Brain-derived Neurotrophic Factor Increases Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase 2 Activity in Hippocampus*  

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Here we show that brain-derived neurotrophic factor (BDNF) stimulates both the phosphorylation of the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase 2 (CaMK2) and its kinase activity in rat hippocampal slices. In addition, we find that: (i) the time course of BDNF action is not accompanied by a change in the spectrum of either α- and β-subunits of CaMK2 detected by immunoblotting; (ii) both treatment of solubilized CaMK2 with alkaline phosphatase and treatment of immunoprecipitated CaMK2 with protein phosphatase 1 reverse phosphorylation and activation of the kinase; (iii) phospholipase C inhibitor D609 and intracellular Ca\textsuperscript{2+} chelation by 1,2-bis-(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetra(acetoxymethyl)ester or 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate but not omission of Ca\textsuperscript{2+} or Ca\textsuperscript{2+} chelation by EGTA, abolish the stimulatory effect of BDNF on phosphorylation and activation of CaMK2. These results strongly suggest that the conversion of CaMK2 into its active, autophosphorylated form, but not its concentration, is increased by BDNF via stimulation of phospholipase C and subsequent intracellular Ca\textsuperscript{2+} mobilization.

Ca\textsuperscript{2+}/calmodulin-dependent protein kinase 2 (CaMK2) is a major neuronal mediator of calcium signaling that integrates multiple related functions. It has been implicated in a variety of events in neurons, ranging from the neurotransmitter synthesis and release, modulation of neurotransmitter receptors and ionic channels, and gene expression to several aspects of synaptic plasticity such as the prevailing model for memory called long term potentiation as well as spatial learning (1–4). The distinctive property of CaMK2 is that the Ca\textsuperscript{2+}/calmodulin-activated enzyme rapidly autophosphorylates, thereby generating a constitutively active, phosphorylated Ca\textsuperscript{2+}-independent kinase that phosphorylates multiple substrates at presynaptic and postsynaptic sites. Studies have shown that certain neurotransmitters are of critical importance in the modulation of this activation reaction (2). However, whether activation of CaMK2 can be due to other signaling factors still remains unclear.

In addition to the characteristic trophic and phenotypic effects of trophic factors, it has been recently shown that neurotrophins strongly enhance synaptic efficacy. For example, brain-derived neurotrophic factor (BDNF) induces an increase of synaptic transmission in rat hippocampal cultures and slices (5, 6). Furthermore, consistent with BDNF knockout studies (7), a critical level of BDNF activity was found to be important for long term potentiation in hippocampus (8). However, little is known about how the neurotrophins might modulate synaptic plasticity. It is therefore of great interest to examine whether CaMK2 might be also capable of orchestrating these trophic responses. Here, we have investigated the effects of BDNF on the Ca\textsuperscript{2+}-dependent CaMK2 activity in hippocampal slices of adult rat.

EXPERIMENTAL PROCEDURES

Materials and Reagents—The materials and reagents were obtained as follows: [γ-\textsuperscript{32}P]ATP and ECL Western blotting analysis system, Amersham International; KN-62, D609, U73122, A20187, and calyculin A, LC Laboratories; protein phosphatase 1, Boehringer Mannheim; autocomtide-2 and monoclonal antibody (IgG1) to phosphothreonine, Biomol Research Laboratories, Inc.; BDNF, Alomone Laboratories, Ltd; polyclonal antibodies specific for the α- and β-subunits of CaMK2 (anti-CaMK2a and anti-CaMK2b), Santa Cruz Biotech. Inc; normal mouse IgG, Caltag Laboratories; Mini-Protean 2 Electrophoresis and Trans-blot semi-dry transfer cells, Bio-Rad Laboratories; and P-81 phosphocellulose filters, Whatman International, Ltd. All other chemicals were purchased from Sigma.

Slice and Homogenate Preparation—Transverse hippocampal slices (500 μm) were prepared from adult male CD rats (200–250 g) and submerged in 1 ml of continuously oxygenated (95% O\textsubscript{2}/5% CO\textsubscript{2}) Ringer’s solution at 30 °C for 1 h. For each tested point, five slices were removed and incubated at 30 °C for the indicated times into a glass tube containing 1 ml of continuously oxygenated Ringer’s solution and test drugs. Afterward, slices were homogenized in 1 ml of ice-cold homogenization buffer by 20 strokes of a hand-held glass Telfon homogenizer, and homogenates were stirred for 1 h at 4 °C and then centrifuged two times at 12,000 × g for 15 min to remove nonsolubilized material. The homogenization buffer consisted of 50 mM Tris-HCl, pH 7.3, 1 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 50 mM β-glycerophosphate, 25 mM NaF, 15 mM Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 20 μg/ml aprotinin, and 100 μg/ml benzidine. Protein content of homogenates was determined as described in Ref. 9. When analyzing the effects of insolubilized alkaline phosphatase (APP) on CaMK2, cytosolic extracts were prepared exactly as described above, except that they were dialyzed against 50 mM Tris-HCl, 1 mM Mn\textsubscript{2}O\textsubscript{4}, pH 7.3, and were then adjusted to pH 8.

Dephosphorylation of CaMK2—CaMK2 was treated with insolubilized APP in 200 μl of cytosolic extracts as described (10), and APP was then removed by centrifugation prior to assays for CaMK2 activity. When CaMK2 was treated with protein phosphatase (PP1) in an immunocomplex, fractions (500 μl) of homogenates were preclayed by adding 0.6 μg of normal mouse IgG together with 13 μl of protein A-agarose and were then incubated at 4 °C for 1 h with anti-CaMK2b (1:100 dilution). Protein A-agarose (20 μl) was added to samples, and the mixtures were rocked at 4 °C for 1 h. After that, the immunoprecipitates were washed three times with phosphatase buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 mM NaCl) and were incubated for 2 h at 30 °C in 50 μl of phosphatase buffer containing PP1 (10 milliunits), 0.1% β-mercaptoethanol and 1 mM MnCl\textsubscript{2}. Finally, the immunoprecipitates were washed twice with kinase buffer (10 mM Heps, pH 7.4, 5 mM MgCl\textsubscript{2}) and resuspended in 20 μl of this buffer prior to assays for CaMK2 activity.

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Assays for CaMK2 Activity—Phosphorylation reactions were initiated by adding 20 μl (about 40 μg) of solubilized homogenate or 20 μl of resuspended CaMK2 immunoprecipitate to 25 μl of kinase buffer containing 2 mM CaCl2, 1 μM calmodulin (CaM), 20 μM ATP, 4 μCi of [γ-32P]ATP (3,000 Ci/mmol), and autocamtide-2 at 40 μg/ml. To monitor Ca2+-independent activity of CaMK2, parallel tests were performed in the absence of Ca2+ and calmodulin and in the presence of 2 mM EGTA. All reactions were carried out for 3 min at 30 °C. Phosphorylation was then stopped either by spotting 50 μl of soluble mixtures on P-51 phosphocellulose filters or by sedimenting the immunocomplexes by centrifugation prior to addition of 50 μl of supernatants to filters. Filters were washed, and radioactivity was determined as described in Ref. 11. Each sample was analyzed in quadruplicate, averaged, and normalized to the control fractions. Differences between means of paired comparisons were evaluated by Student’s t test. A p value of < 0.05 was considered significant.

Western Analysis—CaMK2 was immunoprecipitated using anti-CaMK2α and anti-CaMK2β (1:100 dilution) as described above. The immobilized immunocomplexes were washed three times in buffered detergent solution (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride) and treated with SDS sample buffer as described in Ref. 9. Supernatants were then run on 10% SDS-polyacrylamide minigels and transferred to nitrocellulose membrane using Mini-Protean 2 Electrophoresis and Trans-blot semi-dry transfer cells. Finally, filters were incubated at 4 °C overnight in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 2% bovine serum albumin, followed by incubation with primary antibody in TBS-T/4% bovine serum albumin for 2 h at room temperature, using anti-CaMK2α, anti-CaMK2β (1:1000 dilution), or the anti-phosphothreonine antibody (1:500 dilution). Filters were then washed six times for 5 min each in TBS-T, incubated for 1 h with anti-mouse IgG conjugated to peroxidase (1:1000 dilution) in TBS-T/4% bovine serum albumin, and washed again three times for 6 min each in TBS-T. Immunoblotted bands were detected using the ECL system. Autoradiograms were scanned with a BIOCOM COMPAQ computer.

RESULTS AND DISCUSSION

In the basal state, the average Ca2+/CaM-dependent CaMK2 activity with autocamtide-2 detected was 3.5 ± 1.2 nmol/mg/min. The average Ca2+/CaM-independent activity from the same control samples was 0.19 ± 0.07 nmol/mg/min. The ratio of Ca2+/CaM-independent activity to Ca2+/CaM-dependent activity in the basal state was about 5.5%, which is consistent with data reported by others (12, 13). Interestingly, treatment of slices with 50 ng/ml BDNF resulted in a 4-fold increase in the Ca2+/CaM-independent activity of CaMK2 (Fig. 1A). This increase in activity was transient, being maximal within about 4 h and slowly decreasing by 6 h. In contrast, no significant stimulation in CaMK2 activity was observed when slices were treated with BDNF in the presence of KN62, a drug designed to inhibit autophosphorylation of CaMK2 (Fig. 1A). Because BDNF rapidly activates its signaling pathways in hippocampal neurons through activation of the trkB receptor (14), the time of BDNF incubations (1–4 h) of slices necessary to enhance the activity level of CaMK2 left open the question of why activation of BDNF incubations (1–4 h) was necessary to enhance the activity level of CaMK2. To verify this hypothesis, an immunoblot procedure was employed to monitor the level of CaMK2 protein over a time course of BDNF action. CaMK2 was immunoprecipitated from extracts using anti-CaMK2α and anti-CaMK2β, and after separation on SDS-PAGE and transfer of proteins to nitrocellulose, blots were probed with the anti-CaMK2 antibodies. Immunoblot analysis revealed that the migrations of α- and β-subunits are not substantially altered over this time course (Fig. 1B). For example, quantification of immunoband intensities indicated that immunoreactivity of α- and β-subunits at 4 h post-BDNF treatment was 95 ± 10.9 and 109 ± 12.7% of controls, respectively (values obtained from three experiments). In fact, recent studies have emphasized that immunohistochemical labeling for BDNF is only detectable in hippocampal slices after about 1 h and continues to increase gradually over several hours (7). It seems likely, therefore, that the time observed here for the CaMK2 activation results from the time required for the penetration of BDNF into slices.

It is clearly established that formation of the active Ca2+/CaM-independent form of CaMK2 is dependent on autophosphorylation of Thr-286/287 and Thr-308 residues of the enzyme (α/β subunits, respectively) (15). To address whether CaM-sensitive CaMK2 activation in response to BDNF also involved an autophosphorylation event, cytosolic extracts were treated with insolubilized APP. Following the removal of APP, extracts were then assayed for CaMK2 activity. As shown in Fig. 2A, treatment with APP of extracts from BDNF-treated slices greatly depressed the CaMK2 activity. In contrast, no significant effect of phosphatase was observed when the APP inhibitors orlovamidate and EDTA were present. These results strongly suggested that activation of CaMK2 by BDNF resulted from an autophosphorylation of the kinase. The relative levels of observed activities, however, could not be completely valid in crude extracts because autophosphatase 2 may not be necessarily entirely specific for CaMK2 as a kinase substrate. To ensure that the level of CaMK2 activity strictly correlated with the level of Thr phosphorylation of the kinase, we performed control experiments in which both Thr phosphorylation and activ-
ity of CaMK2 were analyzed following treatment with PP1 of CaMK2 immunoprecipitates. To this end, Thr phosphorylation was evaluated by Western blotting, and activity was measured in an immunocomplex kinase assay. In accordance with our initial experiments, Thr phosphorylation was dramatically reduced and CaMK2 activity was substantially depressed in BDNF-stimulated extracts treated only with the phosphatase (Fig. 2, B and C). However, both phosphorylation and activity of CaMK2 were not significantly decreased when the extracts from BDNF-treated slices were incubated with PP1 in the presence of PP1 inhibitors okadaic acid or calyculin A (Fig. 2, B and C).

Phospholipase C (PLC) was shown to bind specific phosphotyrosines in the trk receptors (16), but it was still unclear

![Fig. 2](image2.png)

**Fig. 2.** Effects of protein phosphatases on the activity of BDNF-induced activity and phosphorylation of CaMK2. A, slices were incubated for 3 h in Ringer’s solution with (+) or without (−) 50 ng/ml BDNF. Cytosolic extracts were incubated with (+) or without (−) insolubilized APP beads for 30 min at 25 °C, in the presence (+) or the absence (−) of APP inhibitors orthovanadate (Orthov; 50 μM) and EDTA (2.5 mM). Phosphatase beads were then removed by centrifugation, and Ca2+–independent CaMK2 activity of extracts was calculated as in Fig. 1A. Data are the means ± S.E. of three independent experiments in quadruplicate. B, slices were treated (+) or not (−) with BDNF as described for A. Clarified extracts were incubated with anti-CaMK2β, and immunoprecipitates were incubated with (+) or without (−) PP1 and the PP1 inhibitors okadaic acid (OA; 1 μM) and calyculin A (Caly. A; 100 nm) as indicated. The washed immunoprecipitates were then assayed for Ca2+–independent CaMK2 activity. Data are the means ± S.E. of three independent experiments in quadruplicate. C, extracts from control and BDNF-treated slices were immunoprecipitated with anti-CaMK2β, and immunoprecipitates were treated as described for B. The same amount of proteins were then submitted to SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphothreonine. Thr phosphorylation of CaMK2β was quantified and expressed as the mean ± S.E. of five independent experiments. The data are presented as phosphorylation relative to levels measured in control slices.

![Fig. 3](image3.png)

**Fig. 3.** Effects of impairment of Ca2+ signaling on BDNF-induced activity and phosphorylation of CaMK2. A, slices were incubated for 2 h in Ringer’s solution with (+) or without (−) 50 ng/ml BDNF. As indicated, BDNF-unstreated and BDNF-treated slices were submitted to EGTA (5 mM) or to intracellular Ca2+ chelators BAPTA-AM (10 μM) and TMB-8 (100 μM) or were incubated in the absence of Ca2+. These drugs were added after a preincubation period of 1 h and were tested for 30 min. In some cases, control slices were also treated with the Ca2+ isophore A23187 (1 μM) for 1 h. Slice extracts were then assayed for Ca2+–independent CaMK2 activity as in Fig. 1A. Results are the means ± S.E. of three independent experiments in quadruplicate. B, slices were treated (+) or not (−) with BDNF as described for A. CaMK2β was then immunoprecipitated with anti-CaMK2β, and the immunoprecipitates were dephosphorylated and analyzed by Western blotting as described in Fig. 2C. Thr phosphorylation of CaMK2β was quantified and expressed as the means ± S.E. of five independent experiments. The data are presented as phosphorylation relative to levels measured in control slices.
whether this docking signal enzyme participated in signaling pathways of BDNF (14). Of particular interest, therefore, was our observation that the selective PLC inhibitor D609 completely suppressed BDNF-induced CaMK2 activation (Fig. 1A). A similar result was observed with the specific PLC inhibitor U73122 (5 μM) (data not shown). These results strongly suggested that BDNF-induced CaMK2 activation was dependent of PLC in hippocampus. Stimulation of PLC leads to the generation of inositol trisphosphate, which is involved in Ca²⁺ mobilization from inositol trisphosphate-sensitive stores. Furthermore, Ca²⁺ channels in hippocampal neurons are modulated by protein kinase C (17) and thereby might be related to PLC activation via the production of diacylglycerol. Because cytoplasmic elevation of Ca²⁺ level by BDNF in hippocampus has been described (18), we were interested in whether the activation of CaMK2 by BDNF was mediated by the release of Ca²⁺ from the cytosolic stores or by the influx of Ca²⁺. After pre-exposure with BDNF, treatment of slices with BDNF in the presence of BAPTA-AM or TMB-8, two drugs commonly used as intracellular Ca²⁺ chelators, resulted in a dramatic decrease of BDNF-induced CaMK2 activity (Fig. 3A). In contrast, omission of extracellular Ca²⁺ or Ca²⁺ chelation by EGTA failed to depress this activity. In addition, treatment with the Ca²⁺ ionophore A23187 resulted in a marked stimulation of CaMK2 activity (Fig. 3A). To determine how CaMK2 was inductively phosphorylated on Thr sites after incubation of slices with the agents described above, CaMK2β immunoprecipitates were transferred to nitrocellulose filters and probed with the anti-phosphothreonine antibody. As shown in Fig. 3B, Thr phosphorylation of CaMK2 was increased after BDNF treatment and did not appear to return toward the basal level in the presence of EGTA. In contrast, the effect of BDNF on Thr phosphorylation was abolished by D609 (data not shown) and markedly attenuated by BAPTA-AM (Fig. 3B). Collectively, therefore, the present results strongly suggest that the PLC-dependent release of Ca²⁺ from intracellular inositol trisphosphate-sensitive stores, rather than Ca²⁺ influx through Ca²⁺ channels, acts as a trigger for the Thr phosphorylation and the subsequent activation of CaMK2 in response to BDNF.

Although the available evidence indicates an incomplete overlap between neurons producing BDNF, trkB, and CaMK2, these proteins appear to be synthesized primarily by granule and pyramidal neurons of rat hippocampus, i.e. the principal hippocampal cells (1, 19). Given that CaMK2, trkB, and the internal Ca²⁺ stores are found to be predominantly concentrated in the postsynaptic densities of rat neurons, it is tempting to speculate that the signaling pathway described here most likely occurs mainly in postsynaptic structures of principal hippocampal neurons (20–22). Recently, several studies reported that Ca²⁺-induced activation of CaMK2 evoked via synaptic activity may be involved in controlling the transcriptional activation of BDNF genes (23). Therefore, the crucial question to answer now is whether such a de novo synthesis of BDNF may potentiate activation of CaMK2 and thereby lead to self-controlled consolidation of synaptic plasticity in the hippocampus.

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