gene regulation during neural and skeletal muscle development (Buonanno & Fields, 1999), cannot be ruled out as the mediator of the signaling cascade under study. Whether CaM kinase-dependent CREB phosphorylation requires enzyme translocation to the nucleus is not known. IGF-1 induces calcineurin activity with the same time course as that observed for CaM kinase. This indicates that the level of CREB phosphorylation is finely tuned by a phosphorylation-dephosphorylation process evoked by IGF-1R activation. That IGF-1-evoked calcineurin activity modulates $DHPR\alpha_{1S}$ gene transcription, results from experiments in which cyclosporine was used. In these experiments, a sustained inhibition of calcineurin activity would lead to higher levels of CREB phosphorylation that are not further significantly enhanced by IGF-1. These results are consistent with the higher level of pCREB detected by immunoblots in cyclosporine-treated myotubes compared to non-treated cells (Fig. 7), and with the levels of luciferase activity reported (Fig. 1).

CaM Kinase- and Calcineurin-Mediated IGF-1 Potentiation of Charge Movement

Charge movements are currents arising from the movement of charged molecules dwelling in the T-tubule (Schneider & Chandler, 1973). The integral of the recordings is directly related to the number of moving charged molecules (Rios, Ma & Gonzalez, 1991). The maximum control charge movement reported here is similar to that reported by us previously (Zheng et al., 2002b), but between 1.35- and 2.3-fold higher than that reported by other groups (Hollingworth & Marshall, 1981; Simon & Beam, 1985) using microelectrode techniques. Although several voltage-gated channels contribute to charge movement, these recordings represent mainly the activity of the DHPR $\alpha 1$ subunit in skeletal muscle (Adams et al., 1990). 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 from the remaining charge not attributable to the L-type Ca^{2+} channel (Wang et al., 1999a). In that publication, 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 CaM kinase and calcineurin inhibition on IGF-1-induced enhancement of charge movements as an indication of CaM kinase and calcineurin participation in the IGF-1R activation/$DHPR\alpha_{1S}$-expression cascade. KN-62 and cyclosporine have an effect on basal CaM kinase activity. Consistently, we found that inhibition of CaM kinase prevents IGF-1-induced CREB phosphorylation and $DHPR\alpha_{1S}$ expression. Also, CaM kinase inhibition does decrease basal $DHPR\alpha_{1S}$ expression or CREB phosphorylation. An explanation for this result is that KN-62 inhibits basal (non-stimulated cells) CaM kinase activity. The phosphorylated status of a transcription factor depends on the balance between the activity of kinases and phosphatases (Latchman, 1995). The increase in charge movement recorded in myotubes incubated in cyclosporine indicates that calcineurin-induced dephosphorylation is depressed, favoring CREB phosphorylation, which results in increased expression of DHPR $\alpha 1$ subunit. Calcineurin could be activated in response to IGF-1-induced increase in intracellular Ca^{2+} (Kazaki, Shibata & Kojima, 1997), however, the precise mechanism whereby IGF-1 evokes calcineurin activity in muscle cells is not known at the present time. The similar charge movement recorded in cyclosporine- and cyclosporine plus IGF-1-treated cells (Fig. 8) indicates that calcineurin exerts a strong modulatory effect on CREB dephosphorylation and subsequently on $DHPR\alpha_{1S}$ transcription.

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