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Calcineurin Enhances Acetylcholinesterase mRNA Stability during C2-C12 Muscle Cell Differentiation

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ABSTRACT

Treatment of C2–C12 mouse myoblasts with the immunosuppressant drug cyclosporin A (CsA) enhances the increase in acetylcholinesterase (AChE) expression observed during skeletal muscle differentiation. The enhanced AChE expression is due primarily to increased mRNA stability because CsA treatment increases the half-life of AChE mRNA, but not the apparent transcriptional rate of the gene. Neither tacrolimus (FK506), an immunosuppressive agent with a distinct structure, nor cyclosporine H, an inactive congener of CsA, alters AChE expression. The enhanced AChE expression is associated with the muscle differentiation process, but cannot be triggered by CsA exposure before differentiation. Myoblasts and myotubes of C2–C12 cells express similar amounts of cyclophilin A and FKBP12, immunophilins known to be intracellular-binding targets for CsA and tacrolimus, respectively. However, cellular levels of calcineurin, a calcium/calmodulin-dependent phosphatase known to be the cellular target of ligand-immunophilin complexes, increase 3-fold during myogenesis. Overexpression of constitutively active calcineurin in differentiating cells reduces AChE mRNA levels and CsA antagonizes such an inhibition. Conversely, overexpression of a dominant negative calcineurin construct increases AChE mRNA levels, which are further enhanced by CsA. Thus, a CsA sensitive, calcineurin mediated pathway appears linked to differentiation-induced stabilization of AChE mRNA during myogenesis.

Differentiation of skeletal muscle from myoblasts to myotubes is globally controlled by the expression of muscle-specific genes of the myo D family. However, following initiation of differentiation, expression of genes encoding proteins important for controlling cellular excitability, such as acetylcholinesterase (AChE) and nicotinic acetylcholine receptors (nAChR), occurs through distinct mechanisms. Although the increased expression of nAChR arises from enhancement of its transcription rate (Evans et al., 1987; Baldwin and Burden, 1988), the expression of AChE appears mainly due to stabilization of a labile AChE mRNA (Fuentes and Taylor, 1993; Luo et al., 1994).

Previous studies indicated that regulation of intracellular Ca^{2+} through L-type Ca^{2+} channels in the plasma membrane or intracellular ryanodine-sensitive Ca^{2+} channels plays an important role in stabilization of AChE transcripts during muscle development in mouse C2–C12 myocytes (Luo et al., 1994) and in intact mouse skeletal muscle (Luo et al., 1996). Other studies revealed that immunosuppressant cyclosporin A (CsA) regulates mRNA stability of interleukin-3 (Nair et al., 1994). Furthermore, FKBP12, an intracellular receptor for another immunosuppressant tacrolimus (FK506), was copurified with ryanodine receptors (Jayaraman et al., 1992) and found to modulate intracellular calcium release by stabilizing ryanodine-sensitive calcium channels in skeletal muscle (Timerman et al., 1993; Brillantes et al., 1994).

CsA and tacrolimus bind to intracellular immunophilins such as cyclophilin A and FKBP12, respectively (Handschumacher et al., 1984; Harding et al., 1989; Standaert et al., 1990). The immediate cellular target for the complexes of CsA-cyclophilin and FK506-FKBP12, but not for the complexes of CsA-FKBP12 or FK506-cyclophilin, is calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase (Liu et al., 1991). The influence of CsA and tacrolimus on regulation of mRNA stability in other systems and the linkage of their intracellular receptors to intracellular Ca^{2+} mobilization prompted us to investigate the functional role of the cal-

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ABBREVIATIONS: AChE, acetylcholinesterase; nAChR, nicotinic acetylcholine receptor; CsA, cyclosporin A; CsH, cyclosporine H; BCA, bicinchoninic acid; α-BTX, α-bungarotoxin; HA, hemagglutinin; DFP, diisopropyl fluorophosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; β-gal, β-galactosidase; CnAΔCaM-AI, constitutively active calcineurin catalytic construct; DRB, 5,6-dichloro-1-β-*d*-ribofuranosylbenzimidazole; BKO, B subunit knock out construct; Adv, adenovirus.

cineurin-mediated pathway in regulation of AChE mRNA stabilization during myogenesis.

Experimental Procedures

Materials. CsA and cyclosporine H (CsH) were from Sandoz Ltd. (Basel, Switzerland). A stock solution of CsA and CsH was dispensed in ethanol and Tween 80 followed by addition of PBS. Tacrolimus was from Fujisawa Ireland, Ltd. (Kerry, Ireland). Ethanol stock solutions were further diluted into culture medium at the time of treatment. Bicinchoninic acid (BCA) protein assay reagents were from Pierce Chemical Co. (Rockford, IL). The creatine kinase assay materials and all other chemicals were from Sigma Chemical Co. (St. Louis, MO). Components of culture medium were from Gibco Laboratories (Grand Island, NY). [α-^{125}I]Bungarotoxin ([α-^{125}I]BTX; specific activity 14.5 μCi/μg) was from NEN Research Products (Wilmington, DE). ^{32}P-α-UTP (specific activity ~800 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). The polyclonal anticyclophilin A antibody was from Affinity BioReagents, Inc. (Neshanic Station, NJ), the monoclonal anticalcineurin antibody was from Transduction Laboratories (Lexington, KY), the anti-FKBP12 antibody was a gift from Dr. Steven J. Burakoff at the Dana-Farber Cancer Institute (Boston, MA) and the antihemagglutinin (HA) antibody was a gift from Dr. Michael Karin at the University of California-San Diego. The secondary antibody and detection reagents were from Amersham Corp. (Buckinghamshire, England). Tris-glycine polyacrylamide gels were from NOVEX (San Diego, CA).

Tissue Culture. C2–C12 cells (American Type Culture Collection, Rockville, MD) were stored at −70°C and cultured at 37°C, with 5% CO_2 in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum; 0.5% chick embryo extract; and 1% penicillin, streptomycin, and amphotericin B stock solution (Antibiotic-Antimycotic; Gibco Laboratories). Cells were passed either two or three times before plating. Differentiation from myoblasts to myotubes was induced at ~70% confluence by replacing the high serum medium with Dulbecco's modified Eagle's medium containing 2% horse serum and 1% of the above-mentioned antimicrobial stock solution. To measure secreted AChE in the medium, cells were differentiated in medium containing serum pretreated with 0.1 mM diisopropyl fluorophosphate (DFP) to inhibit serum esterase activity. The DFP-treated serum was incubated overnight at room temperature, sterile filtered and held for at least 48 h at 4°C, and then assayed for residual DFP and AChE activity. Such treatment results in >99% inhibition of serum esterase activity with no inhibitory activity toward exogenously added AChE activity (Coleman and Taylor, 1996).

RNA Extraction and RNase Protection Assay. Total RNA was extracted from cultured cells with TRIzol reagent from Gibco Laboratories and stored at −20°C. mRNAs encoding AChE and the γ-subunit of nAChR (γ-nAChR) were quantified by RNase protection as described in Luo et al. (1998). The antisense probe of AChE mRNA was made from a mouse *Ache* cDNA subcloned in Bluescript SK II plasmids and linearized with *Xho*I. This probe allows us to distinguish exon 4 to 6 spliced AChE mRNA from other splice variants. A fully protected probe in RNase protection assays indicates the presence of exon 4 to 6 spliced AChE mRNA, whereas the two shorter protected fragments represent other splice variants (Luo et al., 1998). A probe made from a 1.7-kilobase mouse nAChR γ subunit cDNA (kindly provided by Drs. Jim Boulter and Stephen Heinemann, Salk Institute, San Diego, CA), cloned in pSP-65 and linearized with *Xho*I, was used to hybridize with mRNA of the γ-nAChR subunit. To normalize for sample loading, a probe was made from a 316-bp cDNA fragment of the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Ambion, Inc. (Austin, TX). Molecular masses of the protected probes were estimated by electrophoresis on polyacrylamide gels. Densities of bands were quantified by densitometry (UltroScan XL; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and standardized by ratios to the GAPDH bands.

Determination of AChE Activity. AChE was extracted from rinsed C2–C12cells in 0.01 M sodium phosphate buffer containing 1 M NaCl, 1% Triton X-100, 0.01 M ethylene glycol bis(β-aininoethyl ester)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and a spectrum of protease inhibitors, and protein concentrations were determined with BCA reagents. AChE in the media was concentrated >10-fold in Centriprep 30 concentrators (Amicon Corp., Beverly, MA). Enzyme activity was determined at room temperature as described by Ellman et al. (1961) in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic)acid, 0.5 mM acetylthiocholine iodide, and 0.05 to 0.1 ml of cell extract or concentrated medium. More than 90% of the enzyme activity is due to AChE as determined from the presence of 10 μM BW284c51 (data not shown).

Determination of Creatine Kinase Activity. Rinsed C2–C12 myotubes differentiated for 3 days were extracted in PBS containing 0.5% Triton X-100 and a spectrum of protease inhibitors. After sonication and centrifugation, supernatants were assayed for protein concentrations with BCA reagents and for creatine kinase activity with Sigma Diagnostics Procedure 520.

β-Galactosidase (β-Gal) Staining. C2–C12 cells infected with adenovirus containing the lac-Z gene were stained for β-gal activity as described by Sanes et al. (1986). Briefly, infected cells were rinsed with PBS and fixed for 5 min on ice in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. After washing, the cells were overlaid with a PBS reaction mixture containing 1 mg/ml 4-Cl-5-Br-3-indolyl-β-galactosidase, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ and incubated at 37°C until desired color intensity was achieved. The cells were then fixed for 10 min at room temperature, rinsed, and stored in PBS.

Determination of nAChR Expression. Densities of cell surface nAChR were monitored by binding of [α-^{125}I]BTX to intact cells cultured in six-well plates at room temperature. Cells were washed with differentiation medium and incubated for 10 min in the presence or absence of 10 mM carbamylcholine chloride. [α-^{125}I]BTX at a final concentration of 10 nM was added directly to each well and incubated for 2 h. Cells were washed three times gently with K^+-Ringer's buffer (140 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, 25 mM HEPES, 0.03 mg/ml bovine serum albumin, pH 7.4) and 1 ml of 1 M NaOH was added to each well to lyse the cells. Cell lysates were counted in a gamma counter and protein was assayed in the cell lysates with BCA reagents.

Run-On Transcriptional Analysis. Nuclei were isolated from cultured cells and stored at −70°C as described previously (Luo et al., 1994). Nuclei (200 μl) were thawed and mixed with equal volume of 2× buffer containing 10 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 0.3 M KCl; 5 mM dithiothreitol; 1 mM each of ATP, GTP and CTP; and 10 μl of ^{32}P-α-UTP. Radiolabelled mRNA was transcribed, isolated, and hybridized for at least 36 h at 65°C to slot blots containing 5 μg of each plasmid DNA. A 1.7-kilobase cDNA fragment within the coding region of mouse γ-nAChR subunit in a pSP-65 vector was linearized with *Bam*HI. A 1.4-kilobase cDNA fragment of mouse α-tubulin in Bluescript SK II$^+$ was linearized with *Kpn*I. Bluescript SK II$^+$ plasmid DNA linearized with *Eco*RI or *Kpn*I was used as control for the dsDNAs. For detecting AChE mRNA, control M13 phage DNA and that containing a 2.3-kilobase single-strand antisense AChE cDNA insert were used. After extensive washing with 2× standard saline citrate buffer, radioactivity was determined by autoradiography and densitometry.

Determination of AChE mRNA Stability. C2–C12 cells differentiated for 3 days in the presence or absence of 1 μM CsA were treated with 30 μg/ml 5,6-dichloro-1-β-*d*-ribofuranosylbenzimidazole (DRB) to block transcription and total RNA was extracted at the designated time after treatment. The decay rate of AChE mRNA was then determined by RNase protection with 50 μg of total RNA per lane. The AChE mRNA half-lives calculated from these data could be overestimated because slight reductions in the amount of total RNA occurred at longer times after DRB treatment; use of a constant amount of total RNA per sample contributes to the relatively high

values in the later time points of the curves (see *Results*). A GAPDH antisense probe was included in all the experiments for normalization of sample loading for each time point.

Western Blots. To examine the cellular levels of immunophilins or calcineurin, C2–C12 cells were washed three times with PBS and extracted in 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 0.5% Triton, 1 mM EDTA, and mixture of protease inhibitors. After removal of an aliquot for protein assay, the extracts were subjected to polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) electrophoretically. After blocking nonspecific binding sites with 5% low-fat milk in PBS containing 0.1% of Tween-20, antibodies against specified proteins or peptides were used to blot the membrane in the same buffer for 1 h at room temperature. After washing the nitrocellulose membrane two times with the same buffer and one time with a buffer containing 150 mM NaCl and 50 mM Tris-HCl (pH 7.5), the antibody-protein complexes were blotted for 1 h at room temperature with secondary antibodies labeled with horseradish peroxidase in the buffer containing 5% low-fat milk. After extensive washing, the protein-antibody complexes were detected with chemiluminescent reagents (Amersham Corp.).

Construction of Recombinant Adenoviruses Containing Calcineurin cDNAs. Conventional transfection methods, such as calcium phosphate or lipofectamine-mediated transfection, resulted in low transfection efficiencies for C2–C12 cells. More importantly, the transfected cells lost CsA responsiveness (Z.D.L., unpublished data), presumably due to transient permeabilization and influx of Ca^{2+}. To overcome the transfection limitations, adenoviruses containing either the constitutively active or the dominant negative recombinant calcineurin constructs were used to infect these cells. The constitutively active construct encodes a truncated calcineurin catalytic subunit (CnAΔCaM-AI) that lacks the sequences encoding the functional calmodulin-binding and autoinhibitory domains (O'Keefe et al., 1992). This construct was designed to mimic proteolysed forms of calcineurin known to have constitutive calcium-independent phosphatase activity in vitro (Hubbard and Klee, 1989; Werlen et al., 1998). Overexpression of this truncated calcineurin subunit in human Jurkat cell line also exhibits Ca^{2+}-independent, constitutive phosphatase activity (O'Keefe et al., 1992; Werlen et al., 1998).

The dominant negative construct lacks the autoinhibitory domain, calmodulin-binding domain, and most of the catalytic core, but contains the binding domain for the B (regulatory) subunit (Muramatsu and Kincaid, 1996). Overexpression of this "B-subunit knock out" (BKO) construct in Jurkat cells results in sequestration of the endogenous B subunit and suppression of calcineurin-mediated activity (Muramatsu and Kincaid, 1996).

Plasmids of pAdv/HA-CnAΔCaM-AI and pAdv/HA-BKO were constructed by inserting an *Eco*RI fragment containing HA-CnAΔCaM-AI or HA-BKO coding sequences from the pSRα3/HA-CnAΔCaM-AI or pSRα3/HA-BKO plasmids, respectively, into the *Eco*RI site of the pAC/CMV vector. The HA epitope was used to detect overexpression of these constructs in Western blots because commercially available anticalcineurin A antibodies recognize an epitope in the deleted region of these constructs and therefore were not useful in our study. The HA tag at the N terminus does not affect the function of the constructs (data not shown) (Werlen et al., 1998). These recombinant adenoviruses were generated through homologous recombination between cotransfected pJM17 plasmid (Wang et al., 1996) and pAdv/HA-CnAΔCaM-AI or pAdv/HA-BKO in 293 cells (American Type Culture Collection). The resulting adenoviruses were plaque purified and amplified in 293 cells. The genomic structure of the recombinant adenovirus was confirmed by polymerase chain reaction analysis with oligonucleotides specific for the insertion site at the E1a region. Recombinant adenoviruses were prepared from CsCl density gradient ultracentrifugation. Titers were determined from A_{260} with 1.0 absorbance being equivalent to $\sim 10^{12}$ particles/ml.

Infection of Calcineurin-Containing Recombinant Adenoviruses in C2–C12 Myocytes. Different dosages of adenovirus were added into culture media to achieve specified multiplicity of infection. The optimal time of infection during muscle differentiation was determined by detecting HA peptide expression in C2–C12 cells infected with pAdv/HA-CnAΔCaM-AI between day 0 and day 2. Differentiating myocytes were infected more efficiently than undifferentiated myoblasts (see *Results*), consistent with findings reported in other rodent muscle cell lines (Quantin et al., 1992). In our experiments, cells infected at day 1 of differentiation express the largest amounts of HA peptides (see *Results*). This may result from two factors. First, expression of integrin receptors, essential for the attachment of adenovirus to host cells (Kohout et al., 1996), is developmentally regulated in C2–C12 cells (Collo et al., 1993; Ziober and Kramer, 1996). The difference in infection efficiencies may result from different expression levels of integrin receptor subtypes mediating the adenovirus infection. Second, when cells were infected at later stages of differentiation (day 2 or 3) and harvested 2 days later, some mature infected myotubes may have detached and died. Thus, cells at day 1 of differentiation were used for all the infection experiments. Adenovirus vector expressing β-gal was used in parallel infections (Wang et al., 1998). After 16 h of infection, medium was removed and cells were incubated for an additional 2 days in fresh medium before extraction of total RNA.

Statistical Analyses. Unpaired Student's t tests were performed where significance is indicated by a two-tailed P value $< .05$.

Results

Enhancement of AChE Expression by CsA in C2–C12 Cells during Myogenesis. CsA treatment caused concentration-dependent increases in the expression of AChE in differentiating C2–C12 cells (Fig. 1). The half-maximal enhancement occurred at ~300 nM, a concentration that correlates well with the K_d value in binding studies (200 nM;

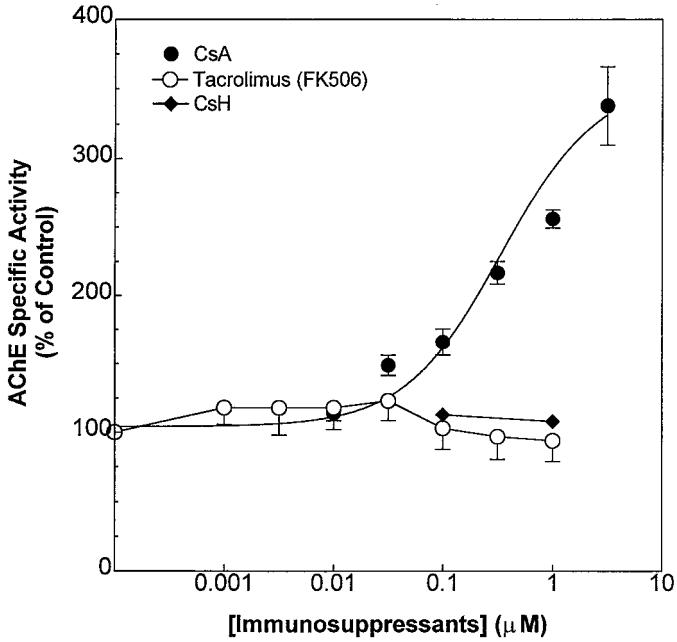

Fig. 1. Concentration dependence of immunosuppressants on differentiation-induced AChE expression in C2–C12 cells. C2–C12 myoblasts at ~70% of confluence were differentiated into myotubes for 3 days in low serum medium in the presence or absence of CsA, CsH, or tacrolimus. AChE was extracted and activity was determined by the method of Ellman et al. (1961). Data are presented as percentage of control within each experiment and the values reported are means ± S.E. from at least six independent determinations.

Handschumacher et al., 1984). In contrast, tacrolimus, another immunosuppressive agent active in the subnanomolar concentration range in other systems (Bierer et al., 1990), had little influence on AChE expression. Treatment with rapamycin, a structurally distinct immunosuppressant, in the subnanomolar concentration range resulted in inhibition of cell growth and differentiation followed by substantial reduction of AChE expression (data not shown). Furthermore, treatment with CsH, an inactive analog of CsA, did not alter AChE expression (Fig. 1).

The effects of CsA are not due to a greater differentiation of the C2–C12 cells because control and CsA-treated (1 μM, 3 days) cells displayed similar morphology (Fig. 2,A and B). In addition, muscle creatine kinase activities for differentiating cells treated with 1 μM CsA for 3 days were similar to control cells (control: 3.2 $\pm$ 0.6 A_{520}/ng protein/min; +CsA: 3.3 $\pm$ 0.2 A_{520}/ng protein/min, $n = 4$). Finally, nAChR expression levels were unchanged (Fig. 3B).

The enhancement of AChE expression by CsA was examined at various stages of muscle differentiation (Fig. 3A). Fractional increases in AChE expression induced by CsA were more evident after initial differentiation. However, following wash out of CsA after 1 day of treatment, the increase in AChE expression was again minimal at day 3. Furthermore, when cells were treated with CsA before terminal differentiation, no enhancement of AChE expression was observed (data not shown). The increased cellular AChE expression is not due to reduction of AChE secretion because AChE found in the medium showed a comparable fractional increase with CsA treatment. The majority of AChE is associated with the cells (data not shown). In contrast to AChE, cell surface ^{125}I-α-BTX binding sites on the nAChR, whose increased expression roughly parallels that of AChE upon differentiation of C2–C12 cells, were not altered by CsA treatment (Fig. 3B).

As shown in Fig. 4 and summarized in Fig. 5, AChE and nAChR mRNA increase substantially during muscle terminal differentiation. The parallel sequential increases of functional AChE and nAChR indicate that the increased expression of both proteins reflects the availability of their transcripts (compare Figs. 3 and 5). CsA treatment during muscle differentiation only augments AChE mRNA levels, but not mRNA levels of the γ-nAChR subunit (Figs. 4 and 5). The mouse *Ache* gene has two polyadenylation signals separated by 1.1 kilobase (Li et al., 1993). With a probe that extends across the first poly A site, we can detect both species. The most 3′ signal is in predominant use during differentiation (69–74%) and this percentage is unchanged by CsA treatment (data not shown). The increased AChE mRNA is mainly the exon 4 to 6 spliced species because only fully protected AChE probe (458 bp) was observed after CsA treatment. The inactive analog CsH (Fig. 4) and another immunosuppressive agent tacrolimus (data not shown) were without effect on both AChE and nAChR mRNA levels.

Run-On Transcriptional Rates of Ache Gene Were Not Altered by CsA. To examine whether the CsA-induced increases in AChE expression are due to enhanced transcription of the *Ache* gene, transcriptional rates of *Ache* and γ-*nAchR* genes were examined. As indicated in Fig. 6, treatment of 1 μM CsA in differentiating C2–C12 cells did not affect run-on transcriptional rates of the *Ache* and γ-*nAchR* genes.

CsA Increases AChE mRNA Stability in C2–C12 Cells. Because CsA treatment did not enhance run-on transcriptional rates of the *Ache* gene, the CsA-induced AChE mRNA expression may be due to increased stability of the transcripts. To test this hypothesis, rates of AChE mRNA degradation were examined in cells treated with or without CsA after blocking transcription with DRB, an adenosine analog that specifically inhibits RNA polymerase II (Tamm et al., 1976). As indicated in Fig. 7, the estimated AChE mRNA half-life in 3-day-differentiated myotubes was ~8 h, a value similar to that reported previously (Fuentes and Taylor, 1993). CsA treatment increased the estimated half-life of AChE mRNA to ~16 h.

Precise determinations of mRNA turnover in this system

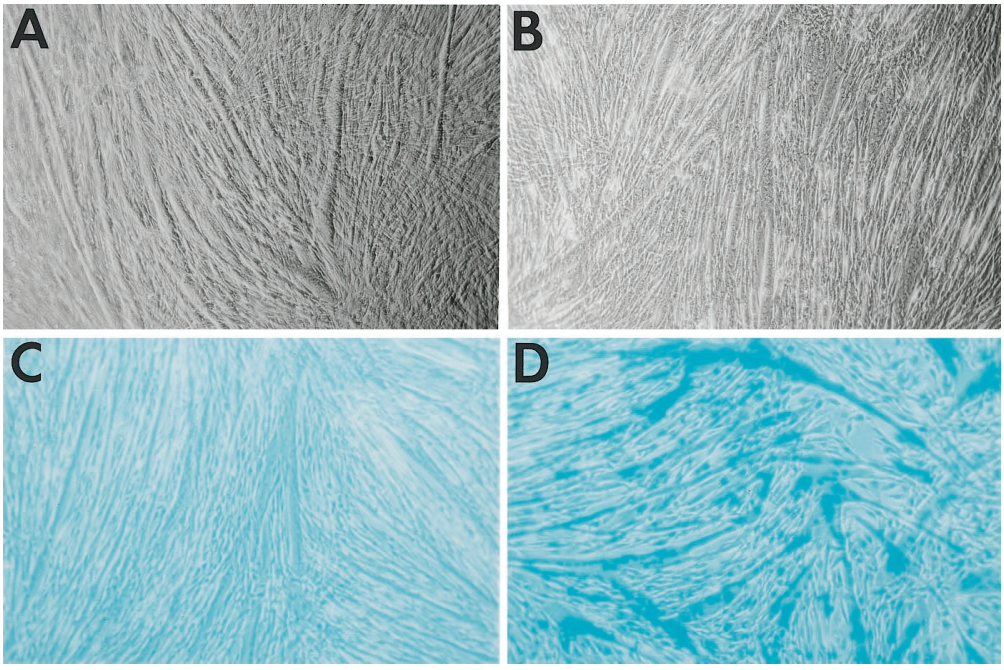

Fig. 2. Morphology of differentiated C2–C12 cells treated with CsA or infected with adenovirus. C2–C12 cells were differentiated for 3 days in the absence (A) or presence (B) of 1 μM CsA; or infected overnight at day 1 without (C) or with (D) adenovirus containing the lacZ gene as described in the text. At day 3 of differentiation, cells were fixed and stained for β-gal activity (C and D), and photos were taken with a 10× phase contrast objective.

are likely to be confounded by several factors. DRB and cycloheximide treatments during muscle differentiation promote a superinduction of AChE mRNA suggesting that destabilizing factors with a rapid turnover may influence AChE mRNA stability in differentiating C2–C12 cells (Fuentes and Taylor, 1993). The potential for superinduction, along with the asynchrony inherent in the differentiation process, precludes measurements of mRNA turnover at an earlier stage when AChE mRNA stabilization may be more evident. Also, asynchrony of differentiation even at day 4 may result in multiphasic decay. Finally, the extended half-life of AChE mRNA in myotubes may be influenced by the generalized inhibition of transcription. Nevertheless, the enhanced mRNA levels in the presence of CsA appear to parallel the diminished initial rate of mRNA degradation and cannot be accounted for by changes in transcriptional rates.

Intracellular Immunophilin and Calcineurin Levels in C2–C12 Cells. Certain cell lines are known to be deficient in tacrolimus binding proteins (Kaye et al., 1992). To ascertain whether the differential effects of CsA and tacrolimus on AChE expression were due to different levels of intracellular receptors for these agents in C2–C12 cells, we examined the concentrations of cyclophilin A, the receptor for CsA, and FKBP12, the receptor for tacrolimus, in C2–C12 cells. Western blot analyses indicate that myoblasts and myotubes express similar levels of cyclophilin A and FKBP12. However, calcineurin expression is low in myoblasts, but increases >3-fold upon muscle differentiation (Fig. 8).

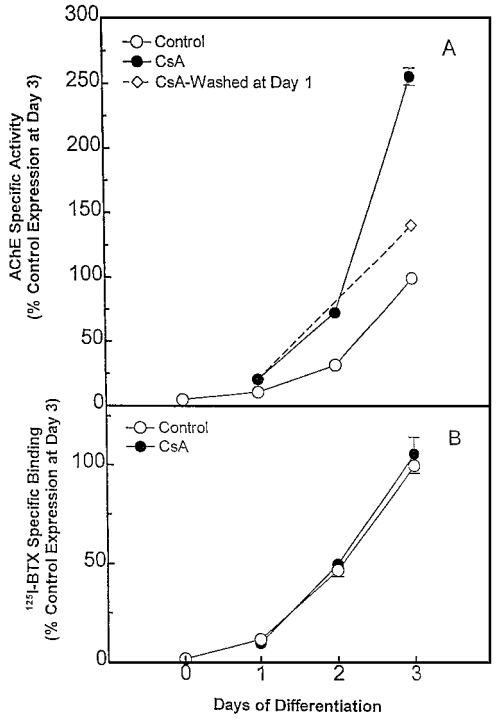

Fig. 3. Kinetics of the influence of CsA on AChE and nAChR expression during myoblast-to-myotube differentiation in C2–C12 cells. Cells were differentiated for 3 days in the presence or absence of 1 μM CsA. Cells from three plates per experimental point were harvested at designated times and either AChE activity or α-BTX binding sites on the nAChR were determined. A, AChE activity in C2–C12 cells with or without CsA treatment. B, α-BTX binding sites in C2–C12 cells treated with or without CsA treatment. AChE activity or α-BTX binding sites on the nAChR at day 3 of differentiation were chosen as maximum (100%) values. Values reported are means ± S.E. from at least six independent determinations.

Influence of Overexpressed Calcineurin Mutants on AChE Expression. Because cyclophilin is expressed in myoblasts and myotubes at comparable high cellular concentrations, its regulation is less likely to be responsible for the CsA induction than calcineurin, whose expression associated with muscle differentiation is enhanced >3-fold. To test this hypothesis, overexpression of constitutively active (HA-CnAΔCaM-AI) or dominant negative (HA-BKO) calcineurin constructs was sought. As indicated in Fig. 2, C and D, adenovirus infect nearly 100% of the C2–C12 cells and infection does not affect cell differentiation as indicated by the normal muscle morphology of infected cells. Infected differentiating C2–C12 cells express the calcineurin constructs well as indicated by expression of a large amount of the HA peptide (Fig. 9). Muscle morphology also was not altered in cells overexpressing calcineurin (data not shown). If CsA enhancement of AChE mRNA level is mediated by calcineurin inhibition, then calcineurin, perhaps through its phosphatase activity, may play a role in destabilizing AChE mRNA during myogenesis. If this is the case, overexpression of HA-CnAΔCaM-AI should reduce AChE mRNA levels in differentiating cells, and its effect should be blocked by CsA treatment. Conversely, overexpression of HA-BKO should increase AChE mRNA levels in differentiating cells and its effect should be further enhanced by CsA treatment. These predictions are generally supported by our findings shown in Fig. 10. Overexpression of HA-CnAΔCaM-AI in untreated differentiating myotubes resulted in a 59% reduction of AChE mRNA compared with mock-infected, untreated myotubes ($P < .001$). This calcineurin-induced inhibition was blocked by CsA treatment ($P < .001$) at concentrations that should be expected to inhibit both the endogenous and mutant calcineurins expressed after infection. In contrast, overexpression of HA-BKO in untreated differentiating myotubes

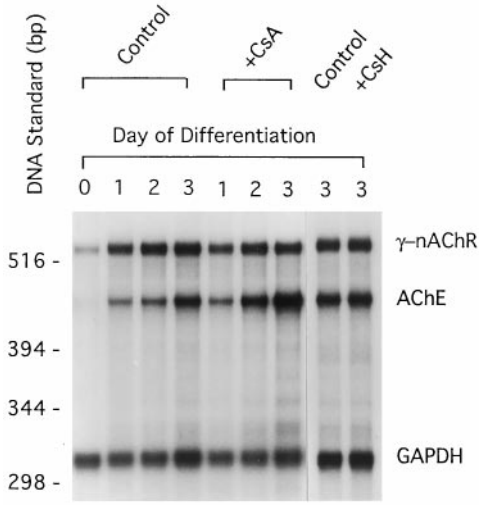

Fig. 4. Representative autoradiogram showing the influence of CsA and CsH on differentiation-induced expression of AChE transcripts in C2–C12 cells. Cells were differentiated in the presence or absence of 1 μM CsA or CsH for 3 days, total RNA was extracted at the indicated times, and analyzed by RNase protection. The AChE probe extended between bp 1651 and 2107 in the cDNA. Protection by a mRNA species splicing between exons 4 and 6 should yield a 456-bp band. The receptor probe extended between bp 1168 and 1716 in the cDNA and the γ-nAChR subunit mRNA should protect a 548-bp species. To standardize for loading differences, a GAPDH probe was included that gave a protected band of 316 bp. To ensure complete RNA digestion, protection was examined in the presence of transfer RNA.

caused a 48% increase of AChE mRNA compared with mock-infected, untreated myotubes ($P < .05$), which was further enhanced by CsA treatment ($P < .05$) (a total of 140% increase over mock-infected, untreated myotubes), indicative of CsA possessing the capacity to inhibit the residual calcineurin activity.

Discussion

Our findings indicate that treatment of CsA in differentiating C2–C12 muscle cells enhances differentiation-induced expression of AChE, but not nAChR. The enhancement is seen at both the level of AChE mRNA and gene product (Figs. 1, 3–5) indicating that CsA primarily influences transcript production or its stabilization. Previous studies analyzing run-on transcription rates, transcription rates of *Ache*-luciferase reporter gene constructs, superinduction of AChE mRNA by inhibition of protein synthesis, and AChE mRNA degradation rates all suggest that mRNA stabilization plays a critical role in enhanced AChE expression associated with muscle differentiation (Fuentes and Taylor, 1993; Li et al., 1993; Luo et al., 1994). CsA treatment does not increase the apparent transcriptional rate of the *Ache* gene (Fig. 6), suggesting that CsA regulation is due mainly to further enhancement of the stability of AChE mRNA during differentiation. This is confirmed by the finding that CsA treatment increases the estimated half-life of AChE mRNA (Fig. 7). Thus, a CsA-sensitive pathway plays a role in the differentiation-induced stabilization of AChE mRNA.

The action of CsA appears to be specific for AChE expression and requires its interaction with intracellular cyclophilins. These conclusions are supported by the following observations. The EC_{50} of the CsA action (300 nM) correlates well with the K_d value (200 nM) for its binding to cyclophilins (Fig. 1; Handschumacher et al., 1984). In addition, CsA treatment selectively enhances expression of AChE, but not nAChR whose expression is enhanced by transcriptional activation during myogenesis (Evans et al., 1987; Baldwin and Burden, 1988). Furthermore, the action of CsA is reversible because treated cells show a normal rate of AChE expression during myogenesis after CsA was removed (Fig. 3). Finally, CsH, an inactive analog of CsA, and tacrolimus, another immunosuppressive agent, neither of which bind to cyclophilins (Handschumacher et al., 1984; Harding et al., 1989; Siekierka et al., 1989), do not influence AChE expression (Fig. 1). The inability of tacrolimus to enhance AChE expression is not due to the absence of intracellular receptors as

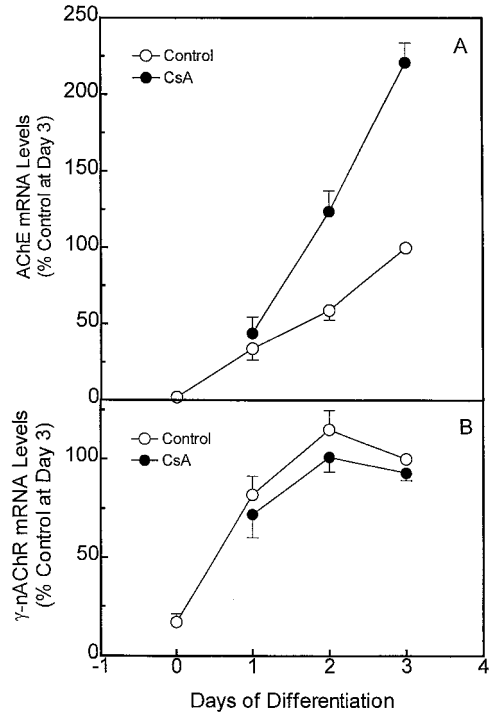

Fig. 5. Influence of CsA on differentiation-induced expression of AChE and nAChR mRNAs in C2–C12 cells. Cells were differentiated for 3 days in the presence or absence of 1 μM CsA, total RNA was extracted, and mRNA levels for AChE or the γ-subunit of nAChR were determined as described in Fig. 4. The mRNA levels were quantified by taking the ratio of AChE or nAChR band densities over that of GAPDH to correct for sample loading errors and then compared with values from 3-day control samples, which were chosen as 100%. Values reported are means ± S.E. from at least nine independent determinations. A, AChE transcript levels during differentiation. B, γ-nAChR subunit transcript levels during differentiation.

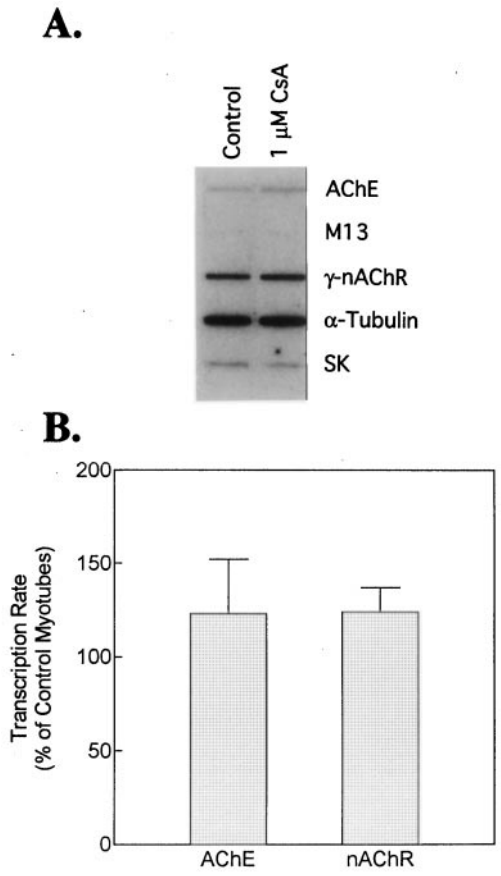

Fig. 6. Influence of CsA on transcriptional rates of the *Ache* and *nAchR* genes measured by run-on transcription. ^{32}P-α-UTP labeled transcripts were synthesized in nuclei isolated from C2–C12 cells differentiated for 3 days in the presence or absence of 1 μM CsA and hybridized to slots containing 5 μg each of the appropriate cDNAs. These cDNAs include double-stranded γ-nAChR and α-tubulin cDNAs in Bluescript II SK (SK) plasmids and single-stranded AChE antisense cDNA in M13 phage (M13). Vector DNAs were used as controls. A, representative autoradiogram. B, quantification of the signals by densitometric analysis. Transcriptional rates of the genes in control myotubes were chosen as 100% values. Densities were normalized with respect to α-tubulin mRNA densities. Values reported are means ± S.E. from at least three independent determinations.

observed for mast cells (Kaye et al., 1992) because FKPB12 can be detected in both myoblasts and myotubes (Fig. 8).

Myoblast differentiation is restricted in G_1 phase of the cell cycle (Clegg et al., 1987). Even though CsA is known to arrest the cell cycle in G_0 phase in cells such as lymphocytes (Schreiber and Crabtree, 1997) and keratinocytes (Karashima et al., 1996), CsA action on AChE expression is not likely to be associated with cell cycle arrest because treatment with CsA before the induction of muscle differentiation does not increase AChE mRNA expression (data not shown). In addition, CsA-treated muscle cells display normal differentiation as assessed by their muscle fiber morphology, fusion to myotubes (Fig. 2, A and B), creatine kinase activity, and expression of nAChR (Figs. 3–5). Furthermore, when cells were treated with CsA for 1 day, then allowed to differentiate for two additional days in CsA-free medium, the AChE expression rate is similar to that of control cells (Fig. 3A), consistent with CsA action being associated with muscle differentiation, rather than the immunosuppressant triggering an early event in differentiation.

It is known that CsA binds to its immediate downstream target cyclophilin A and inhibits *cis-trans* proline isomerase activity of cyclophilin (Harding et al., 1989). However, similar to immunosuppressant activities of this drug (Bierer et al., 1990), the influence of CsA on AChE mRNA seems independent of the isomerase activity of cyclophilin. This is supported by the findings that expression of cyclophilin A is independent of muscle differentiation (Fig. 8), whereas the CsA action appears differentiation-dependent. Furthermore, overexpression of cyclophilin A does not increase AChE mRNA levels during myogenesis (Z.D.L., unpublished data).

CsA-cyclophilin and FK506-FKBP complexes are known to bind to a common target calcineurin and therein inhibit its phosphatase activity (Liu et al., 1991). The correlation between differentiation-induced expression of calcineurin (Fig. 8) and AChE (Figs. 3–5) suggests a role of calcineurin in regulation of AChE mRNA. The down- or up-regulation of AChE mRNA upon overexpression of the constitutively active or dominant negative calcineurin constructs, respectively, in untreated cells indicates a role for calcineurin in destabilizing AChE mRNA during myogenesis (Fig. 10). This regulatory mechanism may serve to control enhanced biosynthesis of AChE during myogenesis, which has a long half-life of ~50 h (Rotundo and Fambrough, 1980). Because the effects of constitutively active or dominant negative calcineurin on AChE mRNA can be reversed or enhanced, respectively, by CsA treatments (Fig. 10), it is likely that the

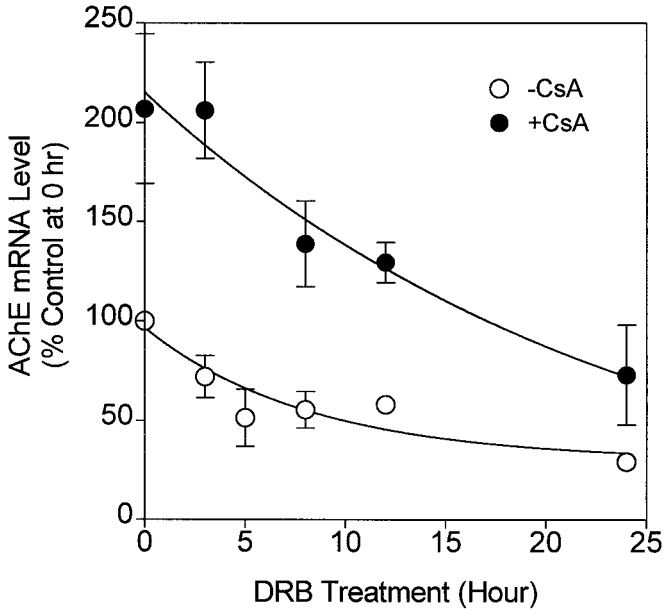

Fig. 7. Influence of CsA on AChE mRNA stability in C2–C12 cells. AChE mRNA degradation was measured by RNase protection at different time points after blocking cellular transcription with 30 μg/ml DRB in differentiating C2–C12 cells treated with or without 1 μM CsA for 3 days. At this stage, cells have differentiated into myotubes with greatly increased AChE mRNA. Data are presented as percentage of values from control myotubes immediately after DRB treatment. The values reported are means ± S.E. from four to six independent determinations.

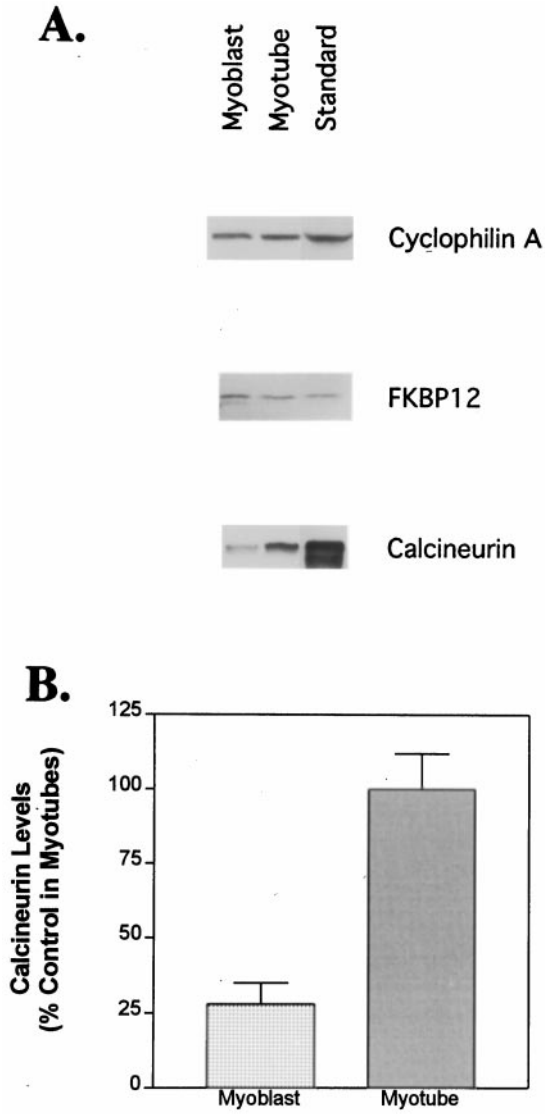

Fig. 8. Cellular levels of cyclophilin A, FKBP12, and calcineurin catalytic subunit in C2–C12 cells at the myoblast and myotube stages. Cell extracts from myoblasts and 3-day differentiated myotubes were subjected to Western blot analysis with the indicated antibodies as described in *Experimental Procedures*. A, representative blots from at least two independent experiments. Positive controls were cyclophilin A and FKBP12 from Jurkat cell extracts (a gift from Dr. Michael Karin, University of California-San Diego) and purified calcineurin A subunit (Sigma Chemical Co.). B, tabulation of cellular calcineurin A subunit levels. Data represent means ± S.E. from three independent determinations.

CsA-induced stabilization of AChE mRNA is mediated through inhibition of calcineurin. The similar increases of AChE mRNA in CsA-treated cells with or without overexpression of the calcineurin constructs indicate that CsA at this concentration (1 μM) may have elicited close-to-maximal inhibition of endogenous and exogenous calcineurin activities, thus, achieving a near maximal increase in AChE mRNA (Fig. 10).

The inability of tacrolimus to produce the same response as CsA (Fig. 1) suggests that the phosphatase activity of calcineurin per se may not be the sole determinant of CsA-induced AChE expression. Specificity for a particular phosphoprotein and/or a downstream association responsive to the CsA-cyclophilin-calcineurin complex, but not to that of FK506-FKBP12-calcineurin, may govern the CsA response in muscle. This hypothesis is supported by the notion that phosphatases exhibit substrate selectivity by recognizing three-dimensional patches on phosphoproteins remote from the dephosphorylation site (Schreiber, 1992). For example, these drug-immunophilin complexes inhibit phosphatase activity of calcineurin toward phosphopeptide and phosphoprotein substrates, yet they cause a 2- to 3-fold activation of phosphatase activity toward a small substrate, *p*-nitrophenyl phosphate (Liu et al., 1991). Thus, the catalytic site of the inhibited calcineurin is still accessible to small substrates, suggesting that the binding of drug-immunophilin complexes to an allosteric rather than the active site of calcineurin modulates substrate selectivity. In addition, CsA-cyclophilin A and FK506-FKBP12 bind to distinct, highly conserved regions of calcineurin A (Cardenas et al., 1995), which may cause distinct conformational changes of the enzyme and result in different binding affinities for downstream targets. This behavior was found for phosphatase 2A where replacement of its regulatory subunit by the small T antigen of simian virus 40 results in alteration of its substrate specificity and cellular localization (Mumby and Walter, 1991).

Collectively, our studies suggest that calcineurin-mediated dephosphorylation is an important component in the calcium-sensitive regulation of AChE mRNA stability (Luo et al., 1994, 1996). Dephosphorylation may serve to activate destabilizing factors or inactivate stabilizing factors, which are involved in governing AChE mRNA lifetimes during myogenesis. These factors, however, may be influenced by certain conformations of calcineurin complexes induced by CsA, but not FK506. In fact, emerging data support an integrated, calcium-calmodulin-dependent pathway in regulation of AChE mRNA stability. For example, intracellular immunophilins are found to serve as accessory proteins of ryanodine receptors to modulate intracellular calcium (Timerman et al., 1993; Brillantes et al., 1994). In addition, calmodulin regulates intracellular calcium channel activity by interacting with several cytoplasmic domains of the ryanodine receptors (Wagenknecht et al., 1997), which are known to play an

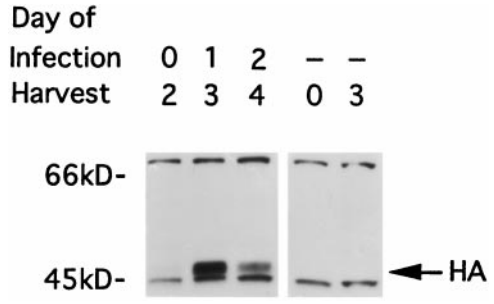

Fig. 9. Optimal time for overexpression of calcineurin constructs in differentiating C2–C12 cells. Cells were differentiated at day 0 and infected overnight with pAdv/HA-CnAΔCaM-AI as indicated. Cells were extracted 2 days after infection and subjected to Western blot analysis with anti-HA antibodies. Uninfected myoblasts (at day 0 of differentiation) and myotubes (differentiated for 3 days) were used as controls. Bands at 45- and 70-kD positions are backgrounds of the HA antibody that also are seen in other cells. The HA-CnAΔCaM-AI protein has a molecular mass of ~48 kD. Data are representative from two independent infections.

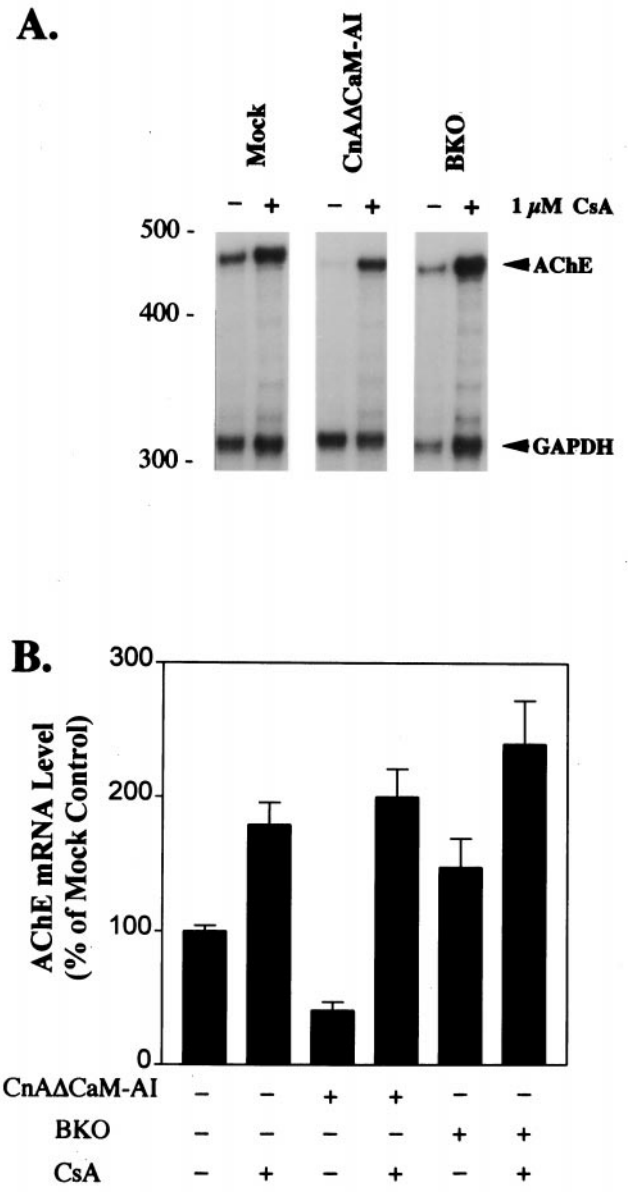

Fig. 10. Influence of overexpression of calcineurin mutants on AChE mRNA levels. Control and CsA-treated C2–C12 cells were infected with control adenovirus vectors, or vectors containing a constitutively active calcineurin construct, pAdv/HA-CnAΔCaM-AI, or a dominant negative calcineurin construct, pAdv/HA-BKO, at day 1 of myoblast differentiation and harvested 2 days later. Total RNA was extracted, treated with DNase, and subjected to RNase protection. A, representative autoradiogram. B, tabulation of the influence of HA-CnAΔCaM-AI or HA-BKO overexpression on AChE mRNA expression. Data represent means ± S.E. from 12 independent determinations coming from four separate platings of C2–C12 cells.

important role in the calcium-sensitive regulation of AChE mRNA stability during myogenesis (Luo et al., 1994). Our study suggests that calcineurin may be an important mediator of the regulation.

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