Depolarization of Neural Cells Induces Transcription of the Down Syndrome Critical Region 1 Isoform 4 via a Calcineurin/Nuclear Factor of Activated T Cells-dependent Pathway*

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In this study we showed that the transcriptional regulation of Down syndrome critical region isoform 4 (DSCR1.4) is mediated by the calcineurin/nuclear factor of activated T cells (NFAT) pathway in neural cells. Stimuli that elicit an increase in the intracellular concentrations of calcium, such as membrane depolarization, induced de novo transcription of DSCR1.4, with mRNA expression peaking after 4 h and then declining. Action via the physiologically relevant L-type calcium channel was confirmed by blockade with nifedipine and verapamil. This calcium-dependent transcription of DSCR1.4 was inhibited by the calcineurin inhibitors cyclosporin A and FK506. Deletional analysis showed that the calcium- and calcineurin-dependent activation is mediated by the promoter region between nucleotides −350 and −166, a region that contains putative NFAT-binding motifs. Exogenous NFATc2 potently augmented the DSCR1.4 promoter transcriptional activity, and the involvement of endogenous NFAT signaling pathway in DSCR1.4 transcription was confirmed by the suppression of depolarization-inducible promoter activity with the NFAT inhibitor peptide VIVIT. Exogenous overexpression of DSCR1 protein (calcipressin 1) resulted in the inhibition of the transcription of DSCR1.4 and NFAT-dependent signaling. These findings suggest that calcineurin-dependent induction of DSCR1.4 product may represent an important auto-regulatory mechanism for the homeostatic control of NFAT signaling in neural cells.

Calcium signaling is essential for neuronal survival and function (1–3). Membrane depolarization opens L-type voltage-gated calcium channels (L-VGCC), leading to an influx of calcium (Ca$^{2+}$) into cells, and the resulting increases in intracellular Ca$^{2+}$ concentration activate a wide array of signaling pathways (reviewed in Ref. 4). A key element of the cellular response to Ca$^{2+}$ signals is the action of the phosphatase calcineurin (CN). CN is a Ca$^{2+}$ - and calmodulin-dependent phosphatase that was first discovered in neuronal tissue, where it has its greatest abundance (5), and has since been found in many other tissues (reviewed in Refs. 6 and 7). CN controls a wide variety of physiological processes, such as hippocampal long term potentiation and long term depression, lymphocyte activation and apoptosis, development of the heart, differentiation of skeletal muscle fiber type, and cardiac functions (6, 8).

Signaling pathways controlled by CN have been the most studied in lymphocytes, where the main pathway of action for this phosphatase so far characterized has been the regulation of nuclear factor of activated T cells (NFAT) family of transcription factors (9). Calcineurin-mediated dephosphorylation promotes translocation of NFAT proteins to the nucleus, where they bind specific elements within the target gene promoters, in association with other transcription factors (10–13). The pharmacological action of immunosuppressive drugs such as cyclosporin A (CsA) and FK506 is based on inhibition of CN in immune effectors cells (14).

The CN/NFAT pathway can be activated by increases in intracellular Ca$^{2+}$ concentrations in the central nervous system that regulates the expression of the inositol 3-phosphate receptor and brain-derived neuronal factor genes in neurons (15–17). Furthermore, CN/NFAT signaling has been shown to be necessary for axonal outgrowth during mouse development (18). Despite the wide importance of CN/NFAT signaling and its role in the central nervous system, little is known about the specific genes regulated by this pathway in neural cells.

CN signaling is tightly regulated within the cell. Several endogenous cellular CN modulators have been identified, among them AKAP79 (19), Cabin/Cain (20, 21), the calcineurin B homology protein (22), and the calcipressin family of proteins (reviewed in Ref. 23). In humans, this last family includes the

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products of the Down syndrome critical region 1 (DSCR1) gene, also known as calcipressin 1, MCIP1, and Adapt 78 (24–26); the protein products of the DSCR1L1 gene, calcipressin 2; the protein product of the DSCR1L2 gene, calcipressin 3 (28). Calcipressins 1 and 2 are known to bind and negatively regulate CN (26, 29–31).

**DSCR1** transcripts are expressed in diverse cell types and tissues, including heart/cardiac muscle (32–35), striated muscle (26, 36), brain/neuronal cells (33, 37), and T cells (38). In the adult brain, **DSCR1** is expressed in the same regions as calcium and magnesium (33, 39, 40).

The human **DSCR1** gene consists of 7 exons, of which exons 1–4 can be alternatively spliced, resulting in a number of different mRNA isoforms. Exons 1 and 4 containing transcripts encode for distinct protein isoforms that differ at their N-terminus, giving rise to a 252-amino acid protein, calcipressin 1-L, and a 197-amino acid protein, calcipressin 1-S, respectively (41). For the mouse orthologous gene, only isoforms containing equivalent exons 1 and 4 have been detected. These isoforms have different expression patterns and different regulation mechanisms that control their expression. For example, the transcriptional regulation through a calcineurin-dependent pathway is exclusive of an internal promoter placed between exons 3 and 4 that regulates the expression of the **DSCR1.4** variant (36). Very recently it has been described as a down-regulation of the **DSCR1 exon 1 promoter via a Notch-dependent pathway** (42).

The analysis of the internal promoter, located upstream exon 4, shows a dense cluster of consensus NFAT-binding motifs (36). In endothelial cells, stimulation of the cells with vascular endothelial growth factor (VEGF), thrombin, and Ca²⁺ ionophore induces an up-regulation of the **DSCR1.4** mRNA due to an activation of the promoter, through a CN/NFAT-dependent pathway (43, 44). Noteworthy, this pathway appears not to induce any of the other **DSCR** isoforms or other **DSCR** family members (31, 36, 43).

Transcription induction of the **DSCR1.4** mRNA has been reported in hearts subjected to pressure overload (45) and in neonatal rat cardiac myocytes in response to mechanical stress or in response to a number of hormones, cytokines, and stress (25, 34, 46, 47). However, very little is known about the mechanisms that control the expression of **DSCR1** and their role in neural cells.

In this work, we have identified the induction of **DSCR1.4** mRNA in a screen of candidate genes activated by the CN/NFAT pathway in neural cells in response to stimuli that increase concentrations of intracellular Ca²⁺ such as membrane depolarization. By using the PC12 cells as a model neural system, we show that **de novo DSCR1.4** transcription is induced by these stimuli in a CN/NFAT-dependent manner. We show the existence of a regulation loop that controls NFAT-dependent transcription, suggesting a role in the homeostasis of this signaling pathway in neural cells.

**MATERIALS AND METHODS**

**Cell Culture and Reagents**—Rat pheochromocytoma PC12 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 100 units/ml penicillin and streptomycin. Cells were maintained under the conditions defined by the ATCC. Phorbol 12-myristate 13-acetate (PMA) and the Ca²⁺ ionophore A23187 were from Calbiochem. Actinomycin D (ActD), cycloheximide, nifedipine, verapamil, and KCl were all purchased from Sigma. CsA and FK-506 were purchased from Sandoz (East Hanover, NJ) and LC Laboratories (Woburn, MA).

**Cell Fractionation and Immunoblot Analysis**—Confluent PC12 cells in 100-mm culture dishes were exposed to vehicle or to a specific inhibitor for 1 h and then treated with pharmacological stimuli for different times as indicated under “Results.” After stimulation, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in 150 µl of hypotonic buffer A (10 mU Tri-HCI, pH 7.5, 10 mM NaCl, 3 mg MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 0.1 mM EGTA, and 2 µg/ml each leupeptin, aprotinin, and pepstatin) supplemented with 0.05% Nonidet P40, 0.75 mM spermine, and 10 mM Na₂Mo₄. Lysates were centrifuged for 10 min at 13,000 × g, and the supernatants, containing the cytosolic extracts, were each mixed with an equal volume of 2× Laemmli buffer containing 2-mercaptoethanol. The nuclei-containing pellets were washed twice in hypotonic sample buffer without detergent and lysed for 30 min on ice with occasional mild agitation in 50 µl of hypotonic buffer C (20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM Na₂Mo₄, 2 µg/ml each of leupeptin, aprotinin, and pepstatin, and 0.1% Triton X-100). The resulting nuclear protein extract was then centrifuged at 13,000 × g for 10 min, and the supernatant, containing most of the nuclear proteins, was mixed with an equal volume of 2× Laemmli buffer. For whole-cell extracts, cells grown and stimulated in 35-mm dishes were lysed with 150 µl of hypotonic buffer C and resuspended in 2× Laemmli buffer as described above.

Extracts were boiled for 5 min and resolved by SDS-PAGE under reducing conditions (8% polyacrylamide for NFATc2). Proteins were transferred to nitrocellulose membranes that were then blocked overnight at 4 °C in Tris-buffered saline with 0.05% Tween 20 (TBS-T) containing 5% (v/v) skimmed milk. The membranes were washed twice with TBS-T and incubated for 2 h at room temperature with a 1:3000 dilution of rabbit polyclonal antisera 672, raised against the NH₂-SPPSPGAYPDVD-LDYGLK² peptide (residues 53–70 of human NFATc2), as described previously (48). The membranes were then washed four times with TBS-T and incubated for 1 h at room temperature with the secondary antibody (peroxidase-labeled goat anti-rabbit IgG, Pierce) and washed four times with TBS-T. Bound antibodies were detected with ECL Western blotting analysis kit (Amersham Biosciences).

**Nuclear Extracts and EMSA**—Nuclear protein extracts were obtained as described above (without dilution in Laemmli buffer); the protein concentration was quantified by the Bradford procedure (Bio-Rad), and the extracts were snap-frozen and stored at −70 °C. Electrophoretic mobility shift assays (EMSA) were performed as follows. Three to five µg of nuclear protein were incubated in a reaction mixture containing 5% (v/v) rabbit liver extract for 10 min in a final volume of 15 µl with 5× NFAT DNA binding buffer (10% polyvinylalcohol, 12.5% glycerol, 50 mM Tris, pH 8.0, 2.5 mM EDTA, 2.5 mM DTT). 2 µl (1 ng/µl) of a 5′-labeled double-stranded oligonucleotide (5×10⁹–1×10¹⁰ cpm/µg) was then added to this reaction mixture, and the reaction was continued at 4 °C for a further 30 min. The sequence of the oligonucleotide probe used was 5′-gatcGAGGAAGAAAATCCTATACAGAAGCGGT-3′ (the distal NFAT site from the human IL-2 promoter). The NFAT binding site is underlined. DNA-protein complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels (49).

**Immunofluorescence**—PC12 cells were grown on poly-L-lysine-coated glass coverslips. After stimulation, the cells were washed twice with cold PBS and fixed for 15 min with 4% paraformaldehyde (w/v) in PBS. Cells were permeabilized twice with PBS and permeabilized for 30 min with PBS-containing 0.25% (v/v) Triton X-100. After three washes with PBS, the fixed cells were blocked with 10% bovine serum albumin in PBS (PBS/bovine serum albumin) for 20 min. Cells were incubated for 1 h at room temperature with a 1:1500 dilution of rabbit polyclonal antisera 672. After three washes with PBS, cells were incubated for 30 min at room temperature with the secondary antibody (Alexa Fluor 488-labeled goat anti-rabbit IgG, Molecular Probes, Eugene, OR) in the presence of the nucleic acid dye To-Pro 3 (Molecular Probes). Cells were mounted and analyzed on an LSM510 META confocal laser microscope (Zeiss, Germany).

**RNA Isolation, Reverse Transcription, and Real Time PCR Analysis**—Total RNA was isolated from PC12 cells with TriPure isolation reagent (Roche Applied Science), according to the manufacturer’s instructions. Two µg of the isolated total RNA were reverse-transcribed at 37 °C for 50 min in a 20-µl reaction mixture containing 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen), 100 ng of random primers, and 40 units of RNase Inhibitor (Invitrogen). The reaction was terminated by denaturing the reverse transcriptase at 95 °C for 5 min, and the samples were stored at −70 °C.

Differential gene expression in PC12 cells was assessed by real time quantitative RT-PCR of target genes on a micro-fluidic card assay system developed by Applied Biosystems (Foster City, CA). The card included TaqMan probes and primer sets for the amplification of 24 target genes, thus acting as a low density custom array. RT-PCR was conducted with the TaqMan Universal PCR master mix kit (Applied Biosystems) and the ABI Prism 7900 sequence detection system (Applied Biosystems).
Transcripts encoding rat DSCR1 were further quantified by TaqMan real time quantitative RT-PCR. PCR primers and TaqMan probes were obtained from Applied Biosystems and optimized according to the manufacturer’s protocol. The probe designed for rat DSCR1.4 (MCIPL4 mRNA, GenBank™ accession number NM_153724.2) specifically recognizes the cDNA sequence encoding the short form (197 amino acids, splicing variant 4 or calcipressin 1-S) of DSCR1.4 and the long form (411 amino acids, splicing variant 1 or calcipressin 1-L) of DSCR1.1, DSCR1.2, and DSCR1.3. Depolarization with KCl, and this was accompanied by accumulation of the dep-phosphorylated form in the nucleus. A similar pattern was observed when cells were treated with Ca^{2+} ionophore (Io).

RESULTS

Depolarization Induces NFAT Transcriptional Activity in PC12 Cells—Exposure to high concentrations of KCl, which induces membrane depolarization in PC12 cells, triggers a rise in intracellular Ca^{2+} concentrations (data not shown). Because NFATc2 is expressed in this cell line (55), we decided to explore whether depolarization with KCl induced NFAT activity. We first examined NFATc2 protein expression, phosphorylation status, and translocation in KCl-stimulated PC12 cells. Cytosolic and nuclear extracts were first immunoblotted with a polyclonal anti-NFATc2 antiserum. As shown in the upper panel of Fig. 1A, disappearance of phosphorylated NFATc2 from the cytosol was observed within 5 min of exposure to 50 mM KCl, and this was accompanied by accumulation of the de-phosphorylated form in the nucleus. A similar pattern was observed when cells were treated with Ca^{2+} ionophore (Io).

Pretreatment of cells with the CN inhibitor CsA fully prevented NFAT de-phosphorylation (Fig. 1B). Translocation of NFATc2 was confirmed by immunofluorescence (Fig. 1C). Nuclear localization of NFATc2 was evident as short as 15 min of KCl exposure, and this translocation was inhibited by pretreatment with CsA.

Consistent with these data, EMSA analysis revealed that nuclear proteins in KCl-stimulated PC12 cells formed a specific DNA-nuclear protein complex with an NFAT/AP1 consensus sequence (Fig. 1D, lanes 2–4). Formation of this complex was abolished by pretreatment of cells with CsA (Fig. 1D, lane 5). The presence of NFAT in these complexes was confirmed with the anti-NFAT antibody 674, which recognizes a common epitope in the DNA binding domain of all NFAT family members (56) (data not shown).

Membrane depolarization also induced NFAT-dependent transcriptional activity. PC12 cells were transfected with a reporter plasmid encoding the luciferase gene driven by a min-

![Figure 1](image149x573to473x738)

**FIG. 1.** NFAT nuclear translocation by depolarization in PC12 cells. **A**, immunoblot showing the endogenous expression and activation of NFATc2 in cytosolic (lanes 1–5) and nuclear (lanes 6–10) fractions of PC12 cells treated with 50 mM KCl or 1 μM CsA for the times indicated. NFATc2 was detected with anti-NFATc2 antibody (672), and the positions of phosphorylated NFAT (NFAT-P) and de-phosphorylated NFAT are indicated. The asterisk marks the position of a nonspecific band found in cytosolic fractions, running below the NFAT band. ns indicates samples from nonstimulated control cells. **B**, immunoblot of NFATc2 in total lysates from PC12 cells pretreated for 1 h with vehicle (lanes 1–6) or 100 ng/ml CsA (7–12) before exposure to 50 mM KCl for the times indicated. The asterisk marks the nonspecific band also found in cytosolic extracts. **C**, immunofluorescence analysis of endogenous NFAT protein with anti-NFATc2 antibody (672). PC12 cells were pretreated for 1 h with CsA or vehicle and then exposed for 1 h to 50 mM KCl or were nonstimulated (ns). **D**, EMSA of nuclear extracts from PC12 cells pretreated with vehicle (lanes 1–4) or 100 ng/ml CsA (lane 5) and then exposed to 50 mM KCl for the times indicated. The nuclear extracts were incubated with a probe containing the composite NFAT/AP1 site from the human IL-2 promoter. The position of the specific NFAT-DNA complex is indicated by the arrow.

![Image](image149x573to473x738)
Depolarization-inducible NFAT Activity and DSCR1 Isoform 4 Transcription Are Mediated via L-VGCC—Membrane depolarization of neural cells provokes the increase of intracellular Ca\(^{2+}\) concentration, mainly via L-VGCC. To determine whether these channels are involved in KCl-induced CN/NFAT activation in PC12 cells, we pretreated cells with the L-VGCC inhibitors verapamil and nifedipine. Fig. 4A shows an immunoblot of whole-cell extracts with the anti-NFATc2 antiserum. Verapamil and nifedipine both effectively blocked NFATc2 dephosphorylation elicited by KCl with no effect on the CaA-sensitive NFATc2 de-phosphorylation induced by Io. Immuno-fluorescence experiments confirmed that both verapamil and nifedipine also prevented the nuclear localization of NFAT in PC12 cells (Fig. 4B).

The inhibition of the dephosphorylation and subsequent nuclear localization of NFAT were paralleled by the inhibition of DSCR1.4 gene transcription (Fig. 4C). Pretreatment of PC12 cells with either verapamil or nifedipine prevented the accumulation of DSCR1.4 transcripts induced by membrane depolarization.

CN-dependent Activation of the DSCR1.4 Promoter by Membrane Depolarization Involves L-VGCC—To define the region of the DSCR1 internal promoter that mediates Io- and KCl-inducible DSCR1.4 expression in neural cells, we transfected PC12 cells with luciferase reporter constructs driven by different promoter deletion of the human DSCR1 internal promoter. These deletions spanned the region between −1664 and −166 bp from the TATA box (Fig. 5A) (43). The luciferase activity induced by KCl in cells expressing any of the constructs containing the promoter region 350 bp upstream of the TATA box (−1664/+3, −750/+3, and −350/+3) was 4−6-fold greater than that of nonstimulated control cells (Fig. 5A). In contrast, the −166/+3 promoter fragment only supported basal luciferase activity in membrane-depolarized cells. It thus appears that the regulatory elements between nucleotides −350 and −166 of the human DSCR1.4 promoter are responsible for the KCl induction of its activity. This region includes putative GATA and NFAT regulatory motifs, as indicated in Fig. 5A (43).

Transcription from the −350/+3 construct of the DSCR1 internal promoter (the minimal promoter region that supports membrane depolarization-inducible luciferase activity) was inhibited by pretreatment of cells with CsA before stimulation with KCl or with Io (Fig. 5B).

To determine whether the KCl-induced DSCR1.4 promoter activity was mediated by voltage-operated Ca\(^{2+}\) channels, we pretreated PC12 cells expressing the DSCR1(−350/+83) luc reporter with nifedipine and verapamil for 1 h before stimulation. Consistent with the inhibitory effect of these drugs on DSCR1.4 gene transcription shown in Fig. 4C, both L-type VGCC blockers completely inhibited KCl-induced promoter activity (Fig. 5C). As predicted, Io-induced promoter activity was not affected by the L-type Ca\(^{2+}\) channel blockade.

DSCR1.4 Promoter Activity Is Regulated by Direct Activation or Inhibition of CН/NFAT Signaling Pathway—To obtain direct evidence for the activation of the DSCR1.4 promoter by the CN/NFAT pathway in PC12 cells, CN signaling was activated by exogenous expression of a constitutively active mutant of the catalytic subunit A of human calcineurin (CaA active) together with the regulatory B subunit (CnB). Cells were transfected with constructs encoding these proteins and with the −350/+83 deletion mutant of the DSCR1 internal promoter (Fig. 6A, left panel). Expression of active CN potently induced −350/+83 DSCR1.4 reporter activity in nonstimulated cells. This activation was greater than that achieved by stimulation of cells not expressing active CN with KCl or Io, and equaled

![Fig. 2. Depolarization enhances the transcriptional activation of a NFAT-luciferase reporter gene. A, PC12 cells were transiently transfected with a NFAT/AP1 luciferase reporter gene. Twenty four hours later, cells were exposed to a range of concentrations of extracellular potassium (KCl) or sodium (NaCl) for 4 h. B, PC12 cells transiently transfected as in A were pretreated for 1 h with 100 ng/ml CsA (solid bars) or vehicle (open bars), followed by exposure for 4 h to Plo (20 ng/ml PMA plus 1 μM ionophore), 50 mM KCl, or 50 mM NaCl. Transcriptional activity is expressed as relative luciferase units (RLU). Values are the means ± S.D. of triplicate luciferase determinations for each condition in representative experiments of a minimum of three performed.](https://example.com/fig2.png)
PC12 cells were pretreated or not with 100 ng/ml of CsA and then treated with 50 mM KCl for the times indicated. Target genes mRNAs were amplified from total RNA by TaqMan RT-PCR as described under "Materials and Methods." mRNAs were quantified in arbitrary units normalized to the expression of 18 S. Data are expressed as the relative quantification compared to nonstimulated cells, which were assigned a value of 1. Values are the means ± S.D. of triplicate RT-PCR determinations.

**TABLE I**

Calcineurin-dependent KCl-inducible genes in PC12 cells

| Target genes | TaqMan probe (assay ID) | Relative quantification (mean ± S.D.) |
|--------------|-------------------------|--------------------------------------|
| IL-1 (interleukin 1) | Rn00585432.m1 | ND* |
| IL-2 (interleukin 2) | Rn00585672.m1 | ND |
| IL-3 (interleukin 3) | Rn00584535.m1 | 1.60 ± 0.00 |
| IL-10 (interleukin 10) | Rn00563409.m1 | ND |
| IL-12 (interleukin 12) | Rn00575112.m1 | ND |
| IL-13 (interleukin 13) | Rn00587615.m1 | ND |
| IGFI (insulin growth factor) | Rn00710036.m1 | ND |
| TGF-β (transforming growth factor-β) | Rn00572010.m1 | 0.93 ± 0.18 |
| IP3R-1 (inositol 3-phosphate receptor-1) | Rn00565664.m1 | 1.09 ± 0.13 |
| NTrk-1 (neurotrophic tyrosine kinase receptor, type-1) | Rn00572130.m1 | 1.24 ± 0.15 |
| tPA (tissue plasminogen activator) | Rn00565767.m1 | 1.24 ± 0.11 |
| uPA (urokinase plasminogen activator) | Rn00957755.m1 | ND |
| COX-2 (cyclooxygenase-2) | Rn00568225.m1 | ND |
| Dscr-1 (Down-syndrome critical region-1) | Rn00596606.m1 | 0.34 ± 0.05 |
| Gegr-1 (early growth response-transcription factor 1) | Rn00561138.m1 | 1.10 ± 0.01 |

* ND, nondetectable levels.

**FIG. 3.** Calcineurin-dependent DSCR1 isoform 4 mRNA transcription in PC12 cells. A, PC12 cells were exposed to 50 mM KCl for the times indicated. B, cells were pretreated for 1 h with vehicle (open bars), 10 μg/ml ActD (solid bars), or 10 μg/ml cycloheximide (CHX, gray bars). Cells were then exposed to 50 mM KCl for another 4 h. C, cells were pretreated for 1 h with vehicle (open bars) or 100 ng/ml CsA (solid bars) before and then treated for 4 h with 1 mM or 50 mM KCl. D, cells were pretreated for 1 h with vehicle (open bars) or FK506 (solid bars) before exposure to 50 mM KCl for 4 h. DSCR1.4 mRNA was amplified from total RNA by TaqMan RT-PCR as described under "Materials and Methods." DSCR1.4 mRNA was quantified in arbitrary units normalized to the expression of 18S rRNA, to control for the quantity and integrity of total RNA. Data are expressed as the relative quantification compared to nonstimulated control cells (1), which were assigned a value of 1. Values are the means ± S.D. of triplicate RT-PCR determinations for each condition from a representative experiment of a minimum of three performed.

the levels of luciferase activity observed upon stimulation with Plo. The luciferase activity driven by the –166/+83 deletion mutant of the DSCR1.4 promoter was not affected by the overexpression of active CN (Fig. 6A, right panel). Similar results were obtained when the CN/NFAT pathway was activated by exogenous overexpression of constitutively nuclear NFAT. Cells were transfected with the –350/+83 or –166/+83 DSCR1 luciferase reporters as described above, together with an expression vector encoding the human NFATc2 protein carrying a mutation in the nuclear localization sequence, which results in constitutive nuclear localization (Fig. 6B, left panel) (54). This nuclear NFAT expression strongly induced luciferase activity driven by the –350/+83 DSCR1 promoter in a dose-dependent manner but had no effect on the –166/+83-driven luciferase activity (Fig. 6B, left panel). The nuclear NFAT expression regulates in a similar manner the DSCR1.4 promoter and the transcriptional activity driven by the NFAT/AP1 luciferase reporter (Fig. 6B, right panel).

We confirmed that NFAT transcription factors participate in the regulation of the DSCR1.4 promoter in PC12 cells by inhibiting endogenous NFAT signaling with a GFP fusion protein bearing the VIVIT peptide (GFP-VIVIT). The VIVIT peptide specifically inhibits the CN/NFAT pathway by blocking the binding of calcineurin to NFAT proteins, thereby preventing NFAT de-phosphorylation (52). Expression of GFP-VIVIT effectively inhibited the CN-induced activity of the DSCR1(−350/+83) luciferase reporter construct (Fig. 7), whereas GFP alone had no effect.

**Overexpression of DSCR1 Proteins (Calcipressin IL and S) Inhibit the DSCR1.4 Promoter Activity**—The protein products of DSCR1 variants are all able to bind and to inhibit calcineurin, and thus they are potential regulators of CN/NFAT signaling (23, 29, 30). To analyze whether expression of DSCR1-encoded proteins (calcipressins) regulates the transcription of CN/NFAT-dependent genes, we co-transfected PC12 cells with expression plasmids for GFP protein (GFP), GFP-DSCR1.1 fusion protein (GFP-CALP1L), or GFP-DSCR1.4 fusion protein (GFP-CALP1S) and with the DSCR1(−350/+83) luciferase reporter plasmid. As shown in Fig. 8A, exogenous overexpression of both isoforms of calcipressins inhibited the transcriptional activity of the DSCR1(−350/+83) luciferase promoter induced by Plo or KCl treatment. A similar inhibitory effect was observed when calcipressins were co-transfected in PC12 cells with the NFAT/AP1 luciferase reporter construct (Fig. 8B). The inhibitory effect of calcipressins seems to be specific for NFAT-dependent transcriptional activity, because the overexpression of DSCR1 protein failed to block the Plo-induced luciferase activity driven by either an NFκB-dependent or by an AP1-dependent luciferase reporter plasmid (41) (data not shown).

**DISCUSSION**

The current study provides the first description of CN/NFAT-dependent induction of DSCR1 isoform 4 gene (DSCR1.4) expression in neural cells. Moreover, our results show that this gene is induced by Ca2+ signaling via the physiologically relevant L-VGCC. Depolarization with high concentrations of extracellular KCl induces de novo transcription of the DSCR1.4...
gene and activated transcription from the DSCR1 gene internal promoter; these events were blocked by L-VGCC receptor antagonists. Because DSCR1 proteins can act as endogenous inhibitors of CN, the finding of specific regulation of DSCR1.4 in neural cells by CN/NFAT suggests a role for this variant in the homeostatic regulation of the CN/NFAT pathway in the nervous system.

Accumulation of DSCR1.4 mRNA in response to KCl was transcriptionally mediated, because it was blocked by the general transcriptional inhibitor actinomycin D (Fig. 3B). Furthermore, this transcriptional regulation was direct, because protein synthesis was not necessary for the KCl-inducible up-regulation of the gene (Fig. 3B). Ca²⁺-mediated transcript expression and promoter activity were both inhibited by pretreatment with the CN inhibitors CsA or FK506, and co-transfection analysis with constitutively active CN induced transcription from the DSCR1.4 internal promoter. Confirmation of the involvement of NFAT was obtained by activating and in-
their biological functions through calcineurin-dependent nuclear translocation of NFAT proteins (49, 56), it seems likely that increased intracellular Ca\(^{2+}\) concentrations in neural cells will also act via the NFAT-response element in this promoter region. Indeed, our experiments show that exogenous expression of nuclear NFAT proteins is sufficient to strongly activate transcription of a reporter gene regulated by this promoter region in neural cells (Fig. 6B).

NFAT usually cooperates with other transcription factors to elicit maximum target gene expression (10–13, 57), so it is possible that in this case additional signals are necessary. In fact, in our experiments with the Ca\(^{2+}\) ionophore, the induction of DSCR1.4 transcription achieved with ionophore alone was doubled when cells were co-stimulated with phorbol ester (PJo) (Fig. 6A). Because pretreatment with CsA and FK506 completely blocked the transcription induced by PJo (Fig. 3C), it could be assumed that these extra signals depend on the CN component. Whether the presence of the GATA site is indicative of a GATA-related factor being involved in the fully transcriptional activation remains to be explored.

DSCR1 was initially proposed to be a stress-inducible gene, after it was identified (as Adapt 78) in a screen of genes induced by agents such as peroxide and Ca\(^{2+}\) ionophore (25, 58, 59). Up-regulation of DSCR1 has subsequently been reported in hearts subjected to pressure overload (45) and in neonatal rat cardiac myocytes subjected to mechanical stress or treated with hormones and cytokines such as phenylephrine, endothelin 1, angiotensin II, tumor necrosis factor-\(\alpha\), and leukemia inhibitory factor (34). Specific regulation of DSCR1.4 was first described in cardiac cells from CnA-activated transgenic mice, where a strong up-regulation of the DSCR1.4 expression by a CN-dependent pathway, without altering transcription of other DSCR1 variants, was described (36). In light of the implication of CN/NFAT signaling in DSCR1.4 expression, it is interesting

![Diagram](image)

**Fig. 6.** Calcineurin/NFAT\(^{2+}\) signaling activates transcription from the DSCR1(−350/+83) luc reporter but not from the DSCR1(−166/+83) luc reporter. A, PC12 cells were co-transfected with 500 ng of luciferase reporter plasmids, DSCR1(−350/+83) luc (left-hand chart) or DSCR1(−166/+83) luc (right-hand chart), together with either 200 ng of empty vector (open bars) or constructs encoding active calcineurin (100 ng of CnB + 100 ng of constitutively active CnA, solid bars). The positions of NFAT-response elements in the reporter constructs are indicated by ovals in the schematic below each chart (see Fig. 5). After 24 h cells were stimulated for 4 h with Ca\(^{2+}\) Io (1 \(\mu\)M), KCl (50 mM), or Plo (1 \(\mu\)M Io plus 20 ng/ml PMA). Transfected, nonstimulated controls are shown as ns. B, cells were co-transfected with 500 ng of luciferase reporter constructs and different concentrations (30, 65, and 125 ng) of expression plasmid encoding constitutively nuclear NFATc2. The left-hand panel shows the results of co-transfection with DSCR1(−350/+83) luc (open bars) or DSCR1(−166/+83) luc (closed bars). The right-hand panel shows the results of co-transfection with the NFAT/AP1 luciferase reporter. Each plasmid was co-transfected with the GFP and Renilla plasmids to normalize for transfection efficiency. Total DNA was adjusted to 1 \(\mu\)g per cultured plate with the appropriated empty vector. Transcriptional activity is expressed as RLU. Values are the means ± S.D. of triplicate luciferase determinations for each condition from representative experiments of a minimum of three performed.

![Diagram](image)

**Fig. 7.** The DSCR1.4 internal promoter is positively regulated by the CN/NFAT pathway. PC12 cells were co-transfected with 200 ng of the reporter plasmids-DSCR1(−350/+83) luc or DSCR1(−166/+83) luc as indicated, together with 800 ng of expression constructs encoding either GFP (open bars) or GFP-VIVIT (solid bars). Twenty four hours later cells were stimulated with 50 mM KCl for 4 h. The positions of NFAT-response elements in the reporter constructs are indicated by ovals in the schematic below each chart (see Fig. 5). Each plasmid was co-transfected with the GFP and Renilla plasmids to normalize for transfection efficiency. Transcriptional activity is expressed as RLU. Values are the means ± S.D. of triplicate luciferase determinations for each condition from representative experiments of a minimum of three performed.

C/\(\alpha\)/NFAT-dependent DSCR1.4 Expression in Neural Cells

Ca/\(\alpha\)/NFAT-dependent DSCR1.4 Expression in Neural Cells

Calcineurin/NFAT\(^{2+}\) signaling activates transcription from the DSCR1(−350/+83) luc reporter but not from the DSCR1(−166/+83) luc reporter. A, PC12 cells were co-transfected with 500 ng of luciferase reporter plasmids, DSCR1(−350/+83) luc (left-hand chart) or DSCR1(−166/+83) luc (right-hand chart), together with either 200 ng of empty vector (open bars) or constructs encoding active calcineurin (100 ng of CnB + 100 ng of constitutively active CnA, solid bars). The positions of NFAT-response elements in the reporter constructs are indicated by ovals in the schematic below each chart (see Fig. 5). After 24 h cells were stimulated for 4 h with Ca\(^{2+}\) Io (1 \(\mu\)M), KCl (50 mM), or Plo (1 \(\mu\)M Io plus 20 ng/ml PMA). Transfected, nonstimulated controls are shown as ns. B, cells were co-transfected with 500 ng of luciferase reporter constructs and different concentrations (30, 65, and 125 ng) of expression plasmid encoding constitutively nuclear NFATc2. The left-hand panel shows the results of co-transfection with DSCR1(−350/+83) luc (open bars) or DSCR1(−166/+83) luc (closed bars). The right-hand panel shows the results of co-transfection with the NFAT/AP1 luciferase reporter. Each plasmid was co-transfected with the GFP and Renilla plasmids to normalize for transfection efficiency. Total DNA was adjusted to 1 \(\mu\)g per cultured plate with the appropriated empty vector. Transcriptional activity is expressed as RLU. Values are the means ± S.D. of triplicate luciferase determinations for each condition from representative experiments of a minimum of three performed.

While inhibiting it directly; overexpression of exogenous NFAT protein transactivated the DSCR1.4 internal promoter, and the peptide VIVIT (a specific inhibitor of calcineurin-NFAT binding) blocked transactivation in response to depolarization with KCl.

The DSCR1 internal promoter between exons 3 and 4 contains as many as 15 putative NFAT-response elements (36, 43); our promoter deletion experiments located CN-dependent induction of DSCR1.4 to the region between base pairs −350 and −166 (Fig. 5A), the same region involved in the regulation of DSCR1.4 expression in endothelial cells treated with VEGF or thrombin (43). Because VEGF has been shown to elicit some of their biological functions through calcineurin-dependent nuclear translocation of NFAT proteins (49, 56), it seems likely that increased intracellular Ca\(^{2+}\) concentrations in neural cells will also act via the NFAT-response element in this promoter region. Indeed, our experiments show that exogenous expression of nuclear NFAT proteins is sufficient to strongly activate transcription of a reporter gene regulated by this promoter region in neural cells (Fig. 6B).

NFAT usually cooperates with other transcription factors to elicit maximum target gene expression (10–13, 57), so it is possible that in this case additional signals are necessary. In fact, in our experiments with the Ca\(^{2+}\) ionophore, the induction of DSCR1.4 transcription achieved with ionophore alone was doubled when cells were co-stimulated with phorbol ester (PJo) (Fig. 6A). Because pretreatment with CsA and FK506 completely blocked the transcription induced by PJo (Fig. 3C), it could be assumed that these extra signals depend on the CN component. Whether the presence of the GATA site is indicative of a GATA-related factor being involved in the fully transcriptional activation remains to be explored.

DSCR1 was initially proposed to be a stress-inducible gene, after it was identified (as Adapt 78) in a screen of genes induced by agents such as peroxide and Ca\(^{2+}\) ionophore (25, 58, 59). Up-regulation of DSCR1 has subsequently been reported in hearts subjected to pressure overload (45) and in neonatal rat cardiac myocytes subjected to mechanical stress or treated with hormones and cytokines such as phenylephrine, endothelin 1, angiotensin II, tumor necrosis factor-\(\alpha\), and leukemia inhibitory factor (34). Specific regulation of DSCR1.4 was first described in cardiac cells from CnA-activated transgenic mice, where a strong up-regulation of the DSCR1.4 expression by a CN-dependent pathway, without altering transcription of other DSCR1 variants, was described (36). In light of the implication of CN/NFAT signaling in DSCR1.4 expression, it is interesting
that separate studies report that Ca\textsuperscript{2+} ionophore induces DSCR1 expression (25, 36, 44, 60). However, our results are the first to show that Ca\textsuperscript{2+} mobilization via physiologically relevant ion channels in neural cells activate DSCR1.4 transcription via this pathway. This fact gains more relevance because, even though it has been shown previously that NFAT activity is present in the nervous system (15–17), very few target genes for this signaling pathway have been described in this tissue.

The protein products of DSCR1 isoform 1 (calcipressin 1L) and isoform 4 (calcipressin 1S) are all able to bind and inhibit calcineurin and are thus potential regulators of CN/NFAT signaling (26, 29, 30). The different regulation of DSCR1.4 could imply a special regulatory role for this variant, yet there is currently no direct evidence that the interaction of this protein (calcipressin 1S) with CN leads to distinct physiological outcomes compared with the other isoforms. Calcipressin 1 proteins bind directly to the catalytic subunit of calcineurin (CnA) without affecting the interaction of CnA with the regulatory subunit (CnB) or calmodulin (26, 29). Calciopressins interfere with the interaction between calcineurin and the peptide VIVIT in two-hybrid analysis, suggesting that calcipressins compete directly with NFATs for the same docking site on CN (61). Binding of the Cryptococcus neoformans DSCR homologue, CBP1, to full-length CnA is dependent on Ca\textsuperscript{2+} concentration (62), but this dependence is eliminated when a constitutively active form of CnA is used, from which the C-terminal autoinhibitory domain has been removed (similar to the one used in the experiments presented in Fig. 6A). The size and secondary structure of calcipressin 1 proteins are highly conserved in evolution, so this characteristic might be maintained in higher eukaryotes, although it has been described that in mammalian cells calcipressin 1 can bind to endogenous CN in quiescent and stimulated cells (29). More detailed studies on the binding affinity of these two proteins remain to be done. Despite the capacity of calcipressins to inhibit calcineurin, in nonstimulated cells the NFAT signaling pathway is functional, because DSCR1 expression in the brain does not prevent NFAT-mediated transcription upon activation (32). In our system we observed a strong activation of NFAT (Fig. 6) that can be interpreted as reflecting that calcipressins present in the unstimulated cells are not able to inhibit the spike of CN activation provoked by depolarization. However, it might be possible that the presence of different levels of calcipressins modulate the pattern of calcineurin-dependent transcription and may influence calcineurin activity beyond calcium to integrate a broad array of signals into the cellular response (38). On the other hand, homozygous disruption of the DSCR1 gene in mouse resulted in diminished calcineurin function in the heart under normal circumstances, and in response to certain stresses such as pressure overload (63).

In any case, the coincidence of peak DSCR1.4 induction with the return of NFAT to the phosphorylated state (4 h after stimulation, Fig. 3A and Fig. 1B, lane 6) is consistent with the hypothesis that induced calcipressin inhibits CN. Furthermore, the overexpression of exogenous calcipressins is able to inhibit the transcription of NFAT-dependent genes in PC12 cells as shown in Fig. 8. However, additional experiments are needed to reveal if this indeed is the mechanism or whether the cessation of NFAT activation is due more to the specific activation of kinases such as GSK3, p38, or others (65, 66).

The induction of DSCR1.4 by CN/NFAT may be a homeostatic mechanism to increase calcipressin 1 expression to concentrations that can shut off NFAT signaling. In this model, basal calcipressin 1 expression either would be insufficient to inhibit activated CN or would be supporting CN activity. Only the induced expression of variant 4 (calcipressin 1S) would inhibit CN. Such an autoregulatory loop would serve to protect against persistent CN activation, preventing excessive activation of downstream transcription.

Our experiments demonstrate a clear up-regulation of DSCR1.4 in neural cells in response to Ca\textsuperscript{2+} influx via physiologically relevant channels and on a time scale coincident with the cessation of NFAT activation. Given the importance of CN/NFAT signaling in the brain and the up-regulated expression of DSCR1 in the brains of patients with Alzheimer disease and Down syndrome (29, 67, 68), we believe that the regulation of endogenous inhibitors of the CN pathway has the potential to contribute to the understanding of these neurodegenerative processes.

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