Differential Effects of Ca\(^{2+}\) and cAMP on Transcription Mediated by MEF2D and cAMP-response Element-binding Protein in Hippocampal Neurons*

Johanna L. Belfield, Chris Whittaker\(^1\), M. Zaeem Cader, and Sangeeta Chawla\(^2\)

From the Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, United Kingdom

In neurons, the second messengers Ca\(^{2+}\) and cAMP are mediators of transcriptional responses that are important for the development and function of the nervous system. The pro-survival neuronal transcription factors cAMP-response element-binding protein (CREB) and myocyte enhancer factor-2 (MEF2) both stimulate gene expression in response to activity-dependent increases in the concentration of intracellular Ca\(^{2+}\) ions. CREB is also activated by increases in intracellular cAMP. Here we have investigated whether the MEF2 family member MEF2D, similar to CREB, is also activated by cAMP in hippocampal neurons. We have shown that, unlike CREB, MEF2D is not activated by agents that increase intracellular cAMP. Moreover, increases in cAMP inhibit Ca\(^{2+}\)-activated MEF2D-mediated gene expression. We have also shown that cAMP inhibits Ca\(^{2+}\)-induced nuclear export of the MEF2 co-repressor HDAC5 and prevents Ca\(^{2+}\)-stimulated nuclear import of the MEF2 co-activator NFAT/c4. Our results suggest that cAMP interferes with MEF2D-mediated gene expression at multiple levels by antagonizing the derepression of MEF2D by HDAC5 and by inhibiting recruitment of the co-activator NFAT.

The transcription factors cAMP-response element-binding protein (CREB)\(^3\) and myocyte enhancer factor-2 (MEF2) regulate gene expression in neurons that is required for neuronal differentiation, survival, and plasticity. During development of the central nervous system and in the adult brain, gene expression is regulated by various stimuli such as neurotrophins and synaptic activity. Often, the interaction between different intracellular signaling cascades initiated by concurrent exposure to a range of stimuli elicits specific transcriptional responses that determine neuronal fate. Although CREB regulation in neurons has been the subject of many investigations that have demonstrated CREB activation by the second messengers Ca\(^{2+}\) and cAMP and by neurotrophins (reviewed in Ref. 1), the regulation of MEF2 transcription factors by similar stimuli is not yet fully understood. MEF2 proteins belong to the MADS box family of transcription factors, which contain a conserved N-terminal domain (present in MCM1, Agamous, Deficiens, and serum response factor) and which were initially identified in muscle where they regulate many muscle-specific genes and influence myogenesis. Subsequent studies revealed high expression of MEF2 factors in the brain, where they have emerged as regulators of activity-dependent neuronal survival and differentiation, and in immune cells, where they are involved in mediating T cell activation (reviewed in Ref. 2). Four MEF2 family members (MEF2A, MEF2B, MEF2C, and MEF2D), each encoded by a different gene, have been identified in mammalian cells. The pro-survival effects of MEF2 have been demonstrated in response to activity-induced Ca\(^{2+}\) signals and in response to neurotrophins, which regulate MEF2 by distinct mechanisms. Although neuronal activity-dependent survival of adult cortical and cerebellar neurons mediated by MEF2 is regulated by the Ca\(^{2+}\)-dependent kinases p38 mitogen-activated protein kinase (MAPK) (3) and Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) (4), the survival-promoting actions of neurotrophins in developing cortical neurons (5) and cerebellar granule neurons (6) are attributed to the activation of MEF2-dependent gene expression by ERK5.

The second messenger cAMP has also been implicated in promoting the survival of many types of neurons (7, 8). This survival-promoting effect of cAMP is likely due to its effect on CREB, because CREB-mediated gene expression is induced by cAMP as well as by pro-survival signals such as neurotrophins and Ca\(^{2+}\). In addition to CREB, cAMP may also influence MEF2 activity. Because MEF2, similar to CREB, is clearly activated by synaptic activity-induced Ca\(^{2+}\) transients (3) and by neurotrophic factors (5, 6), here we have investigated whether MEF2 family members similar to CREB are also activated by increases in intracellular cAMP in hippocampal neurons. We compared the activation of CREB with the MEF2 isoform MEF2D that is expressed highly in the hippocampus (9, 10). We report that, unlike CREB, MEF2D-mediated gene expression is not induced by increases in intracellular cAMP in hippocampal neurons, although an increase in intracellular Ca\(^{2+}\) stimulates both transcription factors. Not only did cAMP fail to activate MEF2D, it antagonized Ca\(^{2+}\)-activation of MEF2D-mediated

---

*This work was supported by a Biotechnology and Biological Sciences Research Council David Phillips Fellowship (to S. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the King’s College Durham fund for a vacation scholarship.

2 To whom correspondence should be addressed: Dept. of Pharmacology, Tennis Ct. Rd., Cambridge CB2 1PD, UK. Tel.: 44-1223-334060; Fax: 44-1223-334100; E-mail: sc10008@cam.ac.uk.

3 The abbreviations used are: CREB, cAMP-response element-binding protein; HDAC, histone deacetylase; MEF2, myocyte enhancer factor-2; GFP, green fluorescent protein; NFAT, nuclear factor of activated T cells; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase; ERK, extracellular signal-regulated kinase; CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate; LTP, long term potentiation; GSK, glycogen synthase kinase; MADS, MCM1 Agamons Deficiens serum-response factor.
gene expression. CREB, however, was activated synergistically by concurrent increases in Ca2+ and cAMP.

MEF2 transcription factors are regulated by several Ca2+-dependent mechanisms that operate at many levels. In quiescent cells, MEF2 proteins are bound to and repressed by class II histone deacetylases, which include HDAC4, -5, -7, and -9. This repression is relieved during synaptic activity in hippocampal neurons when Ca2+ influx through L-type voltage-gated calcium channels or synaptic NMDA receptors induces nuclear export of HDAC4 and 5 (11). MEF2 proteins freed from HDAC repression are subject to further Ca2+-dependent regulation by p38 MAPK, ERK5, and the Ca2+/calmodulin-dependent phosphatase calcineurin (reviewed in Ref. 2). Calcineurin acts to control MEF2-mediated gene expression in several ways. Calcineurin can act directly and maintain MEF2 in a hypophosphorylated state, which enhances the DNA binding affinity of MEF2 (12) and also protects it from caspase-mediated cleavage (13). Calcineurin can also influence MEF2-mediated gene expression indirectly by regulating the subcellular localization of the nuclear factor of activated T cells (NFAT) family of transcription factors, which act as MEF2 co-activators (14, 15). Dephosphorylation of NFAT proteins in the cytoplasm by calcineurin allows their translocation into the nucleus where they interact with MEF2A and -D (15) and allow recruitment of p300/CREB-binding protein (14) to activate MEF2-mediated gene expression.

We have shown here that the inhibition of MEF2 activity by cAMP also occurs at many levels and may involve multiple mechanisms. First, we observed that cAMP inhibits the Ca2+-activated nuclear export of the MEF2 co-repressor HDAC5. Second, we found that cAMP also antagonizes Ca2+-induced nuclear import of the MEF2 co-activator NFAT3/c4.

**MATERIALS AND METHODS**

**Cell Culture and Plasmids**—Hippocampal neurons from newborn Wistar rats were cultured as described previously (16), except that the growth medium was supplemented with 2% (v/v) B27 (Invitrogen). Cytosine arabinoside (2.4 μM) (Sigma) was added to the cultures 2 days after plating to inhibit proliferation of non-neuronal cells. Expression plasmid for HDAC5-GFP was a gift from Prof. Stuart Schreiber (Harvard University) (17). The expression plasmid encoding the Gal4 DNA binding domain fused with full-length rat CREB was as described previously (18). Expression plasmids for Gal4 DNA binding domain, Gal4-MEF2D, Gal4-MEF2D(C), and the firefly luciferase reporter gene plasmid containing five Gal4 DNA binding sites (5XGal4-E1bLuc) have been described previously (15). Plasmid pKL-TK expressing Renilla luciferase was from Promega (Madison, WI). The −316/−252 Nur77Luc reporter plasmid was provided by Talal Chatila (UCLA) (15) and contains −316 to −252 bp of the rat Nur77 promoter cloned into pGL2Luc basic vector. The plasmid expressing FLAG-tagged NFAT3/c4 was provided by Dr. Chi-Wing Chow (Albert Einstein College of Medicine, Bronx, NY) (19).

**Transfections and Reporter Gene Expression**—Hippocampal neurons were transfected using Lipofectamine 2000 (Invitrogen) after 8–9 days in culture. 24–36 h after transfection, cells were either left untreated or stimulated for 6 h as indicated.

Transfections and subsequent stimulations were carried out in transfection medium containing 90% buffered salt-glucose-glycine solution (16), 10% minimum essential medium (Invitrogen), insulin (7.5 μg/ml), transferrin (7.5 μg/ml), sodium selenite (7.5 ng/ml), penicillin (50 units/ml), and streptomycin (50 μg/ml). Luciferase activity was assayed with the Promega Dual Glo assay kit according to the instructions of the manufacturer using a TD-20e Turner luminometer. Firefly luciferase activity was normalized to the Renilla luciferase signal, and all measurements were made in duplicate. Experiments were performed 3–5 times on independent neuronal cultures.

**Ca2+ Imaging**—Hippocampal neurons grown on glass coverslips for 10–11 days were loaded with Fura-2 by incubation with 2 μM Fura-2/AM (Molecular Probes) in salt-glucose-glycine solution for 20 min followed by a further incubation for one hour in the absence of Fura-2/AM. Coverslips were mounted in an optical cuvette in a Hitachi F4500 fluorescence spectrometer and alternately excited with light of wavelengths 340 and 380 nm, while collecting emitted light at 510 nm every 0.2 s. Fluorescence ratios 340/380 nm were calculated and are shown as F340/F380 in Fig. 2C.

**Immunofluorescence**—Transfected neurons were fixed in 3% paraformaldehyde in PBS containing 4% sucrose for 20 min, washed twice with 10 mM glycine in PBS for 10 min, and followed by permeabilization in 0.5% Nonidet P-40 in PBS for 5 min at room temperature. After fixation, the cells transfected with FLAG-tagged NFAT3/c4 were incubated with the anti-FLAG M2 monoclonal antibody (Sigma) diluted 1:200 in PBS overnight at 4 °C. Incubations with biotinylated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) and with fluorescein-avidin (Vector Laboratories, Burlingame, CA), both diluted 1:200 in PBS, were for 1 h each at room temperature. Cells were washed with PBS and mounted in Vectashield (Vector Laboratories). Cells expressing HDAC5-GFP fusion protein were washed twice with 10 mM glycine in PBS for 10 min after fixation and mounted in Vectashield. Images of GFP and fluorescein fluorescence were captured using an inverted Zeiss laser scanning confocal system (Zeiss LSM-510, Zeiss, Germany) with an ×63 oil immersion objective lens. For quantitative analysis, transfected cells were scored as displaying either cytoplasmic, or both nuclear and cytoplasmic, or nuclear localization. The percentage of cells in each category was then averaged over 3–5 independent experiments. To detect endogenous HDAC5, neurons (day 10–11 in vitro) were fixed and permeabilized after the indicated treatments by incubating the cells in pre-cooled 100% methanol at −20 °C for 10 min. The cells were subsequently washed twice with PBS for 5 min and incubated with rabbit polyclonal anti-HDAC5 antibody (Cell Signaling Technology) diluted 1:100 in PBS for 2 nights at 4 °C.

The primary antibody was removed, and cells were washed twice for 10 min with PBS followed by incubation with anti-rabbit Alexa Fluor 488 (Molecular Probes) secondary antibody diluted 1:200 in PBS for 1 h at room temperature. Cells were washed with PBS and mounted in Vectashield.

**Live Cell Imaging**—HDAC5GFP-transfected cells were imaged in transfection medium 36 h after transfection at 37 °C on a Zeiss laser scanning confocal system (Zeiss LSM-510,

**Ca2+ and cAMP Differentially Regulate MEF2D**
Zeiss, Germany). Images of GFP-transfected cells were captured every 5 min.

RESULTS

Ca\(^{2+}\) and cAMP Have Distinct Effects on CREB- and MEF2D-mediated Gene Expression—We studied the effects of Ca\(^{2+}\) and cAMP on CREB- and MEF2D-mediated gene expression by using a luciferase reporter gene system. Hippocampal neurons were transiently transfected with a plasmid encoding a MEF2D fusion protein containing the Gal4 DNA binding domain fused to full-length MEF2D along with a luciferase reporter gene construct containing five Gal4 DNA binding sites in the promoter (15). CREB-mediated gene expression was assayed similarly by expressing a Gal4-CREB fusion protein along with the Gal4-luciferase reporter plasmid. Neurons were depolarized with 30 mM extracellular KCl to activate Ca\(^{2+}\) influx into the cells through L-type calcium channels. To activate Ca\(^{2+}\) influx through synaptic NMDA receptors, neurons were treated with \(\gamma\)-aminobutyric acid, type A, antagonist bicuculline in the presence of the K\(^{+}\) channel blocker 4-aminopyridine. This treatment triggers bursts of action potential firing that are accompanied by NMDA receptor-dependent Ca\(^{2+}\) transients (20). We found that Ca\(^{2+}\) influx through L-type calcium channels or synaptic NMDA receptors resulted in a 4.8- and 2.8-fold induction, respectively, of MEF2D-mediated gene expression when compared with control untreated cells (Fig. 1A, 5th and 6th bars). CREB-mediated gene expression was similarly induced by Ca\(^{2+}\) influx through both routes (Fig. 1A). These increases in reporter gene expression can be attributed to MEF2D and CREB, respectively, as the Gal4 DNA binding domain on its own failed to induce expression of the Gal4-luciferase reporter gene in response to increases in intracellular Ca\(^{2+}\) (Fig. 1A, 7th–9th bars).

We next compared the effects of increased intracellular cAMP on CREB- and MEF2D-mediated gene expression. Surprisingly, treatment of neurons with the adenylyl cyclase activator forskolin failed to activate MEF2D-mediated gene expression (Fig. 1C, 3rd bar), although this resulted in a robust activation (21.3 ± 2.2-fold induction) of CREB-mediated transcription (Fig. 1B). When cells were treated with both forskolin and KCl, forskolin inhibited the induction of MEF2D by membrane depolarization (Fig. 1C, 4th bar). Forskolin similarly inhibited MEF2D-mediated gene expression following activation of synaptic NMDA receptors with bicuculline (data not shown). In contrast, forskolin and KCl acted synergistically to induce CREB-mediated gene expression (Fig. 1B). The co- operative induction of CREB-mediated gene expression by simultaneous increases in intracellular cAMP and Ca\(^{2+}\) has been observed on earlier occasions. In PC12 cells, cAMP and Ca\(^{2+}\) have been shown to act synergistically to induce transcription of the cholecystokinin gene (21). A similar synergistic action of these two second messengers has been observed on the promoter of the pituitary adenylate cyclase-activating peptide gene in cortical neurons (22).

CAMP Inhibits Ca\(^{2+}\)-activated MEF2D-mediated Gene Expression—To confirm that cAMP has an inhibitory effect on Ca\(^{2+}\) activation of MEF2D, we used other pharmacological methods to increase intracellular cAMP levels. Treatment of cells with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine inhibited KCl-induced MEF2D-mediated gene expression to 40 ± 3.2% of the KCl-induced response. This is similar to the level of inhibition seen with forskolin, which reduced the KCl-activated luciferase activity to 35 ± 2.5% (Fig. 2A). Because hippocampal neurons express \(\beta\)-adrenergic receptors (23) that activate adenylyl cyclase, we used the adrenergic receptor agonist isoproterenol as an alternative way of increasing cAMP levels. Isoproterenol treatment inhibited the KCl-induced MEF2D-mediated increase in luciferase reporter gene expression by ~50% (Fig. 2A). Increasing cAMP levels by direct application of the cell-permeant CAMP analogue CPT-cAMP similarly inhibited depolarization-induced activation of MEF2D. These experiments indicate that intracellular cAMP antagonizes Ca\(^{2+}\) activation of MEF2D in hippocampal neurons.

Because the experiments above used a Gal4-MEF2D fusion protein, we next investigated the effect of CAMP on transcrip-
Ca\textsuperscript{2+} and cAMP Differentially Regulate MEF2D

transient of a MEF2 target gene in neurons. Transcription of the nuclear orphan receptor gene Nur77 is regulated by MEF2 transcription factors in immune cells (14, 15) and in cerebellar granule neurons (24). We therefore tested whether the inhibition of MEF2D by cAMP is reflected in the regulation of Nur77. We examined the expression of a luciferase reporter gene driven by −316 to −252 bp relative to the transcription start site of the rat Nur77 gene, which contains two MEF2 binding sites (13). As expected, Nur77 expression was induced 3.1-fold following depolarization of hippocampal neurons with 30 mM KCl (Fig. 2B, 2nd bar). Forskolin treatment on the other hand failed to activate transcription of Nur77 (Fig. 2B, 3rd bar). Furthermore, forskolin inhibited depolarization-induced Nur77 expression when cells were treated simultaneously with KCl and forskolin (Fig. 2B, 4th bar).

Cross-talk between the two second messengers Ca\textsuperscript{2+} and cAMP can occur directly, where the intracellular concentration of one can affect the concentration of the other through proteins involved in their homeostasis. For example, increased Ca\textsuperscript{2+} can activate Ca\textsuperscript{2+}-sensitive adenylyl cyclases leading to elevated cAMP levels (reviewed in Refs. 25 and 26). Conversely, levels of cAMP can influence Ca\textsuperscript{2+} concentrations through the actions of cAMP-dependent protein kinase (PKA) on proteins such as voltage-gated calcium channels (27), ryanodine receptors (28, 29), inositol 1,4,5-trisphosphate receptors (30), and Ca\textsuperscript{2+} ATPases (31), which are involved in Ca\textsuperscript{2+} homeostasis. Additionally, cross-talk between Ca\textsuperscript{2+} and cAMP can also occur downstream of the second messengers through signaling cascades activated by them. For example, PKA is required for Ca\textsuperscript{2+}-stimulated ERK nuclear translocation (32), and PKA can inhibit the CaM kinase cascade (33). Thus, increased intracellular cAMP could be exerting its inhibitory effects on MEF2 activation either by interfering with the depolarization-induced Ca\textsuperscript{2+} transients or by antagonizing Ca\textsuperscript{2+}-activated signaling cascades. We tested to see whether cAMP was antagonizing the depolarization-evoked Ca\textsuperscript{2+} transients using the ratiometric calcium indicator Fura-2. Fig. 2C shows an example of calcium transients recorded from a population of cells depolarized with 30 mM extracellular K\textsuperscript{+} in the absence or presence of 10 \mu M forskolin. As reported earlier, depolarization of neurons with KCl elicited a biphasic calcium response with an initial peak followed by a sustained plateau phase (34, 35). Depolarization of hippocampal neurons in the presence of forskolin resulted in a more sustained Ca\textsuperscript{2+} transient, the amplitude of which was similar to that of the initial peak obtained with KCl alone (Fig. 2C). Thus, cAMP does not attenuate the KCl-evoked calcium transient but in fact augments it. This could be due to the activation of PKA by increased intracellular cAMP, which has been shown to phosphorylate both neuronal L-type calcium channels (27) and neuronal ryanodine receptors (28).

cAMP Inhibits Ca\textsuperscript{2+}-induced HDAC5 Nuclear Export—The first step in the activation of MEF2 transcription factors involves derepression by class II HDACs. In hippocampal neurons, increases in intracellular Ca\textsuperscript{2+} induce nuclear export of HDAC5 (11). We therefore tested the effects of increasing intracellular cAMP on HDAC5 subcellular localization. Hippocampal neurons were transfected with a plasmid encoding a HDAC5-GFP fusion protein, and HDAC5 localization was visualized by fluorescence
**Ca\(^{2+}\) and cAMP Differentially Regulate MEF2D**

**A**

Control Forskolin KCl KCl + Forskolin

HDAC5 GFP

**B**

![Graph showing percentage of HDAC5 localization.](image)

**C**

HDAC5 GFP

![Graph showing HDAC5 GFP translocation.](image)

**FIGURE 3. Intracellular cAMP inhibits Ca\(^{2+}\)-induced HDAC5 nuclear export.** A, representative example of hippocampal neurons showing localization of HDAC5 fluorescence following transfection with HDAC5GFP under conditions of spontaneous activity (Control) and in those treated with 30 mM KCl or 10 \(\mu\)M forskolin or KCl and forskolin together for 6 h. Scale bar is 10 \(\mu\)m. B, an average of the percentage of transfected cells showing cytoplasmic, both nuclear and cytoplasmic, and nuclear localization of HDAC5GFP from five independent experiments is shown in the graph (>300 cells analyzed). C, HDAC5GFP translocation visualized in live cells over time. Cells were imaged in transfection medium at time 0 min, and images were collected at the indicated times after the addition of 10 \(\mu\)M forskolin.

microscopy. As reported previously, in untreated hippocampal neurons that exhibit spontaneous synaptic activity, HDAC5 was nuclear in a large proportion (46 ± 11%) of cells, both nuclear and cytoplasmic in 31 ± 5% of cells, and cytoplasmic in 22 ± 9% of cells. Depolarization with high extracellular KCl induced HDAC5 nuclear export with 75 ± 4% cells displaying a cytoplasmic localization of HDAC5 (Fig. 3, A and B). Treatment of cells with forskolin resulted in a redistribution of HDAC5 into the nucleus with 85 ± 4% cells displaying nuclear localization compared with 46% in untreated neurons (Fig. 3, A and B). Live time-lapse imaging revealed that HDAC5 fluorescence could be detected in the nucleus within 20 min after forskolin treatment (Fig. 3C). These results show that cAMP antagonizes spontaneous activity-induced nuclear export of HDAC5. Interestingly, forskolin-induced nuclear HDAC5GFP exhibited a punctate pattern in the nucleus with several bright foci of fluorescence. This speckled pattern of HDAC5 localization in the nucleus is similar to that observed in HeLa cells when HDAC5 was co-expressed with the co-repressor-silencing mediator for retinoic acid and thyroid hormone receptors (SMRTe) (36). The SMRT1-dependent targeting of HDAC5 into subnuclear domains was found to repress MEF2C-mediated gene expression (36). In hippocampal neurons, when SMRT and HDAC5 are co-expressed, they co-localize in discrete nuclear speckles, which can be distinguished from other known promyeloctic leukemia-containing subnuclear domains (37). The cAMP-induced speckled distribution of HDAC5 seen here may thus represent targeting of HDAC5 into discrete nuclear domains by either SMRT or by other proteins. To investigate whether cAMP inhibits depolarization-induced HDAC5 nuclear export, cells were treated with KCl and forskolin together. This also resulted in a nuclear localization of HDAC5 in >75% of cells. We also examined localization of endogenous HDAC5 protein using a rabbit polyclonal antibody to HDAC5 and found that Ca\(^{2+}\)-induced nuclear export of endogenous HDAC5 was similarly inhibited by cAMP (Fig. 4A). Thus cAMP inhibits both spontaneous activity and KCl-induced nuclear export of HDAC5, suggesting that one mechanism by which cAMP inhibits MEF2D is by preventing the Ca\(^{2+}\)-activated derepression of MEF2D from HDAC5.

Class II HDACs bind MEF2 proteins at the N-terminal region encompassing a conserved MADS box and a conserved MEF2 domain. A Gal4-MEF2C fusion protein containing the C-terminal transactivation domain of MEF2C and lacking the N-terminal MADS/MEF2 domains is able to induce transcription of a Gal4-luciferase reporter in response to Ca\(^{2+}\) signals in cortical neurons (3). Phosphorylation of MEF2C by p38 MAPK on serine 387 is necessary for Ca\(^{2+}\) stimulation of Gal4-MEF2C activity. Although p38 does not phosphorylate MEF2D (38), the C terminus of MEF2D is phosphorylated in vitro by CaMKIV (15). We hypothesized that a Gal4-MEF2D protein lacking the N terminus and containing only the C-terminal transactivation domain might be Ca\(^{2+}\)-inducible similar to Gal4-MEF2C but would be resistant to inhibition by cAMP, as it would not bind HDAC5 that had accumulated in the nucleus. We therefore tested the ability of Gal4-MEF2D(C) containing amino acids 93–514 of MEF2D fused to the Gal4 DNA binding domain to enhance expression of the Gal4-luciferase reporter. Fig. 4B shows that, unlike full-length Gal4-MEF2D (Fig. 1A), Gal4-MEF2D(C) was not activated by membrane depolarization. Bicuculline treatment to stimulate Ca\(^{2+}\) influx through synaptic NMDA receptors similarly failed to activate Gal4-MEF2D(C) (data not shown). This indicates that the N terminus of MEF2D is required for Ca\(^{2+}\) activation of MEF2D-mediated gene expression.

**Effect of cAMP on Ca\(^{2+}\)-activated NFAT3/c4 Nuclear Import**—The conserved MADS box and MEF2 domain in the N terminus of MEF2 proteins influences their interactions not only with co-repressors such as HDAC5 but also with co-activators such as NFAT and p300. In T-cells, NFAT recruitment by the MADS domain is an important step in MEF2D-mediated transcriptional activation of the Nur77 promoter by Ca\(^{2+}\) signals (14). Hippocampal neurons express the NFAT isoform NFAT3/c4, which translocates to the nucleus during synaptic activity (39), where it could potentially interact with MEF2D. Because calcineurin activity is required for NFAT to translocate to the nucleus and interact with MEF2D, we tested the effect of calcineurin inhibitors on MEF2D activation in neurons. Treatment of hippocampal neurons with the calcineurin inhibitors cyclosporine A and FK506 strongly inhibited both KCl- and
bicuculline-induced MEF2D-mediated gene expression (Fig. 5A). Ca\(^{2+}\)-activated transcriptional induction of the c-fos gene, which depends on the transcription factors CREB and serum response factor, was unaffected by FK506 and cyclosporine (data not shown).

The inhibitory effect of calcineurin blockers on MEF2D-mediated gene expression, taken together with our finding that the N terminus of MEF2D is required for its Ca\(^{2+}\) activation, suggests that NFAT recruitment may be an important step for Ca\(^{2+}\) activation of MEF2D in hippocampal neurons. Work done in T cells shows that, although Ca\(^{2+}\) activates NFAT-mediated transcription, cAMP inhibits it and that this involves inhibition of NFAT nuclear accumulation by calcineurin (40, 41). We tested the hypothesis that a similar mechanism might be operating in neurons. We examined the effects of cAMP on the localization of the hippocampal isoform NFAT3/c4. To do this, hippocampal neurons were transiently transfected with an expression plasmid for FLAG-tagged NFAT3/c4 and NFAT localization visualized by immunofluorescence. Cells were categorized as dis-
playing nuclear, both nuclear and cytoplasmic, or cytoplasmic immunoreactivity. In untreated hippocampal neurons, NFAT3 was cytoplasmic in >75% of cells (Fig. 5, B and C). Stimulation with 30 mM KCl caused a dramatic increase in the proportion of cells (94 ± 3.5%) showing nuclear localization of NFAT3 (Fig. 5, B and C). When cells were exposed to 10 μM forskolin, most cells displayed NFAT immunofluorescence in the cytoplasm, similar to that seen in untreated neurons. However, when KCl and forskolin were applied together, NFAT3 was found to be largely cytoplasmic. Similar to membrane depolarization, exposure of hippocampal neurons to bicuculline, which gives rise to NMDA receptor-dependent Ca2+ transients, caused a nuclear translocation of NFAT3/c4 that was inhibited by co-application of forskolin (data not shown). Thus, cAMP opposes the Ca2+-induced nuclear translocation of NFAT3/c4 in hippocampal neurons.

DISCUSSION

We have shown here that an increase in intracellular cAMP opposes the Ca2+ activation of MEF2D but acts synergistically with Ca2+ to activate CREB. This differential activation of the two transcription factors may have implications for many neuronal processes that involve transcriptional changes induced by the two second messengers, including long term plasticity, neuronal differentiation, and neuronal viability. For example, long term potentiation (LTP) of excitatory transmission at hippocampal synapses, which is an extensively studied cellular model of neuronal plasticity, involves signaling cascades triggered by both Ca2+ and cAMP (42, 43). Hippocampal LTP has two temporal phases, an early phase where changes in the efficacy of a synapse depend on modification of existing synaptic proteins and a late phase that requires transcription. LTP is observed at three major synapses in the hippocampus, of which the Schaffer collateral pathway/synapse involving the axons of CA3 neurons projecting onto CA1 pyramidal cells is most extensively studied. LTP at this synapse requires an increase in post-synaptic Ca2+ mediated by NMDA receptor activation (44). The increase in intracellular Ca2+ during LTP may elevate cAMP levels by stimulation of Ca2+-activated adenylyl cyclases (26). Additionally, because the hippocampus receives dense adrenergic inputs (45), co-activation of β-adrenergic G protein-coupled receptors by the neuromodulator noradrenaline could also elevate cellular cAMP levels during LTP. The relative amounts of Ca2+ and cAMP under different synaptic stimulations will determine the level of transcription of CREB- and MEF2D-dependent genes. Because some genes are up-regulated and some down-regulated during LTP, the inhibition of MEF2D-mediated gene expression could be an important event for certain forms of plasticity (46). Although a direct role for MEF2 proteins in synaptic plasticity has not yet been established, serum response factor, a related transcription factor belonging to the MADS family, has recently been implicated in hippocampal plasticity (47). The inhibition of MEF2D by cAMP may also have important consequences for neuronal differentiation. A recent study that examined synapse formation in differentiating hippocampal neurons shows that MEF2A and MEF2D activation reduces the number of excitatory synapses (48). Similarly, MEF2A activity promotes synapse disassembly in cerebellar granule neurons, whereas its inhibition by phosphorylation and sumoylation promotes synapse formation (24). Thus, the inhibition of MEF2D by cAMP may have important consequences for synaptogenesis during neuronal development.

Our finding that MEF2D (the activation of which is associated with pro-survival effects) is inhibited by cAMP is unexpected, because cAMP has been reported to have largely neuroprotective effects (7, 8). However, cAMP can be pro-apoptotic under certain conditions. A recent study (49) reports that increases in intracellular cAMP inhibit the ability of brain-derived neurotrophic factor to protect cortical neurons from apoptosis induced by the withdrawal of serum. Poser et al. (49) show that, under these conditions, cAMP also inhibits brain-derived neurotrophic factor-induced transcription of a reporter gene containing DNA binding sites for serum response factor. Because MEF2D and serum response factor belong to the same family of transcription factors, it would be interesting to examine the effects of cAMP on brain-derived neurotrophic factor activation of MEF2D in cortical and hippocam-
Ca\(^{2+}\) and cAMP Differentially Regulate MEF2D

In hippocampal neurons, cAMP signaling in fact enhances gene expression mediated by MEF2 (50). This effect of cAMP was shown to be due to a PKA-mediated phosphorylation of the MEF2 isoform MEF2C, which increases its DNA binding (50). The PKA phosphorylation site identified in MEF2C is conserved in MEF2D; therefore, one might expect a similar regulation of MEF2D by the cAMP/PKA pathway. It is possible that there are mechanistic differences between cerebellar granule cells and hippocampal neurons. Additionally, the two types of cells are maintained in vitro under very different conditions, which might account for the differences. Although cerebellar granule neurons need to be chronically depolarized during the culture in high extracellular KCl (25–30 mM) to obtain viable cells in the absence of serum (50), our hippocampal cultures were maintained in the presence of serum for 8 days followed by 2–4 days in serum-free medium (described under “Materials and Methods”), both containing low concentrations of KCl.

We have shown here that the mechanisms by which cAMP inhibits the activation of MEF2D by Ca\(^{2+}\) signals in hippocampal neurons involve both its co-repressor and co-activator. A schematic representation of the mechanisms underlying the opposing effects of Ca\(^{2+}\) and cAMP on MEF2D activity is shown in Fig. 6. Although Ca\(^{2+}\) signals trigger nuclear export of the MEF2 co-repressor HDAC5, cAMP inhibits its cytoplasmic translocation. The Ca\(^{2+}\)-dependent nuclear export of HDAC5 occurs following phosphorylation by CaM kinases on two serine residues (Ser-259 and -498) in the N terminus that are conserved in HDAC4, -5, and -7 (reviewed in Ref. 2) and subsequent association with the 14-3-3 family of chaperone proteins. The inhibition of HDAC5 nuclear export by cAMP may involve dephosphorylation of the sites, which are required for 14-3-3 binding, by a cAMP-activated phosphatase or may involve phosphorylation events by a cAMP-activated kinase on distinct residues that promote nuclear localization. In support of the second possibility, it has been reported that activation of the MAPKs ERK1/2 by expression of oncogenic Ras in C2C12 muscle cells results in redistribution of HDAC4 into the nucleus and that this is accompanied by phosphorylation of HDAC4 by ERK1/2 (51). Increases in intracellular cAMP can lead to ERK1/2 activation via guanine nucleotide exchange factors Epac1 and Epac2 that are directly activated by cAMP and stimulate the small GTPases Rap1 and Rap2 (52). Given that HDAC4 and -5 show similar regulation by CaM kinases, the possibility that HDAC5 is redistributed to the nucleus by activated ERK1/2 in neurons, analogous to HDAC4 in C2C12 cells, needs further investigation.

In addition to inhibiting HDAC5 nuclear export, cAMP also antagonized Ca\(^{2+}\)-activated nuclear translocation of the MEF2 co-activator NFAT3/c4 (Fig. 6). Interestingly, Ca\(^{2+}\)-activated NFAT-mediated transcription is inhibited by cAMP in Jurkat T cells, and this effect of cAMP is mediated by PKA phosphorylation of NFAT proteins on conserved serines in the N-terminal NFAT homology region (40, 41). In the case of NFATC1, phosphorylation by PKA primes it for further phosphorylation events catalyzed by glycogen synthase kinase-3 (GSK-3) that inhibit its nuclear accumulation by Ca\(^{2+}\) signals (41). In the case of NFAT3, phosphorylation by PKA inhibited its transcriptional activity in baby hamster kidney cells but did not alter its subcellular localization in these cells (40). This is in contrast to the cAMP-induced inhibition of NFAT3 nuclear accumulation observed in our hippocampal neurons. Given that NFATc1 nuclear accumulation by cAMP requires both PKA and GSK-3 activity, the reported lack of effect on NFAT3 nuclear export by PKA in baby hamster kidney cells could be due to either low levels of GSK-3 activity or limiting expression of GSK-3 in baby hamster kidney cells. NFAT3 has recently been implicated in neuronal survival in cerebellar granule neurons, where apoptotic stimuli caused its nuclear export (53). When NFAT3 expression was inhibited using RNA interference, neuronal death was observed even under pro-survival conditions of the culture (53). This pro-apoptotic effect of NFAT3 depletion could in part be due to down-regulation of MEF2 target genes. Thus, the interaction between cAMP, Ca\(^{2+}\), and neurotrophin signaling determines the activity of MEF2 transcription factors, which in turn has implications for neuronal differentiation and plasticity and for the survival of different neuronal populations.

Acknowledgments—We thank Profs. Stuart Schreiber and Talal Chatilla and Dr. Chi-Wing Chow for plasmid constructs.

REFERENCES

1. Lonze, B. E., and Ginty, D. D. (2002) Neuron 35, 605–623
2. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) Trends Biochem. Sci. 27, 40–47
3. Mao, Z., Bonni, A., Xia, F., Nadal-Vicens, M., and Greenberg, M. E. (1999) Science 286, 785–790
4. Linseman, D. A., Bartley, C. M., Le, S. S., Laessig, T., Bouchard, R. J., Meintzer, M. K., Li, M., and Heidenreich, K. A. (2003) J. Biol. Chem. 278, 41472–41481
5. Liu, L., Cavanaugh, J. E., Wang, Y., Sakagami, H., Mao, Z., and Xia, Z. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8532–8537
6. Shalizi, A., Lehtinen, M., Gaudilliere, B., Donovan, N., Han, J., Konishi, Y., and Bonni, A. (2003) J. Neurosci. 23, 7326–7336
7. Hanson, M. G., Jr., Shen, S., Wiemelt, A. P., McMorris, F. A., and Barres, B. A. (1998) J. Neurosci. 18, 7361–7371
8. Li, M., Wang, X., Meintzer, M. K., Laessig, T., Birnbaum, M. J., and Heidenreich, K. A. (2000) Mol. Cell. Biol. 20, 9356–9363
9. Ikehima, H., Imai, S., Shimoda, K., Hata, J., and Takano T. (1995) Neurosci. Lett. 200, 117–120
10. Lin, X., Shah, S., and Bullet, R. F. (1996) Brain Res. Mol. Brain Res. 42, 307–316
11. Chawla, S., Vanhoutte, P., Arnold, F. J. L., Huang, C. L., and Bading, H. (2003) J. Neurochem. 85, 151–159
12. Mao, Z., and Wiedmann, M. (1999) J. Biol. Chem. 274, 31102–31107
13. Li, M., Linseman, D. A., Allen, M. P., Meintzer, M. K., Wang, X., Laessig, T., Wierman, M. E., and Heidenreich, K. A. (2001) J. Neurosci. 21, 6544–6552
14. Youn, H. D., Chatilla, T. A., and Liu, J. O. (2000) EMBO J. 19, 4323–4331
15. Blaeser, F., Ho, N., Prywes, R., and Chatilla, T. A. (2000) J. Biol. Chem. 275, 197–209
16. Bading, H., and Greenberg, M. E. (1991) Science 253, 912–914
17. Grozinger, C. M., and Schreiber, S. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7835–7840
18. Hardingham, G. E., Chawla, S., Johnson, C. M., and Bading, H. (1997) Nature 385, 260–265
19. Yang, T. T., Xiong, Q., Eisen, H., Davis, R. J., and Chow, C. W. (2002) Mol. Cell. Biol. 22, 3892–3904
20. Hardingham, G. E., Fukunaga, Y., and Bading, H. (2002) Nat. Neurosci. 5, 405–414
21. Hansen, T. V., Rehfeld, J. F., and Nielsen, F. C. (2004) *J. Neurochem.* **89**, 15–23
22. Fukuchi, M., Tabuchi, A., and Tsuda, M. (2005) *J. Pharmacol. Sci.* **98**, 212–218
23. Davare, M. A., Avdonin, V., Hall, D. D., Peden, E. M., Buret, A., Weinberg, R. J., Horne, M. C., Hoshi, T., and HELL, J. W. (2001) *Science* **293**, 98–101
24. Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J. W., and Bonni, A. (2006) *Science* **311**, 1012–1017
25. Cooper, D. M. (2003) *Biochem. Soc. Trans.* **31**, 912–915
26. Ferguson, G. D., and Storm, D. R. (2004) *Physiology (Bethesda)* **19**, 271–276
27. Hell, J. W., Yokoyama, C. T., Breeze, L. J., Chavkin, C., and Catterall, W. A. (1995) *EMBO J.* **14**, 3036–3044
28. Yoshida, A., Ogura, A., Imagawa, T., Shigekawa, M., and Takahashi. M. (1992) *J. Biol. Chem.* **267**, 1997–2000
29. Soulsby, M. D., and Wojcikiewicz, R. J. (2005) *Biochem. J.* **392**, 493–497
30. Rogue, P. J., Humbert, J. P., Meyer, A., Freyermuth, S., Krady, M. M., and Malviya, A. N. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9178–9183
31. Impey, S., Obrietan, K., Wong, S. T., Poser, S., Yano, S., Wayman, G., Deloulme, J. C., Chan, G., and Storm, D. R. (1998) *Neuron* **21**, 869–883
32. Wayman, G. A., Tokumitsu, H., and Soderling, T. R. (1997) *J. Biol. Chem.* **272**, 16073–16076
33. Hardingham, G. E., Chawla, S., Cruzalegui, F. H., and Bading, H. (1999) *Neuron* **22**, 789–798
34. Dolmetsch, R. E., Pajvani, U., Fife, K., Spotts, J. M., and Greenberg, M. E. (2001) *Science* **294**, 333–339
35. Wu, X., Li, H., Park, E. J., and Chen, J. D. (2001) *J. Biol. Chem.* **276**, 24177–24185
36. McKenzie, G. J., Stevenson, P., Ward, G., Papadia, S., Bading, H., Chawla, S., Privalsky, M., and Hardingham, G. E. (2005) *J. Neurochem.* **93**, 171–185
37. Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., di Padova, F., Olson, E. N., Ulevitch, R. J., and Han, J. (1999) *Mol. Cell. Biol.* **19**, 21–30
38. Graef, I. A., Mermelstein, P. G., Stankunas, K., Neilson, J. R., Deisseroth, K., Tsien, R. W., and Crabtree, G. R. (1999) *Nature* **401**, 703–708
39. Chow, C. W., and Davis, R. J. (2000) *Mol. Cell. Biol.* **20**, 702–712
40. Sheridan, C. M., Heist, E. K., Beals, C. R., Crabtree, G. R., and Gardner, P. (2002) *J. Biol. Chem.* **277**, 48664–48676
41. Nguyen, P. V., and Woo, N. H. (2003) *Prog. Neurobiol. (N.Y.)* **71**, 401–437
42. Malenka, R. C., and Bear, M. F. (2004) *Science* **303**, 5–21
43. Lynch, G., Larson, J., Kelso, S., Barrionuevo, G., and Schottler, F. (1983) *Nature* **305**, 719–721
44. Schroeter, S., Apparsundaram, S., Wiley, R. G., Miner, L. H., Sesack, S. R., and Blakely, R. D. (2000) *J. Comp. Neurol.* **420**, 211–232
45. Matsuo, R., Kato, A., Sakaki, Y., and Inokuchi, K. (1998) *Neurosci. Lett.* **244**, 173–176
46. Ramanan, N., Shen, Y., Sarsfield, S., Lemberger, T., Schutz, G., Linden, D. J., and Ginty, D. (2005) *Nat. Neurosci.* **8**, 759–767
47. Flavell, S. W., Cowan, C. W., Kim, T. K., Greer, P. L., Lin, Y., Paradis, S., Griffith, E. C., Hu, L. S., Chen, C., and Greenberg, M. E. (2006) *Science* **311**, 1008–1012
48. Poser, S., Impye, S., Xia, Z., and Storm, D. R. (2003) *J. Neurosci.* **23**, 4420–4427
49. Wang, X., Tang, X., Li, M., Marshall, J., and Mao, Z. (2005) *J. Biol. Chem.* **280**, 16705–16713
50. Lin, S. L., Johnson-Farley, N. N., Lubinsky, D. R., and Cowen, D. S. (2003) *J. Neurochem.* **87**, 1076–1085
51. Benedito, A. B., Lehtinen, M., Massol, R., Lopes, U. G., Kirchhausen, T., Rao, A., and Bonni, A. (2005) *J. Biol. Chem.* **280**, 2818–2825