# Ca<sup>2+</sup>-dependent Regulation of TrkB Expression in Neurons\*

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The neurotrophin brain-derived neurotrophic factor (BDNF), via activation of its receptor, tyrosine receptor kinase B (trkB), regulates a wide variety of cellular processes in the nervous system, including neuron survival and synaptic plasticity. Although the expression of BDNF is known to be Ca<sup>2+</sup>-dependent, the regulation of trkB expression has not been extensively studied. Here we report that depolarization of cultured mouse cortical neurons increased the expression of the full-length, catalytically active isoform of trkB without affecting expression of the truncated isoform. This increase in protein expression was accompanied by increased levels of transcripts encoding full-length, but not truncated, trkB. Depolarization also regulated transcription of the gene, TRKB, via entry of Ca<sup>2+</sup> through voltage-gated Ca<sup>2+</sup> channels and subsequent activation of Ca<sup>2+</sup>-responsive elements in the two TRKB promoters. Using transient transfection of neurons with TRKB promoterluciferase constructs, we found that Ca<sup>2+</sup> inhibited the upstream promoter P1 but activated the downstream promoter P2. Ca<sup>2+</sup>-dependent stimulation of TRKB expression requires two adjacent, non-identical CRE sites located within P2. The coordinated regulation of BDNF and trkB by Ca<sup>2+</sup> may play a role in activity-dependent survival and synaptic plasticity by enhancing BDNF signaling in electrically active neurons.

The neurotrophin, brain-derived neurotrophic factor (BDNF),<sup>1</sup> mediates numerous functions in both the developing and mature nervous systems, including the survival of postmitotic neurons, axon growth and guidance, and synaptic plasticity (1). These effects of BDNF are mediated by the tyrosine receptor kinase, trkB. Binding of BDNF to trkB initiates

dimerization and trans-autophosphorylation of tyrosine residues in the intracellular domain of trkB (2). These phosphotyrosine residues act as docking sites for effector proteins that activate downstream signaling pathways, leading to the activation of protein kinase cascades,  $Ca^{2+}$  mobilization, and gene expression, which orchestrate the cellular responses to BDNF (3). Excitatory synaptic input and the resulting elevation in intracellular [ $Ca^{2+}$ ] have been shown to increase the synthesis and release of BDNF (4–9). This BDNF activates trkB receptors in the same or neighboring cells to promote their survival and may also enhance synaptic plasticity (1, 10). Although trkB levels change during development and exhibit cell-specific expression patterns (11–13), very little is known about the mechanisms that regulate *TRKB* expression.

At least four isoforms of trkB are produced by alternative splicing of the primary transcripts of the *TRKB* gene (14–16). Of these, only the full-length isoform, which contains an intracellular tyrosine kinase domain, is known to be capable of mediating BDNF signaling. Three truncated isoforms (T1, T2, and T<sub>shc</sub>), which lack the intracellular kinase domain but possess the same extracellular BDNF binding domain as fulllength receptors, can also be generated by alternative splicing. T1 is prominently expressed in the brain (14) and can act as a dominant negative inhibitor of BDNF signaling (17-21) by forming heterodimers with full-length trkB (17). These observations raise the possibility that the relative expression of full-length and truncated trkB isoforms in normal neurons can modulate cellular responsiveness to BDNF. Dysregulation of trkB isoform expression may also underlie some nervous system abnormalities. For example, overexpression of truncated trkB has been reported in cortical neurons in Alzheimer's disease brain (22), where it may contribute to neurodegeneration, and in the trisomy 16 mouse model of Down syndrome, where it results in failure of BDNF-mediated neuron survival (20).

The *TRKB* gene can be transcribed from two promoters, P1 and P2 (23). Within the *TRKB* upstream sequence are multiple potential regulatory elements, including several  $Ca^{2+}/cAMP$ -response elements (*CREs*), suggesting that elevated  $Ca^{2+}$  and/or cAMP may regulate *TRKB* expression.

In this report, we demonstrate that depolarization alters the relative expression of full-length and truncated trkB receptors in cultured cortical neurons and identify  $Ca^{2+}$ -dependent regulatory elements in the *TRKB* promoters involved in this response.

### EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Cortical neurons were isolated from embryonic day-16 mouse embryos and plated at a density of  $6 \times 10^{5/}$ well in 24-well dishes for luciferase assays and  $3 \times 10^{6/}$ well in 35-mm dishes for RNA and protein analysis. Neuron cultures were maintained in Neurobasal medium supplemented with 2% B27, 2 mM glutamine, and penicillin-streptomycin and incubated in 5% CO<sub>2</sub> at 37 °C. Neurons were transiently transfected 3–5 days after plating using a Ca<sup>2+</sup>-phosphate protocol (24). TRKB luciferase reporters were introduced at a concentration of 1 µg/well; cells were cotransfected with 0.5 µg/well TK

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>TM</sup>/EBI Data Bank with accession number(s) AY307416.

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<sup>&</sup>lt;sup>1</sup> The abbreviations used are: BDNF, brain-derived neurotrophic factor; APV, DL(-)-2-amino-5-phosphonopentanoic acid; *CRE*, cyclic AMPresponse element; CREB, cyclic AMP-response element binding protein; DNQX, 6,7-dinitroquinoxaline-2,3-dione; trkB, tyrosine receptor kinase B; *TRKB*, gene coding for trkB; UTR, untranslated region; MOPS, 4-morpholinepropanesulfonic acid.

*Renilla* plasmid (Promega). All cell culture reagents were purchased from Invitrogen.

*Plasmids*—Luciferase reporter genes were generated by inserting *TRKB* promoter fragments upstream of the luciferase gene in pGL3Basic (Promega) as *Bgl*II-*Bam*HI fragments into the *Bgl*II site of the vector. Introduction of the *Bam*HI site to clone the P2 promoters into pGL3 Basic regenerates the AT of the ATG translation start codon, therefore placing the 3'-end of the luciferase constructs at +2. Multiple independent PCR promoter products were sequenced and found to contain differences from the previously published sequence (23), including a one-base deletion that shifts the transcription start site for P1 from -1800 to -1799. All but one base substitution could be independently verified from Trace sequences available at Ensembl. The sequence of the 2560 bp used in these studies is available at GenBank<sup>TM</sup>, accession number AY307416.

PCR primers used to generate promoter fragments were: upstream primers -2558, 5'-AAAGATCTCATCTATGTGAAAATCTTG-3'; -2258, 5'-AGATCTCGGTGGTAGCAATGGC-3'; -1429, 5'-AGATCTC-CTATGAGCATGGTGAG-3'; -944, 5'-AAAGATCTGGAGTTCTGCC-CC-3'; and 899, 5'-AGATCTGCCAGCAGTAGCAGAG-3'. Downstream primers were: -1710, 5'-AAGGATCCTAAATGCTTTGCACCGACC-3', and +2, 5'-AAGGATCCCGAGCTGCCAGTGCC-3'.

*CRE* site mutations were generated using site-directed mutagenesis. Forward primer sequences were: cre<sup>1-</sup>, 5'-TGGAGTTTCTGCC-CCTGCTCCACTGCAGCCCTCACGT-3'; cre<sup>2-</sup>, 5'-CCTGCTCTGCG-TCAGCCCCAGCTGCACTTCGCCAGCAGTAG-3'; and cre<sup>1-2-</sup>, 5'-TGGAGTTTCTGCCCCTGCTCCACTGCAGCCCCAGCTGCACTTCG-CCAGCAGTAG-3'. Dominant negative CREB plasmid was provided by Dr. Yibin Wang, University of Maryland School of Medicine.

Protein Analysis—Cells were harvested directly into boiling  $2 \times$  sample buffer and fractionated by SDS-PAGE on 4-12% gels run in MOPS buffer as previously described (20). Western blotting was conducted with ECL (Amersham Biosciences) using antibodies to the extracellular domain of trkB (BD Transduction Laboratories, Fig. 1A, or Santa Cruz Biotechnology, H-181, Fig. 1B) at 1:500, anti-phospho-trkA (Tyr-490; Cell Signaling Technologies) 1:500, and anti-actin (Sigma) 1:5000.

RNA Isolation and Analysis-Cells were stimulated for 5 h by addition of 50 mM KCl. Following stimulation, neurons were harvested and homogenized using Qiashredder (Qiagen), and RNA was isolated using RNeasy RNA extraction kit (Qiagen). RNA was quantified by absorbance at 260 nm, and 0.5  $\mu g$  was used for reverse transcription with Superscript II (Invitrogen) for 50 min at 42 °C. PCR was conducted in an Opticon real-time PCR cycler (MJ Designs) using Platinum Taq (Invitrogen), 0.2 mM dNTP, 30 µM/primer, and 3 mM MgCl<sub>2</sub>. Each PCR cycle consisted of 1 min at 94 °C, 30 s at 62 °C, followed by 1 min at 72 °C. Amplification was monitored using the fluorescent dye, Sybergreen I (Roche Applied Science), diluted 1:100,000. For real-time PCR analysis, each cDNA sample (untreated versus KCl-treated) was diluted 1:100 and assayed in duplicate at 1, .5, .25, and .125 $\times$  dilutions. RNA expression was computed from the slope of the  $C_{\rm T}$  versus ln [cDNA] relation and normalized to the concentration of  $\beta$ -actin as amplified using 5'-ATCGTGGGCCGCCCTAGGCA-3' and 5'-TGGCCTTAGGGT-TCAGAGGGG-3' (25). Full-length-specific primers were: 5'-GACAAT-GCACGCAAGGACTT-3' and 5'-AGTAGTCGGTGCTGTACACA-3'. T1specific primers were: 5'-ATAAGATCCCACTGGATGGG-3' and 5'-CGTATAGTCAAACAGCTCGC-3'. Data are reported as the normalized RNA concentration from KCl-stimulated neurons relative to that of unstimulated neurons.

Conventional RT-PCR to detect short 5'-UTRs of P1 and P2 was conducted using upstream primers in the unique 5'-UTR sequences (23) as follows: P1-1, 5'-AGGGTCGGTGCAAAGCATTT-3'; P1-2, 5'-TTAG-GGACCAAGGAAGCATC-3'; P1-3, 5'-AGTTTCTGCCCCTGGCTCTG-3'; or P2-4, 5'-AGCGCGGAGGGACTGTGT-3' with the common down-stream primer 5'-TCTTGCTGCTTGGTGCTGG-3'. The PCR amplification protocol consisted of 40 cycles of 1 min at 94 °C, 30 s at 60 °C, followed by 30 s at 72 °C.

Luciferase Assays—Two days after transfection with *TRKB* luciferase reporter constructs, cells were stimulated for 6 h by the addition of 50 mM KCl in the absence or presence of 2 mM EGTA. EGTA was added 5 min prior to KCl stimulation. Cells were washed with phosphatebuffered saline and harvested in 150  $\mu$ l of *Renilla* luciferase lysis buffer (Promega). Twenty  $\mu$ l of extract were used to measure *TRKB* reporters (luciferase assay reagent; Promega) and *Renilla* luciferase activity (*Renilla* luciferase assay system; Promega). *TRKB* luciferase activity was then normalized by dividing by *Renilla* activity to allow comparison among wells and stimulation conditions. Cells transfected with luciferase vector lacking the *TRKB* promoter exhibited less than 3% of the activity of  $T\!R\!K\!B$  -luciferase-transfected neurons, and this activity was unaffected by depolarization.

## RESULTS

Depolarization Increases the Level of Full-length trkB in Cortical Neurons-Because of the importance of BDNF/trkB signaling in activity-dependent changes in neurons, we investigated the ability of depolarization to regulate trkB expression. Embryonic mouse cortical neurons grown in culture for 5-7 days were depolarized with 50 mM added KCl to induce Ca<sup>2+</sup> influx. Cells were then harvested for Western blot analysis. As previously reported for hippocampal neurons (20), cortical neurons expressed primarily full-length trkB (Fig. 1). In the presence of 50 mM added KCl, the level of full-length trkB protein was elevated by 5 h and continued to increase up to 16 h, the longest time studied (Fig. 1A). The low level of truncated trkB did not change following KCl treatment. Depolarization also increased the amount of phospho-trkB observed in response to BDNF stimulation (Fig. 1B), demonstrating that the additional full-length trkB was functional.

Depolarization Increases the Level of mRNA Encoding Fulllength trkB in Cortical Neurons—In light of the effects of depolarization on full-length trkB expression, real-time PCR was conducted to determine the effect of depolarization on TRKB RNA expression. Following reverse transcription of total RNA, TRKB transcripts were analyzed using primer pairs specific for either full-length or truncated T1 trkB isoforms (Fig. 2A) (25). Five hours of stimulation with elevated KCl produced an  $\sim$ 3fold increase in full-length trkB transcripts, whereas there was no increase in T1 transcripts (Fig. 2B). The increase in fulllength transcripts was sensitive to EGTA (data not shown).

 $Ca^{2+}$  Regulates TRKB Expression—The presence of three CRE sites in the TRKB promoter region (23) suggested that  $Ca^{2+}$  and/or cAMP can modulate TRKB.  $Ca^{2+}$ -dependent TRKB transcription was investigated using a TRKB-luciferase reporter gene. Approximately 2.5 kb of the TRKB promoter region, including both P1 and P2, was cloned upstream of the luciferase gene and transiently transfected into cortical neurons using a  $Ca^{2+}$ -phosphate method (24). Depolarization of the neurons by the addition of 50 mM KCl resulted in a 2-fold increase in TRKB-dependent transcription as measured by luciferase activity (Fig. 3A). The presence of EGTA eliminated the response to KCl, indicating that  $Ca^{2+}$  influx was required to stimulate TRKB-luciferase expression.

To determine the pathway of  $Ca^{2+}$  entry in response to depolarization, neurons transfected with the -2558/+2 luciferase plasmid were stimulated with KCl in the absence and presence of  $Ca^{2+}$  channel or glutamate receptor (GluR) block-



FIG. 1. Depolarization induces full-length trkB protein expression. A, cortical neurons were incubated in medium supplemented with 50 mM KCl for 5 or 16 h as indicated and harvested for Western blot analysis using antibodies to the extracellular domain of trkB or to actin. (-) indicates neurons incubated in normal medium for 16 h. Depolarization increased full-length (*trkB.FL*) but not truncated (*trkB.TR*) trkB expression. The exposure was optimized to show the increase in fulllength trkB. *B*, cortical neurons were maintained in either normal or elevated KCl for 16 h and then exposed to 50 ng/ml BDNF for 10 min. Western blotting was carried out using anti-phospho-trkA, which recognizes phosphorylated Tyr-515 in trkB. The level of BDNF-stimulated phospho-trkB (*P-trkB*) was enhanced by exposure to elevated KCl, demonstrating that the additional full-length trkB induced by depolarization was functionally active.



FIG. 2. Depolarization induces full-length trkB mRNA expression. A, diagram illustrating the location of isoform-specific primers (arrows) for full-length and truncated (T1) trkB mRNAs. Both isoforms share a common extracellular BDNF binding domain and transmembrane domain (TM) but have different intracellular domains and their mRNAs have distinct 5' and 3'-UTRs. B, cortical neurons were incubated for 5 h in the absence or presence of 50 mM added KCl. Levels of trkB RNA were quantified using real-time PCR as described under "Experimental Procedures" using primer pairs specific for RNA encoding either full-length (trkB.FL) or T1 truncated (trkB.TR) receptors (arrows in panel A). Data shown are mean  $\pm$  S.E. (n = 4 experiments) mRNA levels in the presence of elevated KCl relative to unstimulated control.

ers (Fig. 3*B*). The L-type Ca<sup>2+</sup> channel blocker, nifedipine (100  $\mu$ M), completely blocked depolarization-induced activation of *TRKB* luciferase activity, whereas the combination of an NMDA GluR blocker (APV, 80  $\mu$ M) and a kainate/AMPA GluR blocker (DNQX, 20  $\mu$ M) had no effect. Control luciferase activity was not affected by EGTA, nifedipine, or APV/DNQX. Thus, activation of *TRKB* results from depolarization-induced entry of Ca<sup>2+</sup> through L-type, voltage-gated Ca<sup>2+</sup> channels and does not require GluR activation.

P1 and P2 TRKB Promoters Are Differentially Regulated by  $Ca^{2+}$ —Because the upstream regulatory region of TRKB has been shown to contain two promoters, the ability of  $Ca^{2+}$  to separately activate P1 and P2 was tested using TRKB-luciferase reporter genes containing either P1 or P2 (Fig. 4A). Two constructs, -2558/-1710 and -2258/-1710 were generated to test the effects of Ca<sup>2+</sup> on P1. The shorter construct lacked a potential  $Ca^{2+}$ -dependent regulatory site (CRE) located at -2480 within the P1 domain of TRKB. In contrast to the -2558/+2 construct, which contains both P1 and P2 (Fig. 3), the luciferase activity of the P1 constructs was reduced by ~50% following 6 h of depolarization (Fig. 4B). Inclusion of EGTA abolished the inhibition, consistent with a requirement for  $Ca^{2+}$  influx in the inhibition of P1-dependent transcription. The finding that  $Ca^{2+}$  inhibited the -2558/-1710 and -2258/-1710 constructs to the same extent indicates that the *CRE* in P1 is not required for inhibition.

The regulation of P2 was investigated using three constructs, -1429/+2, -944/+2, and -899/+2, which were transiently transfected into cortical neurons. In contrast to the P1 constructs, expression of P2 luciferase constructs was stimulated by KCl depolarization. Depolarization caused  $\sim$ 3- and 2-fold increases in activity of the -1429/+2 and -944/+2 luciferase reporter constructs, respectively (Fig. 4*C*). Increased P2 activity was blocked by extracellular EGTA, demonstrating the re-



FIG. 3.  $Ca^{2+}$  entry stimulates *TRKB* transcription. *A*, cortical neurons transiently transfected with -2558/+2 *TRKB*-luciferase plasmid were stimulated for 6 h with elevated KCl in the absence or presence of 2 mM extracellular EGTA. Luciferase activity was assayed as described under "Experimental Procedures" and is reported relative to unstimulated activity. *B*, transfected neurons were stimulated as in *panel A* in the absence or presence of 100  $\mu$ M nifedipine or 80  $\mu$ M APV plus 20  $\mu$ M DNQX. Data shown are means  $\pm$  S.E. (n = 4). The same results were obtained in five (*A*) or two (*B*) experiments.

quirement for Ca<sup>2+</sup> influx to stimulate P2 *TRKB* expression. Expression of the -899/+2 *TRKB* P2-luciferase construct was not stimulated by depolarization, indicating that promoter elements between -944 and -899, possibly the tandem *CRE* sites, are required for the Ca<sup>2+</sup> dependence of *TRKB* expression (see below). The larger degree of Ca<sup>2+</sup>-dependent stimulation observed with the -1429/+2 *TRKB*-luciferase construct as compared with the -944/+2 construct revealed the presence of an additional regulatory element(s) between -1429 and -944 capable of enhancing Ca<sup>2+</sup>-induced *TRKB* expression.

Both P1- and P2-derived Transcripts Are Present in Cortical Neurons—The transcriptional activity of endogenous TRKB was investigated using RT-PCR analysis of P1- and P2-specific 5'-UTR sequences (23). Alternative splicing of the P1-derived transcript produces three potential 5'-UTRs (Fig. 4D, lanes 1-3), whereas P2 generates a single 5'-UTR (Fig. 4D, lane 4). PCR products corresponding to both P1- and P2-derived transcripts were readily detectable, indicating that TRKB is transcribed from both promoters in mouse embryonic cortical neurons.

Both CRE Sites Are Required for  $Ca^{2+}$ -stimulated Expression of TRKB P2—The sequence between -944 and -899 contains a pair of CRE sites separated by 4 bp (Fig. 5A). CRE sites are binding sites for members of the CREB family of transcription factors (26), which mediate  $Ca^{2+}$ -dependent expression of a wide variety of genes, including BDNF. The role of these tandem CRE sites in mediating P2-initiated TRKB expression following depolarization was examined by introducing substitution mutations (Fig. 5A). Mutation of either CRE site or both in the -1429/+2 TRKB-luciferase construct completely blocked stimulation of luciferase activity (Fig. 5B).  $Ca^{2+}$ -stimulated expression of TRKB, therefore, requires both CRE sites. Mutation of either CRE site in the -944/+2 TRKB-luciferase construct similarly abolished depolarization-stimulated expression (data not shown).

Mutation analysis of the *CRE* sites suggested that CREB, or a closely related family member, is responsible for mediating the Ca<sup>2+</sup>-dependent induction of *TRKB* expression. Cotransfection of a dominant negative CREB construct together with the -944/+2 *TRKB*-luciferase construct inhibited the Ca<sup>2+</sup>-stimulated expression of the *TRKB* reporter (Fig. 5*C*), consistent with a role for a CREB-related transcription factor in the induction of *TRKB* P2 by Ca<sup>2+</sup>.

cAMP Stimulates TRKB Gene Expression—Because CREB is



FIG. 4.  $Ca^{2+}$  differentially regulates P1 and P2. A, schematic of *TRKB* promoters and corresponding *TRKB* reporter constructs. *CRE* sites are indicated by *hatching*. Transcription start sites for P1 (-1799) and P2 (-448) are indicated. *B* and *C*, cortical neurons transiently transfected with P1 (*B*) or P2 (*C*) *TRKB*-luciferase reporters were stimulated for 6 h with 50 mM added KCl in the absence or presence of EGTA. Luciferase activity was assayed as described under "Experimental Procedures" and is reported relative to unstimulated activity. Data shown are mean luciferase  $\pm$  S.E. (n = 4). Similar results were obtained in at least three experiments. *D*, RT-PCR analysis of cultured cortical neurons to detect P1- and P2-specific 5'-UTR sequences. *Reactions 1-3* selectively amplify the three P1 5'-UTR splice variants. *Reaction 4* detects the P2-specific 5'-UTR.

also activated in response to cAMP, we investigated the ability of cAMP to activate the *TRKB* P2 promoter. Cortical neurons transfected with either the -1429/+2 or -944/+2 *TRKB*-luciferase reporter gene were treated with 50  $\mu$ M forskolin, an adenylate cyclase activator, for 6 h in either the absence or presence of 50 mM added KCl. Forskolin stimulated the luciferase activity of each *TRKB* reporter ~2-fold, demonstrating that P2 can be stimulated by cAMP signaling (Fig. 6). The effects of depolarization-induced Ca<sup>2+</sup> signaling and increased cAMP were additive for both -944/+2 and -1429/+2 P2-dependent reporter genes when the neurons were simultaneously treated with elevated KCl and forskolin.

#### DISCUSSION

Depolarization resulted in increased expression of fulllength trkB protein and increased phosphorylation of trkB upon BDNF stimulation (Fig. 1). Depolarization also preferentially increased the level of endogenous full-length trkB mRNA without significantly affecting the level of truncated trkB message (Fig. 2B). These results led us to investigate the role of  $Ca^{2+}$  in the transcriptional regulation of *TRKB*. We show here that the two promoters of *TRKB* are differentially regulated by  $Ca^{2+}$ . P1 reporter constructs were inhibited by  $Ca^{2+}$ , whereas P2 reporters were stimulated by  $Ca^{2+}$  (Fig. 4, B and C). Although the quantitative contribution of P1- and P2-derived transcripts to specific trkB isoform expression has not yet been



FIG. 5. Tandem *CRE* sites are required for  $Ca^{2+}$ -stimulated P2 expression. *A*, sequence of the tandem *CRE* elements in P2 with corresponding point mutations used to inactivate them. *B*, cortical neurons transiently transfected with the -1429/+2 *TRKB*-luciferase reporter containing either wild type or mutated *CRE* sites were stimulated for 6 h in 50 mM added KCl in the absence or presence of 2 mM EGTA. Luciferase activity was assayed as described under "Experimental Procedures" and is plotted relative to unstimulated activity. *C*, cortical neurons cotransfected with either dominant negative (*DN*) *CREB* or control vector were stimulated and assayed as in *panel B*. Data shown are means  $\pm$  S.E. (*n* = 4). Similar results were obtained in at least two additional experiments.

established, these observations suggest that  $Ca^{2+}$ -regulated *TRKB* promoter selection can alter the relative expression levels of trkB isoforms and consequently modulate cellular BDNF responsiveness.

The stimulation of *BDNF* expression by  $Ca^{2+}$  has been extensively studied (4–6).  $Ca^{2+}$ -dependent activation of *BDNF* promoters I and III is mediated by single *CRE* sites acting in concert with additional  $Ca^{2+}$ -dependent regulatory sites. We show here that *TRKB* P2 contains a pair of *CREs*, separated by 4 bp, both of which are required for  $Ca^{2+}$ -dependent expression of *TRKB* (Fig. 5*B*) via the activation of CREB or a related transcription factor (Fig. 5*C*). Because single *CRE* sites are sufficient to mediate  $Ca^{2+}$ -dependent transcription in genes such as *BDNF*, it is unclear why both *CREs* in *TRKB* are necessary. Interestingly, a similar requirement for two tandem *CRE* sites has been previously reported in the *nNOS* promoter (27). In addition to the *CRE* sites, our data indicate that an additional upstream element located between -944 and -1429 functions to promote the  $Ca^{2+}$ -stimulated expression of P2



FIG. 6. Cyclic AMP stimulation of TRKB expression. Cortical neurons transiently transfected with -1429/+2 TRKB-luciferase construct were stimulated with elevated KCl and/or 50 µM forskolin as indicated. Luciferase activity was assaved as described under "Experimental Procedures" and is plotted relative to unstimulated activity. Data shown are mean luciferase  $\pm$  S.E. (n = 4).

(Fig. 4C). Because no  $Ca^{2+}$ -stimulated TRKB expression was observed when either of the tandem CRE sites was mutated in the -1429/+2 TRKB luciferase construct (Fig. 5B), this upstream element cannot function independently of the CRE sites to promote *TRKB* expression.

In contrast to BDNF promoter III, which can be induced by  $Ca^{2+}$  but not by cAMP (28), the P2 promoter of *TRKB* can be activated by either  $Ca^{2+}$  or cAMP signaling (Fig. 6). Because cAMP signaling has also been reported to trigger the recruitment of trkB to the cell surface (29), an increase in cAMP would be expected to lead to more trkB in the plasma membrane due to both increased trkB synthesis and membrane insertion, even in the absence of a rise in  $[Ca^{2+}]$ . The finding that the -1429/+2 and -944/+2 TRKB-luciferase reporters are activated to the same extent by forskolin alone (Fig. 6) shows that the upstream  $Ca^{2+}$ -response element (present only in -1429/+2) is specific for Ca<sup>2+</sup> and does not affect activation of P2 by cAMP. Comparison of -1429/+2 and -944/+2 TRKB-luciferase reporter activity in neurons treated with both KCl and forskolin indicates that cAMP activation of TRKB does not occlude the ability of KCl-induced Ca<sup>2+</sup> signaling to enhance P2 activation via additional upstream sequences, because Ca<sup>2+</sup> activates the -1429/+2 P2 construct to a greater extent than the -944/+2 construct even in the presence of forskolin (Fig. 6).

Neuronal activity, via increased Ca<sup>2+</sup>, has previously been proposed to modulate BDNF/trkB signaling by several mechanisms: (i) The synthesis of BDNF is stimulated by neuronal depolarization via Ca<sup>2+</sup>-dependent regulatory elements in the BDNF promoter (4-6); (ii) Ca<sup>2+</sup> triggers the release of BDNF from neurons (7–9); (iii)  $Ca^{2+}$  induces the trafficking of trkB to the neuronal plasma membrane (30); and (iv) depolarization alters the localization of trkB mRNA within neurons in a Ca<sup>2+</sup>dependent manner, with more trkB message appearing in the neurites of depolarized neurons (31, 32). The results presented here add another level of regulation to this list: Depolarization selectively increases the expression of catalytically active, fulllength trkB via activation of Ca<sup>2+</sup>-responsive elements in the TRKB promoter.

Based on these findings, we propose that the coordination of ligand and receptor expression by Ca<sup>2+</sup> can regulate efficacy of the BDNF/trkB signaling system in those neurons that receive appropriate levels of excitatory stimulation. Activity-dependent regulation of cellular responsiveness to BDNF via Ca<sup>2+</sup>-dependent control of trkB expression could be a novel mechanism by which neuronal activity can modulate not only cell survival but also synaptic plasticity and may play important roles in the pathological as well as the normal nervous system.

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