

Calcineurin activity is required for depolarization-induced, CREB-dependent gene transcription in cortical neurons

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Abstract

Cyclic AMP response element binding protein (CREB) functions as an activity-dependent transcription factor in the nervous system. Increases in intracellular Ca^{2+} due to neuronal activity lead to the phosphorylation and subsequent activation of CREB. Although phosphorylation of CREB at Ser-133 is necessary for the stimulation of transcriptional activity, it is not sufficient. Here we demonstrate that in mouse cortical neurons, inhibition of the Ca^{2+} -dependent protein phosphatase calcineurin by FK506 or cyclosporine A blocks CREB-dependent gene expression induced by depolarization without inhibiting depolarization-induced Ca^{2+} influx or CREB Ser-133 phosphorylation. Over-expression of a constitutively-active allele of the transducer of regulated CREB activity could not

bypass the requirement for calcineurin activity. Stimulation of a *CRE*-luciferase reporter gene by depolarization was sensitive to FK506 throughout the entire time course of the transcriptional response, revealing that calcineurin activity is required to maintain CREB-dependent transcription. Stimulation of *CRE*-luciferase expression by forskolin and 8-Br-cAMP also required calcineurin activity. These results suggest that calcineurin functions as a critical determinant in shaping genome responses to CREB activation in cortical neurons.

Keywords: activity-dependent transcription, calcineurin, calcium, cyclic AMP, cyclic AMP response element binding protein, FK506.

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The cyclic AMP response element binding protein (CREB) regulates the expression of a wide variety of genes necessary for the development and function of the nervous system (Lonze and Ginty 2002) via its interaction with cyclic AMP/ Ca^{2+} response elements (*CREs*) (Montminy and Bilezikjian 1987). In neurons, increases in intracellular Ca^{2+} generated by neuronal activity stimulate the CREB-dependent gene expression necessary to mediate activity-dependent neuron survival and synaptic plasticity (Sheng *et al.* 1990; Moore *et al.* 1996; Bito and Takemoto-Kimura 2003). Mice in which CREB function is disrupted by expression of a dominant-negative allele of CREB exhibit memory defects (Kida *et al.* 2002; Pittenger *et al.* 2002; Josselyn *et al.* 2004). Mice harboring targeted deletions in *CREB α* and *CREB Δ* exhibit defects in learning and memory (Bourtchuladze *et al.* 1994). In the absence of CREB, the closely related CREB-family member cAMP response element modulator (CREM) is over-expressed (Hummler *et al.* 1994) and largely compensates for the loss of CREB; deletion of CREB in a CREM null background leads to the dramatic loss of neurons in the central nervous system and perinatal lethality (Mantamadiotis *et al.* 2002). Deletion of CREB postnatally in the CREM null results in progressive neurodegeneration (Mantamadiotis *et al.* 2002).

Activation of CREB by Ca^{2+} or cAMP requires the phosphorylation of serine-133. Although necessary for activation, Ser-133 phosphorylation is not sufficient to activate CREB-dependent gene transcription (Bito *et al.*

1996). Additional factors, such as the co-activators CREB binding protein (Kwok *et al.* 1994) and transducers of regulated CREB activity (TORCs) (Conkright *et al.* 2003) also influence the transcriptional status of CREB, as do promoter-specific interactions between CREB and other transcription factors (Mayr *et al.* 2001).

The Ca^{2+} -dependent protein phosphatase, calcineurin (protein phosphatase type 2B), has been reported to exert either stimulatory or inhibitory effects on CREB-dependent gene expression, depending upon the experimental system. For example, in cultured rat hippocampal neurons, calcineurin activation of protein phosphatase type 1 has been reported to limit the duration of CREB phosphorylation and thus restrict the transcriptional responses of CREB activation

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Abbreviations used: APV, 2-amino-5-phosphonopentanoic acid; BDNF, brain-derived neurotrophic factor; CaMKII, calmodulin-dependent kinase II; cAMP, 3,5-cyclic adenosine monophosphate; *CRE*, cAMP response element; CREB, cAMP response element binding protein; CREM, cAMP response element modulator; CSA, cyclosporine A; CsA, cyclosporine A; DMSO, dimethyl sulfoxide; FK506, tacrolimus; SDS, sodium dodecyl sulfate; Ser, serine; TORC, transducer of regulated CREB activity; *TRKB*, tropomyosin-related kinase B.

(Bito *et al.* 1996). In contrast, inhibition of calcineurin in non-neuronal cells, such as pancreatic islet cells or chromaffin cells, can block CREB-dependent gene expression (Schwaninger *et al.* 1993; Hahm *et al.* 2003). It is not clear whether these opposite effects of calcineurin are due to a fundamental difference in signaling mechanisms between neurons and non-neuronal cells or if they reflect stimulus-specific or promoter-specific differences in the signaling requirements for CREB-dependent transcriptional activation.

In the central nervous system, two important targets of CREB are the genes encoding brain-derived neurotrophic factor (BDNF) (Shieh *et al.* 1998; Tao *et al.* 1998) and its receptor, tropomyosin-related kinase B (trkB) (Kingsbury *et al.* 2003), which promote neuron survival, differentiation, and synaptic plasticity (Huang and Reichardt 2001; 2003). In this report we show that depolarization-induced activation of *BDNF* and *TRKB* transcription is sensitive to the calcineurin inhibitor FK506. Although depolarization-induced Ca^{2+} entry and Ser-133 phosphorylation were unaffected by calcineurin inhibition, FK506 also blocked the activation of a *CRE*-luciferase reporter gene, suggesting that calcineurin is required for CREB-dependent transcription in these neurons.

Experimental procedures

Cell culture and transfection

Cortical neuron cultures were prepared from mouse brains on embryonic day 16 (E16). Neurons were dissociated by trituration and plated onto 35 mm dishes, 24-well dishes, or 25 mm coverslips coated with 15 $\mu\text{g}/\text{mL}$ polyornithine (Sigma, St Louis, MO, USA) and 8 $\mu\text{g}/\text{mL}$ laminin (Invitrogen, Carlsbad, CA, USA) and cultured in Neurobasal medium supplemented with 2% B27, 1 mmol/L glutamine and penicillin/streptomycin (Invitrogen). Neurons were transiently transfected 3–5 days *in vitro* using calcium phosphate as previously described (Xia *et al.* 1996).

Luciferase assays

Plasmids used for luciferase assays were as follows: pCRE-Luc PathDetect cis-reporter plasmid (Stratagene, La Jolla, CA, USA), mouse BDNF promoter IV-luciferase (Martinowich *et al.* 2003), -944/-325 *TRKB* P2-luciferase (Kingsbury and Krueger 2007) and TK Renilla (Promega, Madison, WI, USA). Two days following transfection, cells were stimulated with either 50 mmol/L KCl, 10 $\mu\text{mol}/\text{L}$ forskolin (Sigma), or 100 ng/mL BDNF (Regeneron, Tarrytown, NY, USA). Except where indicated otherwise, cells were pre-treated for 15 min with either 100 nmol/L FK506 (Sigma) or dimethyl sulfoxide (DMSO). For time course of addition experiments, FK506 and 0.5 $\mu\text{g}/\text{mL}$ actinomycin D (Sigma) were added when indicated. DL-2-amino-5-phosphonopentanoic acid (APV) (100 $\mu\text{mol}/\text{L}$) and KN93 (1 $\mu\text{mol}/\text{L}$) were added 15 min prior to KCl-depolarization. Following stimulation, cells were rinsed once with phosphate-buffered saline and then harvested for luciferase assays using the Dual Luciferase Assay kit (Promega). Data presented are the means of either triplicate or quadruplicate

samples with standard error indicated. Each experiment was repeated at least three times with similar results using separate cortical neuron preparations. The solvent, DMSO, alone had no effect on basal luciferase activity of any of the luciferase reporter constructs used in this study.

Western blotting

Cortical neuron cultures were pre-treated with either with FK506 or with vehicle (DMSO) for 15 min prior to depolarization by addition of 50 mmol/L KCl. Following stimulation, the medium was aspirated, cells rinsed once with phosphate-buffered saline and then harvested in boiling 2 \times Laemmli buffer (Sigma) in the presence of 'Halt' protease inhibitor cocktail (Pierce, Rockford, IL, USA). Samples were subjected to SDS-polyacrylamide gel electrophoresis using NuPage SDS 3-(*N*-morpholino) propanesulfonic acid buffer system (Invitrogen), proteins transferred to Immobilon-P^{SO} membrane (Millipore, Billerica, MA, USA). Membranes were processed for western analysis using anti-CREB or anti-phospho CREB (Cell Signaling, Danvers, MA, USA) at 1 : 1000 dilution, anti-trkB (Upstate, Lake Placid, NY, USA) at 1 : 1000 or anti-actin (Sigma) at 1 : 5000 in Tris-buffered saline. Horseradish peroxidase-conjugated anti-rabbit secondary (Jackson ImmunoResearch, West Grove, PA, USA) was used at 1 : 10,000 in Tris-buffered saline with Tween-20. Following incubation with ECL Plus (Amersham, Piscataway, NJ, USA), bands were imaged using a Fujifilm LAS-3000 (Fujifilm Life Science USA, Stamford, CT, USA). Western blot analysis was repeated three times with cells from independent neuronal preparations. Representative western blots are shown.

Ca^{2+} imaging

For Ca^{2+} imaging experiments, cortical neurons grown on 25 mmol/L coverslips were loaded with 3 $\mu\text{mol}/\text{L}$ fura-2-AM (Invitrogen) for 30 min at 37°C in tissue culture medium and washed for an additional 30 min at 37°C in physiological saline solution (containing 140 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L NaH_2PO_4 , 1.4 mmol/L MgCl_2 , 1.8 mmol/L CaCl_2 , 11.5 mmol/L glucose, and 10 mmol/L HEPES, titrated to 7.4 with NaOH) to allow complete de-esterification of the Fura-2-AM ester. Coverslips were continuously superfused with physiological saline solution during the experiment. Fura-2 fluorescence was measured using 340 and 380 nm excitation provided by a Til IV monochromator, emission filtered at 510 nm and captured by an ORCA ER camera (Hamamatsu Photonics, Hamamatsu City, Japan). Data from 30–50 individual cells/coverslip were analyzed using MetaFluor (Molecular Devices, Sunnyvale, CA, USA). For depolarization, 50 mmol/L KCl was added in the superfusate. For experiments with FK506, the drug was present in the loading and de-esterification media and in the superfusate. DMSO did not affect the intracellular Ca^{2+} concentration.

RNA analysis

RNA was isolated from cortical neurons using Trizol (Sigma). mRNA was reverse transcribed using oligo-dT primers and Superscript III (Invitrogen). Real time PCR was conducted on an Icyler IQ (BioRad, Hercules, CA, USA) using Finnzymes (New England Biolabs, Ipswich, MA, USA) master mix. The PCR protocol consisted of an initial hold at 95°C for 5 min then 40 cycles of a two-step protocol consisting of 95°C for 30 s step followed by 58°C for 30 s. After 40

cycles, a melting curve was conducted to verify reactions generated a single product. Each primer pair was also validated to result in the amplification of a band of the expected size by gel electrophoresis. The expected reaction products for *BDNF* exon I, *BDNF* exon III, *trkB* full-length, and actin were 133, 112, 103, and 140 bp, respectively. RNA expression was computed from the slope of the C_T versus log [cDNA] and normalized to actin amplification. PCR primers used were designed to span intron/exon junctions to avoid genomic DNA contamination. Sequences were as follows: mouse *BDNF* exon I: GGACAGCAAAGCCACAATGTTCCA and TTGCCTTGCCG TGGACGTTTACT; mouse *BDNF* exon IV: ACCAGGTGAG AAGAGTGATGACCA and AGTTGCCTTGCCGTTGACGTT TA; mouse full-length *trkB*: TTTCCTTGCCGAGTGCTACAAC CT and TGAAAGTCCTTGCGTGATTGTCG; actin: TGTGA TGGTGGGAATGGGTGAGAA and TGTGGTGCCAGATCTTC TCCATGT.

Results

Depolarization-stimulated expression of *BDNF* and *TRKB* requires calcineurin activity

In the central nervous system, activation of CREB stimulates expression of the genes encoding BDNF and its receptor, *trkB*. Depolarization-induced Ca^{2+} influx stimulates CREB-dependent expression of *BDNF* and *TRKB* via either a single *CRE* or two tandem *CREs* located within the *BDNF* and *TRKB* promoters, respectively (Shieh *et al.* 1998; Tao *et al.* 1998; Kingsbury *et al.* 2003). Using mouse *BDNF* promoter IV (Martinowich *et al.* 2003) and a *TRKB* promoter 2-luciferase reporter construct (Kingsbury and Krueger 2007) we tested the requirement for calcineurin in depolarization-induced stimulation of *BDNF* and *TRKB* transcription. Primary cortical neuron cultures were transiently transfected with either the *BDNF* promoter IV or *TRKB* P2-luciferase reporter gene. Two days after transfection, cells were stimulated for 5 h with 50 mmol/L KCl to depolarize the neurons in either the presence or absence of the calcineurin inhibitor FK506. As previously reported, KCl depolarization stimulated both *BDNF* and *TRKB* reporter constructs (Fig. 1). In the presence of KCl and FK506, *BDNF* and *TRKB*-luciferase activity was partially inhibited, exhibiting a 68% and 74% decrease in KCl-stimulated expression, respectively. No effect of FK506 on basal *BDNF* or *TRKB*-luciferase expression was observed. These results indicate that calcineurin participates in Ca^{2+} -stimulated transcription of these two reporter genes.

Calcineurin is required for the stimulation of endogenous *BDNF* and *TRKB* genes

Although our results with reporter genes reveal a role for calcineurin in mediating CREB-dependent transcription, we sought to determine if calcineurin activity is also required for depolarization-stimulated expression of endogenous genes. We investigated the expression of *BDNF* and *TRKB*. As above,

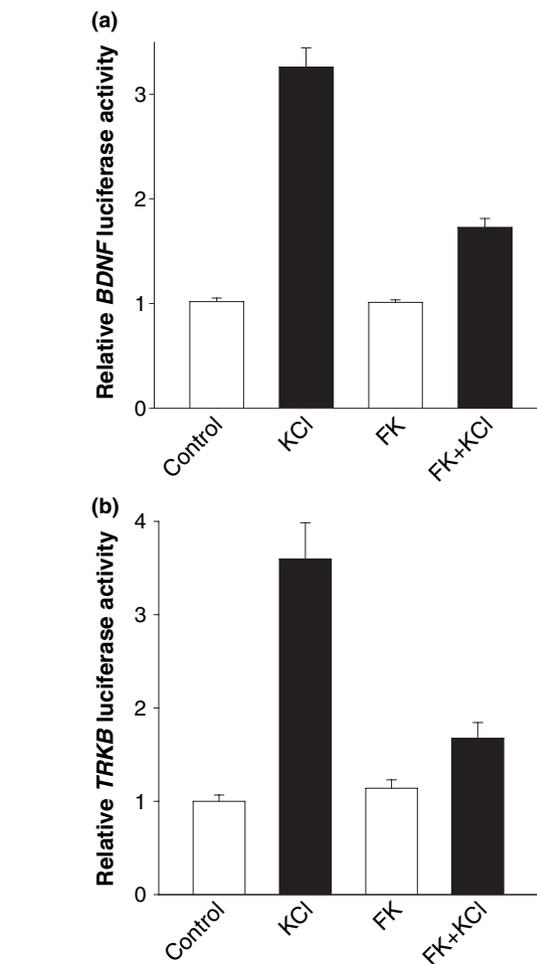


Fig. 1 FK506 inhibits depolarization-induced stimulation of *BDNF* and *TRKB* transcription. (a) Cortical neurons transiently transfected with *BDNF*-luciferase were depolarized with 50 mmol/L KCl for 5 h in either the presence or absence of 100 nmol/L FK506 (FK). Data shown are the means \pm SEM ($n = 4$). Luciferase activity is reported relative to unstimulated activity. (b) *TRKB*-luciferase transfected neurons were stimulated as in (a). Data shown are the means \pm SEM ($n = 4$).

cortical neurons were stimulated with 50 mmol/L KCl in the presence or absence of FK506. Following 5 h of stimulation, cells were harvested for RNA isolation and the levels of *BDNF* and *TRKB* transcripts were assayed using real-time PCR. As expected from previous studies (Tao *et al.* 1998; Tabuchi *et al.* 2000), depolarization increased expression of both *BDNF* exon I and *BDNF* exon IV transcripts (Fig. 2a and b). Mouse *BDNF* exon IV corresponds to rat exon III (Martinowich *et al.* 2003), which has been studied extensively as a CREB-dependent gene (West *et al.* 2001). Depolarization stimulated *BDNF* exon I transcripts approximately 14-fold and *BDNF* exon IV transcripts 12-fold. Inclusion of FK506 reduced the stimulation of the *BDNF* transcripts by about 85% and 75%, respectively. *TRKB* transcripts exhibited a twofold stimulation in response to depolarization (Fig. 2c) that was completely

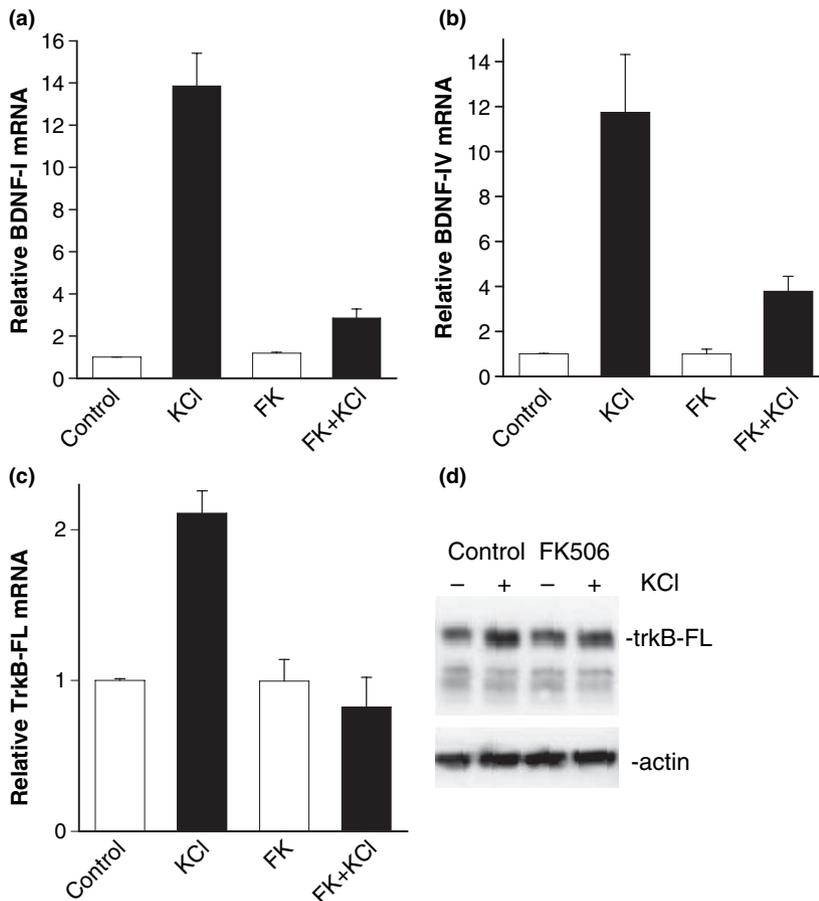


Fig. 2 Calcineurin activity promotes CREB-stimulated expression of endogenous *TRKB* and *BDNF*. Cortical neurons were stimulated for 6 h with 50 mmol/L KCl in either the presence or absence of FK506 (FK) and harvested for RNA. Real-time PCR analysis was conducted to assay the levels of (a) *BDNF* exon I, (b) *BDNF* exon IV, and (c) *TRKB* transcripts. (d) Cortical neurons were stimulated for 18 h with 25 mmol/L KCl in either the presence or absence of FK506. Protein samples were harvested and subjected to SDS-PAGE and western blotting using anti-trkB and anti-actin antibodies. Full-length trkB (trkB-FL) is labeled. Truncated trkB isoforms were also observed.

inhibited by the presence of FK506. Consistent with this observation, FK506 blocked the increase in full-length trkB protein expression induced by depolarization with 25 mmol/L KCl (Fig. 2d). Calcineurin activity is therefore necessary for depolarization-stimulated expression of the endogenous CREB-dependent promoters present in *BDNF* and *TRKB*.

Calcineurin is required for CREB-dependent gene transcription

As the Ca^{2+} -stimulated transcription of both the *BDNF* and *TRKB* promoters requires functional interactions between one or more *CREs* and additional promoter regulatory elements (West *et al.* 2001; Kingsbury and Krueger 2007), the effect of calcineurin may be due to either the inhibition of CREB activation or disruption of the functional cooperation between CREB and neighboring transcription factors bound to the promoters. The role of calcineurin in regulating transcriptional activity of CREB was therefore studied using a simple promoter consisting of four *CREs* driving luciferase expression (Stratagene). Mouse cortical neurons transiently transfected with the 4x*CRE*-luciferase were stimulated with 50 mmol/L KCl for 5 h in the presence or absence of either FK506 (Fig. 3a) or cyclosporine A (CsA) (Fig. 3b) to inhibit calcineurin, and assayed for luciferase activity. Neuron depolarization led to a robust (>30-fold) stimulation of

4x*CRE*-luciferase activity that was blocked more than 95% by either FK506 (Fig. 3a) or CsA (Fig. 3b). Neither FK506 nor CsA affected basal 4x*CRE*-luciferase activity.

Calcineurin activity is required throughout the time course of CREB stimulation

The sensitivity of depolarization-induced *CRE*-luciferase activity to calcineurin inhibitors may reflect a role for calcineurin in either the initiation or maintenance of Ca^{2+} -dependent transcription. To investigate this further, cortical neurons were transfected with 4x*CRE*-luciferase 2 days prior to stimulation. FK506 was added either 15 min prior to depolarization, or at increasing time intervals following depolarization. Cells were harvested for luciferase assays at the end of 5 h KCl stimulation. As a control for the time course of transcriptional activation, actinomycin D treatment was conducted in parallel experiments. As shown in Fig. 4a, pre-treatment with actinomycin D completely blocked the KCl-stimulation of the 4x*CRE*-luciferase activity. The inhibitory effect of actinomycin D decreased at later times of addition, with no effect observed when actinomycin D was added 2 h after KCl. This finding indicates that the transcriptional response to depolarization, measured by luciferase activity, ended within 2 h after depolarization. Addition of FK506 also blocked the increase in *CRE*-

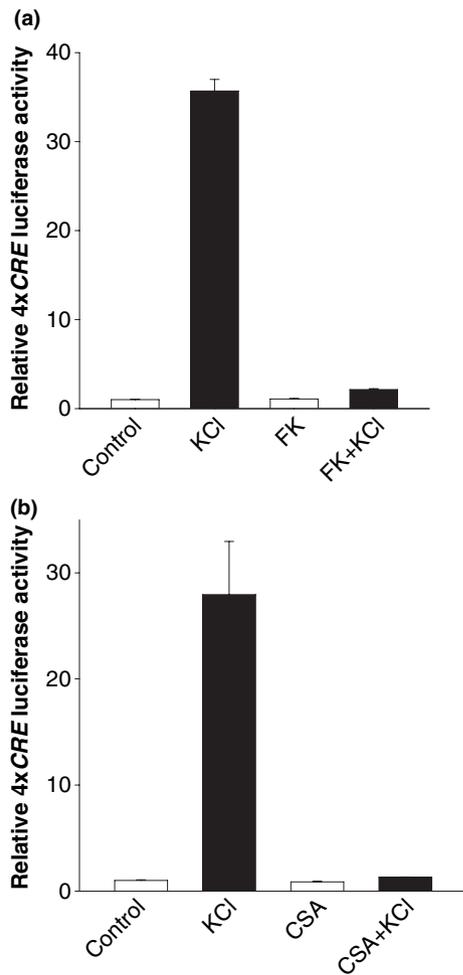


Fig. 3 Inhibition of calcineurin blocks depolarization-induced *CRE*-luciferase activity. Cortical neurons were transiently transfected with 4x*CRE*-luciferase construct. (a) Transfected neurons were stimulated with 50 mmol/L KCl for 5 h in the presence or absence of 100 nmol/L FK506 (FK). Data shown are the means \pm SEM ($n = 4$). (b) Transfected neurons were stimulated by the addition of KCl for 5 h in the presence or absence of cyclosporine A (CSA; 2 μ g/mL). Data shown are the means \pm SEM ($n = 4$).

luciferase activity with decreasing efficacy throughout the time course of the transcriptional response (Fig. 4b). Addition of FK506 within the first 30 min of depolarization blocked the response as effectively as a 15 min pre-treatment with the drug. Addition of FK506 at 30 min, resulted in approximately fourfold stimulation of luciferase activity, compared to the 28-fold stimulation observed in the KCl only control. Addition of FK506 at 60 min resulted in an approximately 12-fold stimulation, indicating that calcineurin function was still required to promote CREB-dependent transcription. As expected from the actinomycin D data, addition of FK506 after 2 h had no effect on depolarization-stimulated luciferase activity. A time course of addition of 2 mmol/L EGTA to block Ca^{2+} -influx mirrored the results obtained with FK506 (data not shown). These results

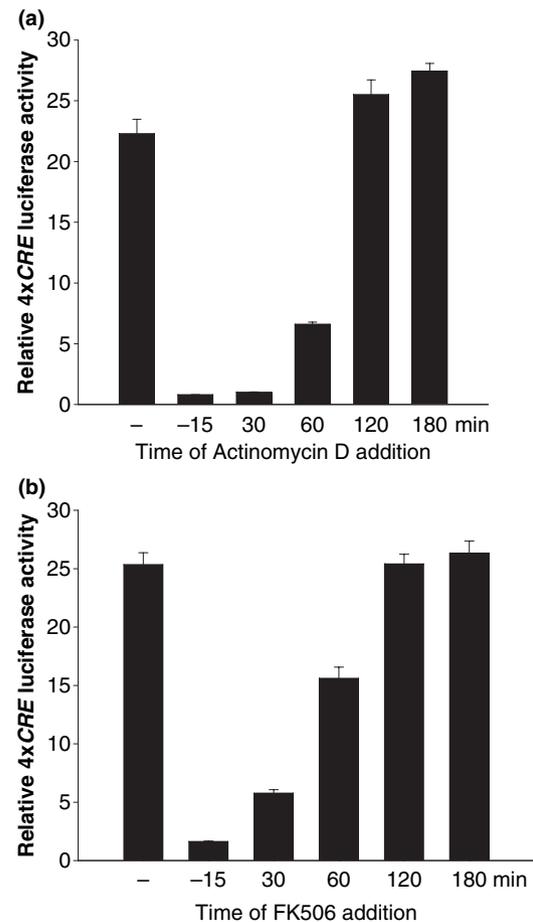


Fig. 4 Calcineurin activity is required throughout the time course of the *CRE* transcriptional response. (a) The time course of the transcriptional activation of the 4x*CRE*-luciferase response to KCl depolarization was determined by the addition of actinomycin D (0.5 μ g/mL) at times ranging from 15 min pre-treatment to 3 h after KCl addition. All samples were harvested after 5 h of KCl treatment and processed for luciferase assays. Data shown are the means \pm SEM ($n = 3$). (b) The requirement for calcineurin function in maintaining the response of 4x*CRE*-luciferase to KCl depolarization was investigated by assaying the effects of the addition of 100 nmol/L FK506 as described in (a) for actinomycin D. Data shown are the means \pm SEM ($n = 3$).

demonstrate that extracellular Ca^{2+} and calcineurin activity is required for the maintenance of *CRE*-stimulated transcription following depolarization. Furthermore, these data demonstrate that during prolonged KCl treatment, calcineurin is not being rapidly inactivated.

FK506 does not inhibit depolarization-induced Ca^{2+} influx

Calcineurin has numerous functions in cells, including the regulation of Ca^{2+} channel activity. As our results would be consistent with a reduction in depolarization-induced Ca^{2+} influx, we examined the effect of FK506 on intracellular Ca^{2+} levels in response to KCl depolarization. Depolarization-induced increases in intracellular Ca^{2+} were measured in

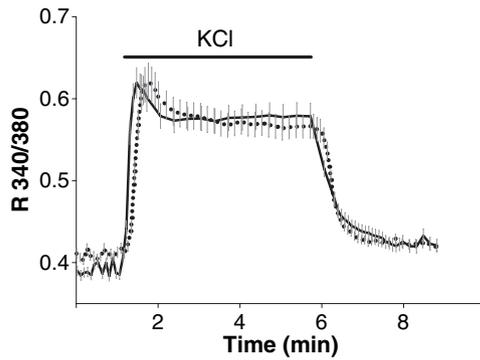


Fig. 5 Inhibition of calcineurin does not block depolarization-induced Ca^{2+} influx. Fura-2 imaging showed that 50 mmol/L KCl induced a similar rise, plateau and recovery in intracellular Ca^{2+} in the absence (solid line) or presence (dotted line) of FK506. Data are means \pm SEM for 32 control and 41 FK506-treated neurons from a single experiment. Data are representative of 5–7 coverslips per condition using cells from two separate cell preparations.

the absence and presence of FK506 in cortical neurons by computer-assisted imaging of fura-2 fluorescence. Figure 5 shows averaged responses of 30–40 cells from two coverslips in a typical experiment. Neither the baseline nor the peak, plateau or recovery of the response to KCl were affected by FK506. Furthermore, although some cells in each field failed to respond to KCl, the fraction of non-responding cells was not changed by FK506 (non-responding cells: $4 \pm 1\%$ and $2.7 \pm 0.5\%$ for control and FK506 treated cells, respectively; not significantly different by *t*-test). Similar results were obtained with 5–7 coverslips for each condition from two independent neuron culture preparations; altogether, 339 control and 344 FK506-treated cells were analyzed. These results demonstrate that the inhibition of CREB-dependent gene transcription by calcineurin inhibition is not due to reduced depolarization-induced Ca^{2+} entry.

Inhibition of calcineurin does not affect Ser-133 CREB phosphorylation

Cyclic AMP response element binding protein activation by Ca^{2+} requires phosphorylation of Ser-133. The ability of depolarization to stimulate Ser-133 phosphorylation was monitored by western blot analysis over the same time course as for *CRE*-luciferase induction. Cortical neuron cultures were depolarized using 50 mmol/L KCl. Cell extracts were harvested over a 3 h time course in the absence or presence of FK506, subjected to SDS–polyacrylamide gel electrophoresis, and processed for western blotting using anti-phospho-CREB or anti-CREB specific antibodies (Fig. 6). Depolarization-induced stimulation of CREB Ser-133 phosphorylation was observed throughout the 3 h time course, and was unaffected by the presence of FK506. This finding indicates that the inhibition of 4x*CRE*-luciferase by FK506 is not due to the inhibition of Ser-133 CREB phosphorylation. Further, this finding suggests that in embryonic cortical neurons, prolonged

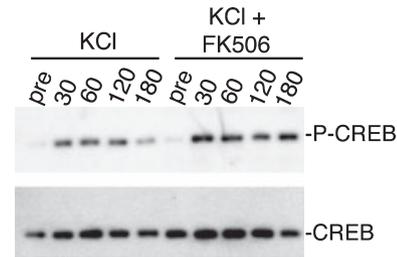


Fig. 6 Inhibition of calcineurin does not block depolarization-induced CREB Ser-133 phosphorylation. Neurons were stimulated in 50 mmol/L KCl in either the presence or absence of 100 nmol/L FK506. Cells were harvested and protein samples collected over a 3-h time course. Proteins were separated by SDS–PAGE and subjected to western blot analysis using either anti-phospho-CREB or anti-CREB.

Ser-133 phosphorylation is not sufficient for CREB-dependent transcriptional activity.

Activated TORC does not bypass the requirement for calcineurin

Dephosphorylation of TORCs by calcineurin can stimulate CREB-dependent transcription by allowing TORC to enter the nucleus and function as a CREB co-activator (Screaton *et al.* 2004). To determine if loss of TORC activation was responsible for the inhibition of CREB activity following FK506 treatment, we co-transfected cortical neurons with the 4x*CRE*-luciferase reporter gene and either empty vector or a vector expressing a constitutively active form of TORC, (TORC2 S171A), which does not require dephosphorylation by calcineurin to enter the nucleus (Screaton *et al.* 2004). If the effect of FK506 were due to the loss of TORC function, expression of the constitutively active TORC2 should bypass the requirement for calcineurin in the activation of 4x*CRE*-luciferase following depolarization. As shown in Fig. 7, depolarization resulted in a 12-fold and 20-fold stimulation of 4x*CRE*-luciferase in the absence or presence of the constitutively active TORC2, respectively. The increase in activation in the presence of activated TORC2 is consistent with previous reports (Screaton *et al.* 2004) and confirms expression of the construct. Inclusion of FK506 blocked depolarization-induced stimulation of *CRE*-luciferase regardless of the presence or absence of TORC2 S171A. Thus, activated TORC was insufficient to bypass the requirement for calcineurin in stimulation of *CRE*-luciferase expression by depolarization. While this observation does not rule out a role for TORC in the activation of 4x*CRE* following depolarization, it demonstrates that the failure to activate TORC is not responsible for the loss of CREB-dependent gene transcription in the absence of calcineurin activity.

Calcineurin activity is necessary for activation of CREB-dependent transcription by cAMP

Cyclic AMP response element binding protein-dependent gene transcription is stimulated in response to activation of

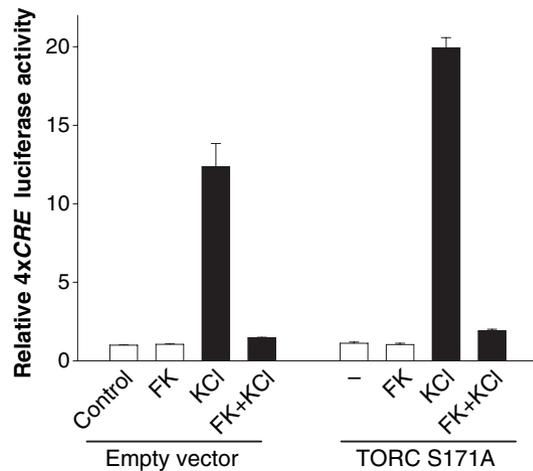


Fig. 7 Constitutively-active TORC cannot bypass the requirement for calcineurin. Neurons were transiently co-transfected with 4xCRE-luciferase and either empty vector or vector expressing constitutively active TORC2 (TORC2S171A). Two days after transfection, cells were stimulated with 50 mmol/L KCl in either the presence or absence of 100 nmol/L FK506 (FK). Data shown are expressed relative to luciferase values obtained for the unstimulated empty vector transfected cells (control). Data shown are the means \pm SEM ($n = 3$).

intracellular signaling cascades in addition to Ca^{2+} signaling. To determine if calcineurin function is a general requirement for CREB-stimulated expression in cultured cortical neurons, we examined the effects of FK506 on CRE-dependent transcription in response to cAMP signaling. Neurons transiently transfected with 4xCRE-luciferase were stimulated for 5 h with either forskolin or 8-Br-cAMP, in the absence or presence of FK506 (added 15 min prior to stimulation) (Fig. 8). Forskolin and 8-Br cAMP caused a 16-fold and sixfold stimulation of luciferase activity, respectively, which were almost completely blocked by FK506.

FK506 does not block depolarization-stimulated CRE-transcription via known CREB inhibitory pathways

One potential explanation of our findings would be that depolarization initiates both a CREB activating pathway and an opposing inhibitory pathway that is normally suppressed by calcineurin. Two potential mechanisms by which depolarization could activate a CREB inhibitory pathway have previously been reported. First, activation of NMDA receptors has been shown to activate a CREB shut-off pathway in hippocampal neurons (Sala *et al.* 2000; Hardingham *et al.* 2002). As depolarization could result in the activation of the NMDA class of glutamate receptors by releasing glutamate, we investigated the possibility that depolarization-induced NMDA receptor activation was initiating a CREB inhibitory pathway normally antagonized by the simultaneous activation of calcineurin. The ability of APV, an inhibitor of the NMDA class of glutamate receptors, to bypass the requirement for calcineurin was therefore tested. As shown in

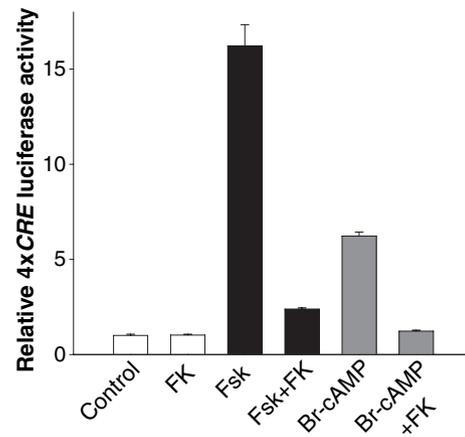


Fig. 8 Cyclic AMP-stimulated gene expression requires calcineurin activity. Cortical neurons transfected with 4xCRE-luciferase were stimulated with either 10 μ mol/L forskolin (Fsk) or 1 mmol/L 8-Br-cAMP for 5 h prior to harvesting for luciferase assays. Data shown are normalized to unstimulated control. Data shown are the means \pm SEM ($n = 4$).

Fig. 9a, inclusion of APV did not overcome the inhibitory role of FK506. This finding demonstrates that NMDA receptor activation was not responsible for the inhibition of CREB in the absence of calcineurin function. Instead, the inclusion of APV partially inhibited KCl-stimulated 4xCRE-luciferase activity. APV had no effect on basal 4xCRE-luciferase expression.

A second potential mechanism reported to inactivate CREB is the phosphorylation of CREB on Ser-142 by calmodulin-dependent kinase II (CaMKII). Ser-142 phosphorylation may inhibit CREB function by disrupting the interaction between CREB and the co-activator CREB binding protein (Sheng *et al.* 1991; Wu and McMurray 2001), although more recently, Ser-142 phosphorylation has been suggested to stimulate CREB transcriptional activation in response to specific stimuli (Kornhauser *et al.* 2002). We examined the effect of KN93, a CaMKII inhibitor, on the ability of FK506 to block depolarization-induced CRE-luciferase expression. As shown in Fig. 9b, KN93 had no effect on either basal expression or the stimulation of 4xCRE-luciferase following depolarization. Inclusion of KN93 did not protect KCl-stimulated luciferase expression from the loss of calcineurin activity. This finding indicates that CaMKII activity is not necessary for the inhibition of CREB-dependent transcription by FK506.

Discussion

Here we show that calcineurin activity is required for CREB-dependent gene transcription in response to depolarization and cAMP signaling in cultured embryonic cortical neurons. Depolarization-induced expression of *BDNF* (Fig. 1a),

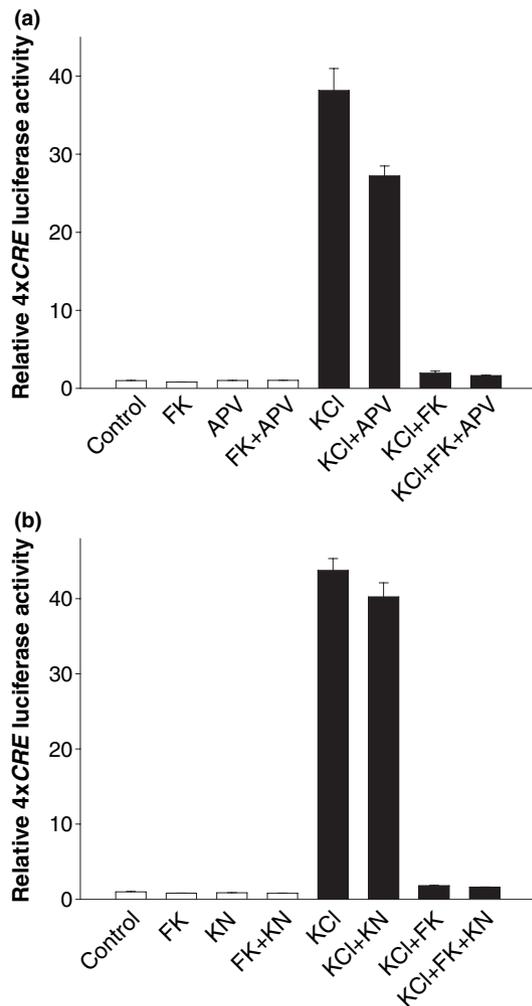


Fig. 9 NMDA receptor and CaMKII signaling pathways do not mediate CREB inhibition in the presence of FK506. (a) Cortical neurons transfected with 4xCRE-luciferase were pre-treated with 100 $\mu\text{mol/L}$ DL-APV. Following 5 h KCl depolarization in the presence or absence of 100 nmol/L FK506 (FK), cells were harvested for luciferase assays. (b) 4xCRE-luciferase transfected cortical neurons were pre-treated with 1 $\mu\text{mol/L}$ KN93 (KN) and stimulated as described in (a). Data shown are plotted relative to the unstimulated control lacking any drugs. Data shown are the means \pm SEM ($n = 3$).

TRKB (Fig. 1b), and synthetic *CRE* (Fig. 3a) reporter genes were blocked by the calcineurin inhibitor, FK506. 4xCRE-luciferase was also blocked by another calcineurin inhibitor, cyclosporine A (Fig. 3b). FK506 also blocked depolarization-induced increases of endogenous *BDNF* and *TRKB* transcripts (Fig. 2a–c), which have previously been reported to be CREB-dependent (Tabuchi *et al.* 2000; Kingsbury *et al.* 2003; Martinowich *et al.* 2003). Ca^{2+} imaging demonstrated that FK506 did not inhibit the depolarization-induced rise in intracellular Ca^{2+} (Fig. 5). Consistent with this observation, FK506 did not block depolarization-induced phosphorylation of CREB at Ser-133 (Fig. 6).

FK506 inhibited depolarization-induced *CRE*-luciferase expression even when added an hour after stimulation (Fig. 4b), indicating that calcineurin is required to maintain CREB-dependent transcriptional activation. Calcineurin also inhibited forskolin- and 8-Br-cAMP-stimulated 4xCRE expression (Fig. 8), demonstrating that calcineurin activity is also required for both Ca^{2+} - and cAMP-stimulated activation of CREB-dependent gene transcription in neurons. We have further observed that forskolin-induced stimulation of 4xCRE-luciferase is similarly inhibited by the presence of extracellular EGTA (unpublished observation). These findings may reflect either a requirement for basal calcineurin activity to promote the activation of CREB-dependent transcription or the ability of cAMP to generate local Ca^{2+} transients that activate calcineurin.

Block of CREB-dependent transcription by calcineurin inhibitors exhibits cell-type specificity, both in terms of outcome and mechanism. FK506 blocks CREB-dependent transcription in the pancreatic islet beta cell line HIT-T15, without affecting Ser-133 CREB phosphorylation following KCl treatment (Schwaninger *et al.* 1995). In chromaffin cells, FK506 inhibits CREB phosphorylation at ser-133 in response to KCl (Hahm *et al.* 2003). In hippocampal neurons (Bito *et al.* 1996), calcineurin has been reported to limit CREB transcriptional activation by activating protein phosphatase type 1, which dephosphorylates CREB. Treatment of hippocampal neurons with FK506 prolonged CREB phosphorylation at Ser-133 following brief electrical stimulation or 1 min of KCl depolarization. The prolonged duration of CREB phosphorylation was shown to correlate with the ability of a previously sub-threshold stimulus (18 s of electrical stimulation) to trigger an increase in *c-fos* and somatostatin expression as measured by immunohistochemistry. Increasing the duration of the electrical stimulus from 18 to 180 s, however, occluded the effects of FK506, possibly due to a relatively rapid inactivation of calcineurin in these cells (Bito *et al.* 1996). Using striatal slice cultures from newborn rats, Liu and Graybiel (1996) showed that inhibition of calcineurin with FK506 could prolong CREB phosphorylation following BAYK 8644 treatment. Inhibition of calcineurin has also been reported to reduce the death of rat cortical neurons following treatment with excitotoxic levels of NMDA by prolonging the time course of CREB phosphorylation (Lee *et al.* 2005). Neither of the two latter studies, however, measured CREB-dependent gene expression.

In contrast to the results reported by Bito *et al.* (1996), we find that addition of FK506 inhibits the stimulation of several CREB-dependent reporter genes, including *CRE*-luciferase. This difference may reflect either neuron-specific (rat hippocampal vs. mouse cortical neurons) or developmental stage-specific differences between the cells (e.g. before or after the formation of functionally mature synapses in the cultures). Stimulation-specific differences (sub-threshold

electrical vs. chronic 50 mmol/L KCl) may further alter the effect of calcineurin inhibition due to the activation of distinct intracellular signaling cascades impinging on CREB. As the effect of FK506 on CRE-dependent transcription can also exhibit promoter-specific differences (Siemann *et al.* 1999), it is possible that the need for calcineurin may be bypassed by the presence of additional transcription factors bound to complex promoters. This may explain our finding that inhibition of CRE-luciferase by FK506 was greater than that observed for the CREB-dependent reporter genes *TRKB* and *BDNF* (compare Figs 1 and 3), both of which possess multiple regulatory elements involved in Ca²⁺-dependent transcription (West *et al.* 2001; Kingsbury and Krueger 2007). Promoter-specific context may partially explain why expression of c-fos and somatostatin by sub-threshold electrical stimulation was enhanced, rather than inhibited, by FK506 in hippocampal neurons (Bito *et al.* 1996).

A key question remaining in our understanding of the regulation of CREB-dependent gene expression is how the activation of CREB by distinct Ca²⁺ signals leads to the stimulation of specific sets of target genes. Our results reveal that calcineurin may play an important role in conferring gene specificity to the genomic response to CREB activation in neurons. Differential activation of calcineurin may determine whether a particular Ca²⁺ signal leads to the global activation of all CREB-responsive promoters or to the activation of a more restricted subset of genes. Ultimately, the dependence of any given promoter on calcineurin activity will be determined by promoter context: promoters with the appropriate configuration of additional regulatory elements may bypass the need for calcineurin to promote CREB function. Modulation of CREB-dependent transcription by calcineurin may play a role in shaping Ca²⁺-dependent gene expression, which is believed to underlie nervous system development and learning and memory. Altered calcineurin activity may contribute to the progression of neurodegenerative and psychiatric diseases by disrupting the normal regulation of CREB-dependent gene expression.

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