THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 7, pp. 4133–4144, February 15, 2008

Neuroprotection by Neurotrophic Factors and Membrane Depolarization Is Regulated by Calmodulin Kinase IV*

Received for publication, July 5, 2007, and in revised form, December 11, 2007 Published, JBC Papers in Press, December 13, 2007, DOI 10.1074/jbc.M705477200

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Neurotrophic factors promote motoneuron (MN) survival through increased intracellular calcium (Ca^{2+}) and regulation of the phosphatidylinositol (PI) 3-kinase/protein kinase B (PKB) pathway by calmodulin (CaM). Activation of the PI 3-kinase/PKB pathway is one of the well established mechanisms involved in MN survival. The Ca^{2+}/CaM complex interacts with and modulates the functionality of a large number of proteins, including serine/threonine protein kinases such as Ca^{2+}/CaM-dependent protein kinases (CaMKs). Using a primary culture of embryonic chicken spinal cord MNs, we investigated the role of CaMKIV in mediating this process. We cloned chicken CaMKIV and demonstrated its expression in purified MNs by means of reverse transcription-PCR, Western blot, and immunofluorescence. Using RNA interference, we show that endogenous CaMKIV mediates cell survival induced by neurotrophic factors or membrane depolarization. The survival effect is independent of CaMKIV kinase activity; however, CaMKIV functionality depends on the presence of Ca^{2+}/CaM. Finally, CaMKIV associates to the p85 subunit of PI 3-kinase in a Ca^{2+}-dependent manner, suggesting a role in regulating PI 3-kinase/PKB activation.

Neurotrophic factors and membrane depolarization promote neuronal survival through the activation of intracellular pathways. Both mechanisms induce a moderate increase in intracellular calcium (Ca^{2+}) concentration; that is, (*a*) neurotrophic factors through intracellular Ca^{2+} mobilization (1, 2) and (*b*) membrane depolarization through Ca^{2+} influx from the extracellular space (3, 4). The intracellular Ca^{2+} increase is detected by the ubiquitous calcium-sensing protein, calmodulin (CaM).[7] CaM becomes activated and mediates some intracellular events related to survival pathways, such as activating the phosphatidylinositol (PI) 3-kinase/protein kinase B (PKB) signaling pathway (2, 5) or directly activating PKB through Ca^{2+}/CaM-dependent kinase kinase (CaMKK) (6). The PI 3-kinase/PKB pathway is one of the well established mechanisms that mediates neuronal survival (7). For example, activation of the specific tyrosine-kinase receptors of the neurotrophin family (8) or the glial cell line-derived neurotrophic factor (GDNF)-family ligands (9) induce neuronal survival through this pathway.

The Ca^{2+}/CaM complex interacts with and modulates the functionality of a large number of proteins, including serine/threonine protein kinases such as Ca^{2+}/CaM-dependent protein kinases (CaMKs). The CaMK cascade consists of CaMKK and its downstream substrates CaMKI and CaMKIV (10, 11). Although CaMKI is broadly expressed in different tissues, CaMKIV is highly expressed in neurons. CaMKIV is mainly localized at the nucleus but is also present in the cytosol (12), suggesting an important role of this kinase in regulating neuronal physiology. In fact, CaMKIV is concentrated in cerebellar granule cells nuclei and catalyzes the phosphorylation of various transcription factors, such as cAMP response element-binding protein (CREB), which is thought to be the downstream effector of the depolarization- and calcium-dependent survival pathway in these cells (13). A similar role of this protein has been described in other neuronal populations, such as spiral ganglion neurons (14). CaMKIV effects on neuronal survival together with the pattern of expression during murine embryonic development (15) suggest an important role of this protein in cellular survival and differentiation during this period.

Brain-derived neurotrophic factor (BDNF) and GDNF promote chicken motoneuron (MN) survival through increased intracellular Ca^{2+} concentration and direct regulation of PI 3-kinase activity by CaM (5, 2). In the present work we investigated the role of CaMKIV in this survival process in a primary culture of embryonic chicken spinal cord MNs. We cloned

* This work was supported in part by Ministerio de Sanidad y Consumo Fondo de Investigaciones Sanitarias Grants PI051445 (to R. M. S.) and PI042537 (to M. Ll.) and by Suport a Grups de Recerca Consolidats from Generalitat de Catalunya (to J. X. 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.

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[7] The abbreviations used are: CaM, calmodulin; CaMK, calcium/CaM-dependent protein kinase; CaMKK, CaMK kinase; BDNF, brain-derived neurotrophic factor; MN, motoneuron; PI, phosphatidylinositol; PKB, protein kinase B; CREB, cAMP response element-binding protein; ERK, extracellular-regulated kinase; BAPTA, 1,2-bis(2-aminophenoxy)ethane *N*,*N*,*N*′,*N*′-tetracetic acid acetomethyl ester; EGFP, enhanced green fluorescent protein; CBD, calmodulin binding domain; AID, autoinhibitory domain; GDNF, glial cell line-derived neurotrophic factor; NS, non-supplemented; RNAi, RNA interference; RNAic, RNA interference control.

chicken CaMKIV and generated a constitutively active form ($CaMKIV_{CA}$) by deleting the calmodulin binding domain (CBD). $CaMKIV_{CA}$ overexpression induces MN survival in the absence of any trophic support. Survival experiments using RNA interference further demonstrated that endogenous CaMKIV mediates MN survival whether induced by neurotrophic factors or membrane depolarization. Finally, we show that CaMKIV associates with PI 3-kinase in a Ca^{2+}-dependent manner and activates PKB. Nonetheless, CaMKIV effects on MN survival and PKB activation are independent of its kinase activity. Taken together these results implicate CaMKIV in the survival process and PI 3-kinase/PKB activation of spinal cord MNs during chicken embryonic development.

EXPERIMENTAL PROCEDURES

Materials—Neurotrophic factors were obtained from Alomone (Jerusalem, Israel); LY294002 was from Calbiochem; EGTA was from Sigma; 1,2-bis(2-aminophenoxy)ethane *N*,*N*,*N*′,*N*′-tetraacetic acid acetomethyl ester (BAPTA-AM) was from Molecular Probes (Eugene, OR); [γ-^{32}P]ATP (10 mCi/ml) was purchased from Calbiochem.

Cloning of Gallus gallus CaMKIV and Site-direct Mutagenesis—The complete sequence of chicken CaMKIV was obtained from two expressed sequence tags (ESTs), ChEST49p9 and ChEST99m17 (Geneservice, Cambridge, UK). Inserts were amplified by PCR using the following primers: forward 5′-CGGGATCCATGCCCTCCACCTCTGCC-3′ and reverse 5′-GCCTTAAGTTTACGCCGGGC-3′ for ChEST49p9 clone and forward 5′-GAAACTTAAGGCTGCCATG-3′ and reverse 5′-CGTCTAGATGCCGCTGGGAGCCGGGCACC-3′ for ChEST99m17 clone. Amplified fragments were subcloned in pcDNA3-FLAG (pcDNA3-FLAG-CaMKIV).

The constitutively active form of CaMKIV ($CaMKIV_{CA}$) was generated from ChEST49p9 clone using the following primers: forward 5′-CGGGATCCATGCCCTCCACCTCTGCC-3′ and reverse 5′-CGAATTCAAAGCTTCTTTTGTGCGTTGTCCAT-3′. PCR fragments were subcloned in pcDNA3-FLAG (pcDNA3-FLAG-$CaMKIV_{CA}$). All inserts in the expression vector were verified by sequencing (3100-Avant Genetic Analyzer, Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Sigma.

A dominant negative form of $CaMKIV_{CA}$ was generated from the constitutively active form by introducing the mutation K60E, which renders it kinase dead (pcDNA3-FLAG-$CaMKIV_{CA}$-KD). Site-direct mutagenesis was performed on the pcDNA3-FLAG-$CaMKIV_{CA}$ plasmid by the PCR method and DpnI digestion of the template. PfU Ultra™ High Fidelity DNA polymerase from Stratagene (La Jolla, CA) was used for PCR amplification. For mutagenesis we used the following primers: forward 5′-TTACGCCATCGAAAAGTTGAAGGAGACAATCGATAAGAAAATTGTCCGCA-3′ and reverse 5′-TGCGGACAATTTTCTTATCGATTGTCTCCTTCAACTTTTCGATGGCGTAA-3′. Mutations were confirmed by DNA sequencing. Truncated forms of CaMKII and CaMKIV were kindly provided by R. A. Maurer, and truncated forms of CaMKK and CaMKI were cloned in pc-DNA3 plasmid according to Matsushita and Nairn (21).

CaMKIV Kinase Assay—HEK293T cells were transiently transfected with pcDNA3-FLAG-CaMKIV, pcDNA3-FLAG-$CaMKIV_{CA}$, pcDNA3-FLAG-$CaMKIV_{CA}$-KD, or the empty vector using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). For CaMKIV immunoprecipitation, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM orthovanadate, 25 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, and Complete™ EDTA-free protease inhibitor mixture. Lysates were immunoprecipitated with anti-FLAG-Sepharose beads (Sigma). Immunoprecipitates were washed 3 times in kinase buffer containing 50 mM Tris, pH 7.4, 0.2% Tween 20, 0.5 mM dithiothreitol, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ or 2 mM EGTA (for kinase assay conditions with or without Ca^{2+}, respectively). The kinase assay was performed in the presence of 1 μM CaM, 25 μM ATP, 10 μCi of [γ-^{32}P]ATP in the kinase buffer using 0.2 μg of CREB (Calbiochem) as substrate for 20 min at 30 °C. The reaction was stopped by adding loading buffer. Samples were resolved by SDS-PAGE and then transferred onto polyvinylidene difluoride Immobilon-P transfer membranes. Radioactive bands were detected by autoradiography using Fuji BAS1000 imaging analyzer and quantified in PCBAS 2.0 software (Fuji Photo Film, Co. Ltd.). The protein levels of phospho-CREB, CREB, and FLAG-CaMKIV were analyzed by probing the membrane with indicated antibodies.

RNA Interference Constructs—For RNA interference experiments (16), constructs were obtained into the pSUPER.retro.puro plasmid (OligoEngine, Seattle, WA) using specific oligonucleotides of the CaMKIV sequence, indicated by capital letters as follows: forward gatccccGGGAGATCAGTACATATATTttcaagagaATATATGTACTGATCTCCCttttt and reverse agctaaaaaGGGAGATCAGTACATATATtctcttgaaATATATGTACTGATCTCCCggg. Oligonucleotides were obtained from Sigma.

PC12 cells were infected using lentivirus obtained from HEK293T cells transfected with pLVTHM, pSPAX2, and pM2G (kindly provided by D. Trono, Geneva, Switzerland) using previously described methods (17, 18). RNAi (RNAi targeting the CaMKIV-specific sequence) and RNAic (RNAi targeting an unspecific RNA sequence, used as a control) were subcloned in pLVTHM from pSUPER.retro.puro using EcoRI and ClaI restriction enzymes. Four hours later, 3 μl of concentrated lentivirus (5×10^8–1×10^9 TU/ml, biological titers expressed as transducing units per ml) were added to pcDNA3-FLAG-CaMKIV-transfected PC12 cultures; 12 h later the medium was changed. RNAi efficiency was monitored by Western blot analysis using anti-CaMKIV antibody (BD Transduction Laboratories).

MN Isolation, Transfection, and Survival Evaluation—Spinal cord MNs were purified from embryonic day 5.5 (E5.5) chick embryos according to Comella *et al.* (19) with minor modifications (35). For survival experiments MNs were transfected 30 min to 1 h after plating using the Lipofectamine 2000™ Transfection Reagent according to the manufacturer's instructions. When indicated, MNs were co-transfected with pEGFP (enhanced green fluorescent protein; Clontech, BD Biosciences) and either pcDNA3-FLAG-$CaMKIV_{CA}$ or pcDNA3-FLAG-$CaMKIV_{CA}$-KD or CaMKIV RNAi or empty

vector. The empty vectors were pcDNA3-FLAG or pSUPER.retro.puro, respectively.

Survival evaluation was performed as described under "Results" for each experiment. Briefly, cell survival was expressed as the percentage of fluorescent cells remaining in the culture dish after 24 or 72 h of treatment with respect to the fluorescent cells present in the same culture dish at the beginning of the treatment. Values are the means ± S.E. of 3–4 wells (total cell number counted per well, 200–250) from a representative experiment that was repeated at least three times. Cell death characterization was evaluated by estimating the percentage of membrane blebbing morphology as described by Edwards and Tolkovsky (20). Twenty-four hours after treatment initiation the percentage of fluorescent cells with membrane blebbing morphology was calculated with respect to the total number of fluorescent cells present in the culture dish. Values are the means ± S.E. of 3–4 wells (total cell number counted per well, 200–250) from a representative experiment that was repeated at least three times. Where applicable, statistical analysis was performed with Student's *t* test.

Reverse Transcription-PCR Analysis—cDNA was reverse-transcribed from RNA extracted from purified or cultured MNs. PCR was performed by co-amplification of CaMKIV and the housekeeping L27 ribosomal protein. Primers used to amplify chicken CaMKIV were 5′-CGGGATCCATGCCCTCCACCTC-TGCC-3′ (forward) and 5′-CGTCTAGATGCCGCTGGGAGC-CGGGCACC-3′ (reverse). The L27 ribosomal protein primers were 5′-AGCTGTCATCGTGAAGAA-3′ (forward) and 5′-CTTGGCGATCTTCTTCTTGCC-3′ (reverse).

Immunoprecipitation and Western Blot Analysis—Western blot analysis was performed as described (2). The following antibodies were used as suggested by the manufacturer: anti-phospho-PKB Ser-473, anti-phospho-ERK, anti-phospho-CREB Ser-133, and anti-CREB (Cell Signaling, Beverly, MA); anti-PKB C-20 (Santa Cruz Biotechnology, CA); anti-α-tubulin (Sigma); anti-p85, anti-CaMKIV, anti-ERK, and anti-EGFP (BD Transduction Laboratories); anti-FLAG (Affinity Bioreagents, Golden, CO).

Co-immunoprecipitation assays were performed as described in Perez-Garcia *et al.* (2) with minor modifications. MNs were lysed in a Nonidet P-40 buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 25 mM NaF, 40 mM glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and Complete™ EDTA-free protease inhibitor mixture). Total protein samples (600 μg) were subjected to immunoprecipitation overnight at 4 °C with an anti-p85 monoclonal antibody in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA. Samples were incubated with protein G for 2 h at 4 °C. Immunocomplexes were washed three times with ice-cold lysis buffer containing $CaCl_2$ or EGTA, resuspended with loading buffer, boiled, resolved in SDS-polyacrylamide gel, and transferred onto polyvinylidene difluoride Immobilon-P membrane filters.

PC12 cells were electroporated with the plasmid encoding pcDNA3-FLAG-CaMKIV or the empty vector using a Gene Pulser (Bio-Rad). HEK293T cells were transfected using Lipofectamine reagent with pcDNA3-FLAG-CaMKIV or pcDNA3-FLAG-$CaMKIV_{CA}$ or the empty vector. After 48 h cells were lysed in a buffer containing 20 mM Tris, pH 7.4, 120 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 25 mM NaF, 40 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 2 mM benzamidine. Total protein samples (1 mg) were subjected to immunoprecipitation overnight at 4 °C with an anti-p85 monoclonal antibody and were recovered with protein G (Sigma) and resolved in SDS-polyacrylamide gel. Blots were probed with an anti-FLAG antibody to detect the transfected CaMKIV or an anti-p85 antibody to check for comparable immunoprecipitation efficiency.

Immunofluorescence—MNs were plated, and 24 h later cells were fixed in 4% (w/v) paraformaldehyde (Sigma) for 20 min, rinsed in phosphate-buffered saline (PBS), and blocked for 1 h at room temperature with 5% fetal bovine serum, 0.1% Triton X-100 in PBS. The primary antibody anti-CaMKIV was used at a concentration of 1:150 in 5% bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline overnight at 4 °C. rhodamine Red X conjugated donkey anti-mouse antibody was from Jackson ImmunoResearch (West Grove, PA). Finally, nuclei were stained with Hoechst 33258 (Sigma) dye and used at a concentration of 0.05 μg/ml. Confocal images were captured with an Olympus FluoView™ FV-500 confocal laser scanning microscope and compatible software (Olympus).

RESULTS

CaMKIV, but Not Other CaMK Family Members, Support MN Survival in Culture—To investigate the molecular mechanisms involved in MN survival induced by intracellular Ca^{2+} increase and CaM activation, we transfected MNs with different constitutive active forms of CaMK family members and analyzed their effects on cell survival. Cultures were then co-transfected with pEGFP and the truncated forms of $CaMKII^{1-290}$ or $CaMKIV^{1-313}$ (kindly provided by R. A. Maurer) or $CaMKK^{1-413}$ or $CaMKI^{1-295}$ (cloned in our laboratory according to Matsushita and Nairn (21))[8] or the empty vector. These truncated forms lack the autoinhibitory-regulatory region and result in constitutively active protein kinases that no longer require Ca^{2+} and CaM (22, 23). Twenty-four hours later cells were washed, the culture medium was replaced, and different experimental conditions were established. MN survival was evaluated 24 h after treatment as the percentage of fluorescent cells with blebbing morphology with respect to the total number of fluorescent cells present in the culture well (Fig. 1). It has been described that 24 h after neurotrophic factor withdrawal, apoptotic dying neurons show a marked blebbing of the plasma membrane, whereas healthy neurons are smooth and have long neurites (20). Cultures transfected with the empty vector in the absence of any neurotrophic support (NS condition) showed 56.5 ± 2.5% of apoptotic cells; however, in the presence of 10 ng/ml BDNF the percentage of blebbing cells was significantly reduced (30.5 ± 2.1%). When apoptotic morphology was evaluated in the culture wells transfected with the constitutively active forms of CaMKs, only $CaMKIV^{1-313}$ (31.5 ± 2.9%) was able to significantly reduce the percentage of

[8] M. J. Pérez-García, J. Egea, Y. de Pablo, M. Llovera, J. X. Comella, and R. M. Soler, unpublished results.

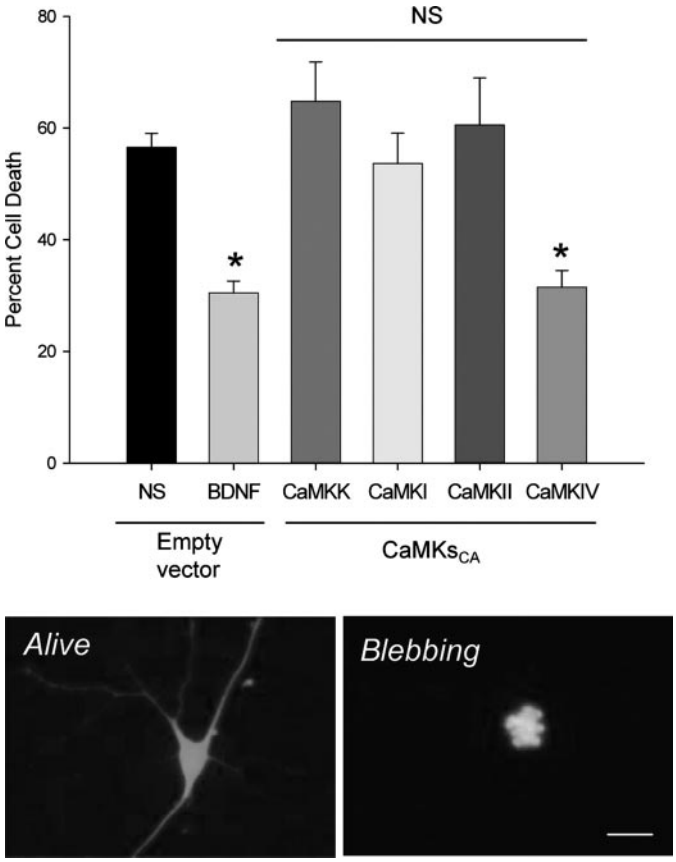

FIGURE 1. **Effect of CaMK constitutively active form transfection on MN survival.** MNs were transiently co-transfected with pEGFP and truncated forms of CaMKs or the empty vector. Twenty-four hours later cells were washed, and different experimental conditions were established; that is, BDNF (10 ng/ml) or NS. Cell death was expressed as the percentage of cells with blebbing morphology with respect to the total EGFP-positive MNs present in the culture dish after 24 h of treatment. Values are the mean ± S.E. of three wells from a representative experiment that was repeated at least two more times. *Asterisks* indicate significant differences between CaMKIV$_{CA}$ and the empty vector cultures in BDNF medium using Student's *t* test (*, $p < 0.001$). Representative images of healthy (*Alive*) and 24-h deprived (*Blebbing*) cultured MNs. *Bar*, 25 μm.

blebbing cells to the same level of BDNF-treated control cultures (Fig. 1). These results indicate that the constitutively active form of CaMKIV, but not the constitutively active forms of CaMKK or CaMKI or CaMKII, protects MNs from the cell death induced by trophic factor deprivation, which further suggests that CaMKIV is involved in the survival signaling pathways of spinal cord MNs.

Molecular Cloning of G. gallus CaMKIV—To understand better the role of endogenous CaMKIV in chicken MN survival, we cloned chicken CaMKIV by PCR. By searching publicly available EST chicken databases (GenBank™, NCBI) we found two clones, ChEST49p9 (GenBank™ accession number BX934362) and ChEST993m17 (GenBank™ accession number BU210024), that contained partial cDNA sequences of CaMKIV identified by homology with CaMKIV cDNA from other species. The first clone contained the cDNA encoding the amino terminus (nucleotide 1–938) and the second the carboxyl terminus (nucleotide 927–1112) of CaMKIV protein (see "Experimental Procedures"). The cloned *G. gallus* sequence is available in GenBank™/EMBL/DDBJ under the accession number NM_001034813. After the full-length fragment was obtained and sequenced, we decided to analyze some general features of CaMKIVs. Fig. 2*A* compares the CaMKIV sequences from *G. gallus* with *Mus musculus* (GenPept accession number NP033923), *Rattus norvegicus* (GenPept accession number NP036859), and *Homo sapiens* (GenPept accession number NP001735). Alignment of *G. gallus* CaMKIV sequence shows 76% identity with *R. norvegicus* and *M. musculus* and 77% identity when compared with *H. sapiens* (Table 1). The carboxyl terminus of *G. gallus* CaMKIV sequence shows less identity (from amino acid 326) compared with the mammalian species. In fact, the entire coding region of cloned CaMKIV encodes a protein with 372 amino acids, whereas CaMKIV amino acid sequences from other species are longer (*M. musculus* is 469 amino acids; *R. norvegicus* is 502 amino acids, and *H. sapiens* is 473 amino acids). Fig. 3*B* shows a Western blot using an anti-CaMKIV primary antibody (generated against the amino-terminal region of human CaMKIV from amino acid 1 to 241) that recognizes an ~40-kDa band in the chicken MN protein extract, whereas in mouse MN the antibody recognizes a ~60-kDa band. According to the expected size deduced from bioinformatics methods, the estimated molecular mass of chicken CaMKIV is 41.3 kDa, and the isoelectric point 8.28 (24).

Using *in silico* methods, we also determined the protein kinase domain of cloned CaMKIV from amino acid 31 to 285, the ATP binding site located at Lys^{60} (25), and the CBD (26). We observed the presence of a subclass of 1–14 motif, the basic 1-8-14 motif, at position 308–327. Compared with CaMKIV sequences from other species, all have the same basic 1-8-14 motif at the carboxyl terminus of the protein (*M. musculus*, position 319; *H. sapiens*, position 323; *R. norvegicus*, position 347), indicating that *G. gallus* CaMKIV also contains the CBD, although its protein sequence is shorter in length (Fig. 2*B*).

We attempted to map the autoinhibitory domain (AID) in chicken CaMKIV. It has previously been described that the carboxyl-terminal region after Leu^{313} contains the AID of CaMKIV (27). Tokumitsu *et al.* (28) expressed and purified a series of carboxyl terminus truncation mutants to map a minimum autoinhibitory sequence of mouse CaMKIV. They concluded that the location of this sequence is between residues Gln^{314} and Lys^{321}. The truncated mutant at Lys^{321} is completely inactive in either the presence or the absence of Ca^{2+}/CaM, indicating the presence of a functional autoinhibitory sequence. However, the truncated mutant at Leu^{313} generated a constitutively active form of the enzyme. Thus, comparing mouse and chicken CaMKIV, we found the same sequence described for mouse minimum AID located between Gln^{303} and Lys^{312} residues of chicken sequence (Fig. 2*B*).

After Ca^{2+}/CaM binding to CaMKIV, it can then be phosphorylated on a specific Thr residue (Thr^{200} in human and Thr^{196} in mouse) by the CaMKK. This event is associated with a marked increase in the total activity of CaMKIV and the generation of a Ca^{2+}/CaM-independent and autonomous kinase activity required for its role in transcription (29). In chicken CaMKIV this specific Thr residue is located in position 185 (Fig. 2*B*). Thus, cloned chicken CaMKIV is shorter in length,

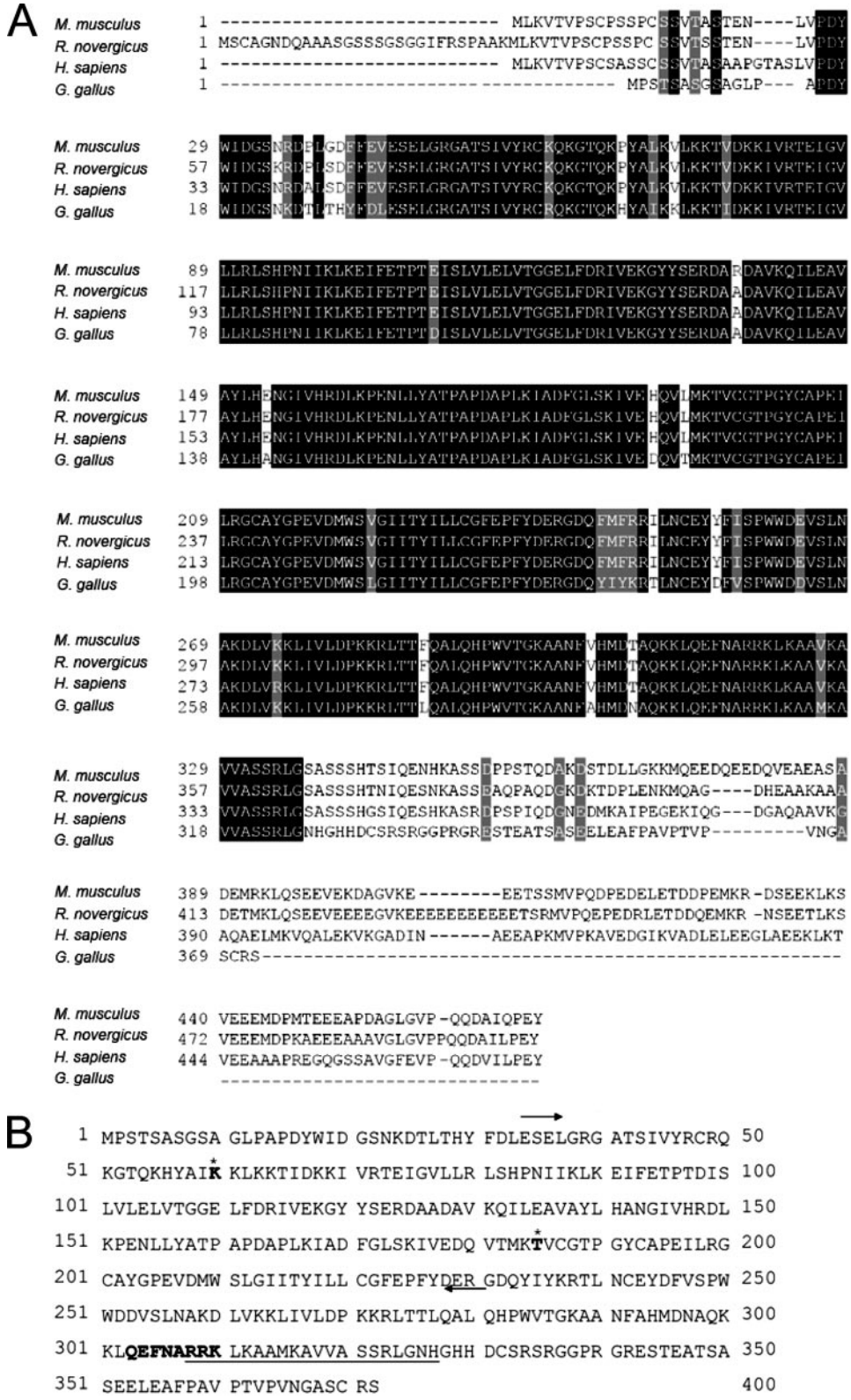

FIGURE 2. **Aligned amino acid sequences of mouse (*M. musculus*), rat (*R. norvegicus*), human (*H. sapiens*), and chicken (*G. gallus*).** *A*, comparison of CaMKIV protein sequences from mouse, rat, human, and chicken. Amino acids identical in all species are *shaded* in *black*, whereas those with conservative changes are *shaded* in *gray*. Mouse, rat, and human protein sequences are available in GenBank™/EMBL/DDBJ under the accession numbers; see "Results." Cloned chicken sequence is now available with accession number NM_001034813. *B*, analysis of the amino acid sequence of chicken CaMKIV. *Arrows* delimited the protein kinase domain of CaMKIV from amino acid 31 to 285. *Underlined amino acids* indicate the basic 1-8-14 motif that corresponds to the CBD. *Bold amino acids* in the carboxyl terminus indicate the putative minimum AID. *Asterisks* indicate Lys^{60} (ATP binding site) and Thr^{185} (amino acid susceptible to be phosphorylated by CaMKK).

recognized by the same antibody as mouse, and contains most of the characteristics of this protein in other species.

Chicken CaMKIV Is Expressed in Purified and Cultured Spinal Cord MNs—To demonstrate that CaMKIV is expressed in chicken spinal cord MNs, reverse transcription-PCR assay was used to analyze the presence of mRNA in these cells. MNs from E5.5 chicken embryos were purified using a density gradient, and RNA was extracted from freshly isolated cells or from 24-h-cultured cells in the presence of 10 ng/ml of BDNF in the culture medium. After semiquantitative reverse transcription-PCR analysis with the specific primers used for the amplification of the complete sequence of CaMKIV (see "Experimental Procedures"), we show the presence of CaMKIV transcripts in both samples (Fig. 3*A*). Cloning and sequencing the PCR products showed the same nucleotide sequence as CaMKIV cloned from ESTs. The immunofluorescence using an antibody against CaMKIV shows a cytosolic (745.2 ± 62 average fluorescence intensity (AFI)) and nuclear (366.6 ± 38.6 AFI; control without primary antibody 2.48 ± 2.6 AFI) distribution in 24-h cultured MNs (Fig. 3*C*). These results demonstrate that CaMKIV is expressed in freshly isolated and cultured embryonic chicken spinal cord MNs.

Chicken CaMKIV Activation Is Ca^{2+}/CaM-dependent and Its Constitutively Active Form Induces MN Survival—One of the characteristic features of CaMKs is the Ca^{2+} and CaM dependence for their activation. Binding of Ca^{2+}/CaM to the CBD alters the conformation of the kinase and , therefore, induces its activation (for review, see Ref. 10). However, maximal CaMKIV activation *in vitro* requires three steps, (*a*) Ca^{2+}/CaM binding, (*b*) phosphorylation by CaM-bound CaMKK in a Thr residue located in its activation loop, and (*c*) autophosphorylation in amino-terminal region (30). Once activated, CaMKIV is

TABLE 1

Percentage of identity in a pairwise alignment between different species

	M. musculus	*R. norvegicus*	*H. sapiens*	*G. gallus*
M. musculus		90	81	76
R. norvegicus	90		79	76
H. sapiens	81	79		77
G. gallus	76	76	77	

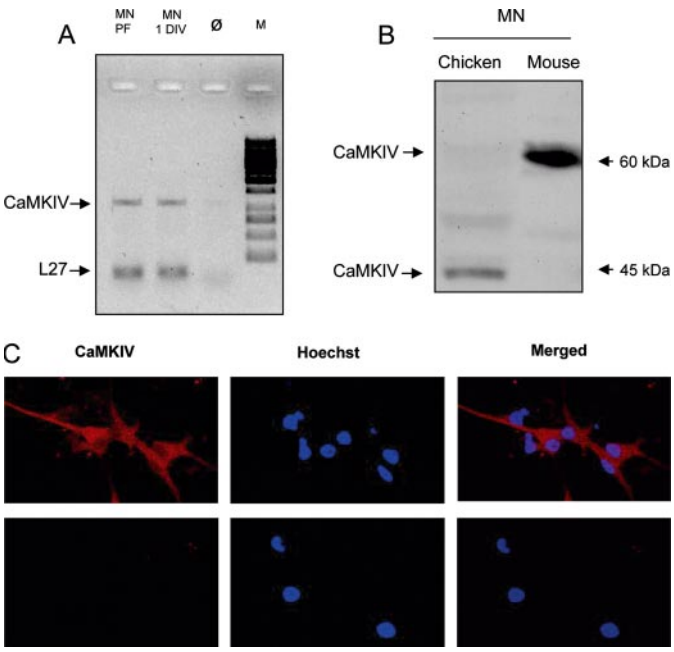

FIGURE 3. **CaMKIV is expressed in chicken spinal cord MNs.** *A*, agarose gel showing the reverse transcription-PCR products using primers for chicken CaMKIV in E5.5-purified chicken spinal cord MNs (*PF*) or 24-h cultured MNs (1 day *in vitro* (*1DIV*)). Co-amplification of the L27 mRNA (*bottom band* in each *line*) serves as an internal control. Ø, control reactions performed without reverse transcriptase. *M*, 1-kilobase DNA ladder marker. *B*, Western blot analysis of protein extracts from freshly purified chicken or mouse spinal cord MNs. The membrane was probed with an anti-CaMKIV antibody. *C*, confocal images of immunofluorescence with a monoclonal antibody against CaMKIV. Representative images of CaMKIV expression in MNs using an anti-CaMKIV antibody (*red*) and Hoescht 33258 (*blue*). No immunoreactivity was detected in the presence of a non-relevant IgG (*bottom panels*).

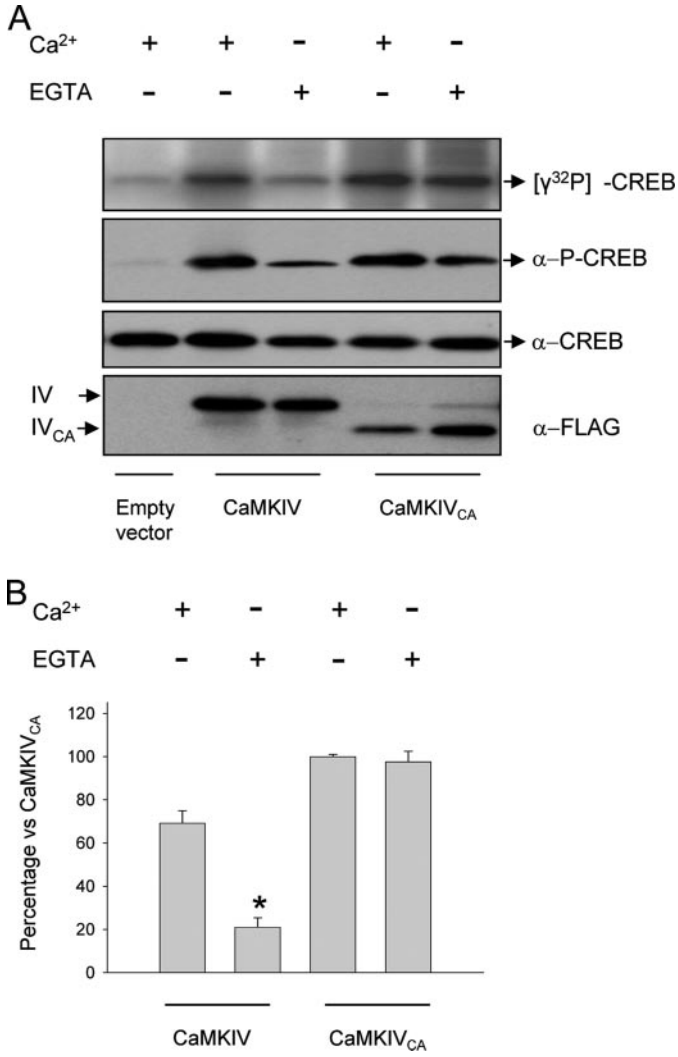

FIGURE 4. **Activity of cloned CaMKIV is Ca^{2+}-dependent.** *A*, HEK293T cells were transfected with pcDNA3-FLAG-CaMKIV or pcDNA3-FLAG-$CaMKIV_{CA}$ or the empty vector and immunoprecipitated with an anti-FLAG-Sepharose beads. Activity was determined in a radioactive kinase assay using CREB as a substrate in the presence (Ca^{2+}) or absence (EGTA) of calcium. [γ-^{32}P]ATP incorporation to the substrate CREB is shown in the *top panel*. The same extracts were used for a Western blot analysis of phosphorylated CREB using a specific (*α-P-CREB*) antibody and CREB protein using an anti-CREB antibody (*α-CREB*) as a control of protein content (*middle panels*). Efficiency of immunoprecipitation was checked by Western blot using a specific anti-FLAG antibody (*bottom panel*). *B*, the graph represents the percentage of [γ-^{32}P]ATP incorporation in different conditions with respect to $CaMKIV_{CA}$-transfected cells in the presence of Ca^{2+}. Values are the mean ± S.E. of three independent experiments. The *asterisk* indicates significant differences when comparing CaMKIV-transfected cells in the presence of EGTA with $CaMKIV_{CA}$-transfected cells in the presence of Ca^{2+} using Student's *t* test (*, $p < 0.001$).

responsible for the physiological Ca^{2+}-dependent stimulation of transcription through the phosphorylation of several transcription factors, including CREB at Ser^{133} (31). In this context we decided to analyze the Ca^{2+}/CaM dependence of cloned CaMKIV for its kinase activity and for CREB phosphorylation. pcDNA3-FLAG-CaMKIV was overexpressed in HEK293T cells, and protein extracts were immunoprecipitated using an anti-FLAG-Sepharose. CaMKIV activity was assayed in those immunoprecipitates using recombinant CREB as a substrate (Fig. 4). We also analyzed the Ca^{2+}/CaM dependence of a constitutively active form of chicken CaMKIV ($CaMKIV_{CA}$). This form is a truncated mutant at Leu^{302}, as predicted by homology with mouse CaMKIV truncated at Leu^{313}, which generates a constitutively active and Ca^{2+}/CaM-independent protein (see above). As shown in Fig. 4, immunoprecipitates containing $CaMKIV_{CA}$ induce kinase activity and CREB phosphorylation. Both outcomes were unaffected by the presence of the Ca^{2+} chelator EGTA, indicating that $CaMKIV_{CA}$ is Ca^{2+}/CaM-independent. On the other hand the presence of 2 mM $CaCl_2$ plus 1 μM CaM in the kinase assay buffer induced 69.2 ± 5.7% activation of CaMKIV compared with $CaMKIV_{CA}$ immunoprecipitates. However, when 2 mM Ca^{2+} chelator EGTA was added, the activation was reduced to 21.2 ± 4.3%, indicating that CaMKIV is Ca^{2+}/CaM-dependent. Using an anti-phospho-CREB antibody, we also observed that chicken CaMKIV induces Ser^{133} phosphorylation in the presence of Ca^{2+} and CaM (Fig. 4*A*). In the presence of EGTA, CREB phosphorylation was less evident than in the immunoprecipitates containing Ca^{2+}, although the level of CREB protein was similar in both lanes.

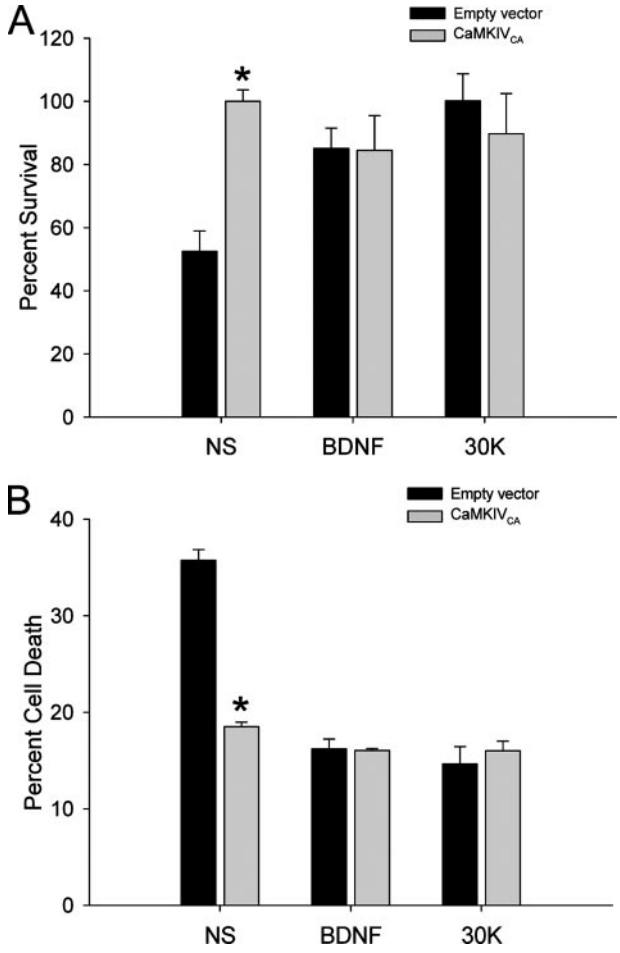

FIGURE 5. **The constitutively active form of CaMKIV prevents cell death induced by trophic deprivation.** Thirty minutes after plating MNs were transiently cotransfected with pEGFP and pcDNA3-FLAG-CaMKIV$_{CA}$ or the empty vector. Twenty-four h later, cells were washed and treated with 10 ng/ml BDNF or 30 mM KCl medium (30K) or NS as indicated. *A*, survival was expressed as the percentage of EGFP-positive cells after 24 h of treatment with respect to the EGFP-positive cells present in the culture surface at the beginning of the treatment. *B*, cell death was expressed as the percentage of cells with blebbing morphology with respect to the total EGFP-positive MNs present in the culture dish after 24 h of treatment. Values are the mean ± S.E. of three wells from a representative experiment that was repeated at least two more times. *Asterisks* indicate significant differences between pcDNA3-FLAG-CaMKIV$_{CA}$ and the empty vector cultures in NS medium using Student's *t* test (*, $p < 0.001$).

To determine whether chicken CaMKIV$_{CA}$ induces MN survival in the absence of neurotrophic support, we co-transfected MN cultures with pEGFP and pcDNA3-FLAG-CaMKIV$_{CA}$ or the empty vector. After 24 h cells were washed and treated with 10 ng/ml BDNF or 30 mM KCl (30K, to induce membrane depolarization) or left untreated (non-supplemented (NS)). Cell survival and cell death were evaluated 24 h later as the percentage of fluorescent cells remaining in the culture well with respect to the fluorescent cells at treatment initiation and the percentage of cells with blebbing morphology with respect to the total fluorescent cells in the culture well, respectively. Results show that CaMKIV$_{CA}$ protected MN from the cell death induced after neurotrophic factor or high potassium withdrawal (Fig. 5). After 24 h of deprivation, CaMKIV$_{CA}$-transfected MN showed ~100% of surviving cells with respect to the EGFP-positive cells present at the beginning of the treatment, whereas empty vector-transfected cells in the same culture conditions showed ~50% of surviving cells (Fig. 5*A*). Otherwise, the percentage of blebbing cells in CaMKIV$_{CA}$-transfected MNs was reduced (~17%) as compared with the empty vector-transfected cultures (~35%) in the absence of any trophic support (Fig. 5*B*). Together, these results indicate that chicken CaMKIV has Ca^{2+}/CaM-dependent kinase activity, and the truncated form in Leu^{313} generates a constitutively active form that protects MNs from the cell death induced by neurotrophic factor or high potassium deprivation.

Endogenous CaMKIV Mediates MN Survival—To ascertain the role of endogenous CaMKIV in MN survival, we generated two RNA interference sequences; that is RNAi, targeting a specific site of CaMKIV sequence (see "Experimental Procedures"), and RNAic, targeting an unspecific RNA sequence, used as a control of the experiment. To check the ability of RNA interference constructs to knock down CaMKIV expression, we used PC12 cells because the efficiency of transfection in chicken MNs with standard methods is not high enough for Western blot analysis of protein expression. For the same reason we used PC12 cells in the signaling experiments described below. PC12 cells do not express CaMKIV (32). Nonetheless, heterologous expression of chicken CaMKIV lacking CBD prevents apoptotic cell death of PC12 cells deprived of any trophic support. Thus, when the percentage of apoptotic nuclei was measured with the fluorescent nucleic acid stain Hoechst 33258 dye (apoptotic cells display a highly condensed DNA that is normally fragmented in two or more chromatin aggregates), we observed that CaMKIV$_{CA}$-transfected cultures showed the same percentage of apoptotic cells than the empty vector-transfected cultures in the presence of trophic support (6.1 ± 0.5 and 4.1 ± 0.5%, respectively). However, the percentage of apoptotic cells in PC12-deprived cultures was found to be significantly higher (14.3 ± 1.7; $p < 0.01$) when compared with their trophic supported or CaMKIV$_{CA}$-transfected counterparts. Therefore, for the experiment we expressed chicken CaMKIV in these cells. Using Lipofectamine we transiently co-transfected PC12 cells with pEGFP and pcDNA3-FLAG-CaMKIV. Four hours later, cells were infected with lentivirus containing the sequence encoding RNAi or the control RNAic or the lentiviral empty construct. RNAi, but not RNAic, dramatically decreased the level of ectopically expressed CaMKIV protein in PC12 cells (Fig. 6*A*).

To analyze the effect of RNAi on MN survival, cultured MNs were co-transfected using Lipofectamine with pEGFP and either RNAi or RNAic or the empty vector. After 24 h cultures were washed, and the medium was replaced with different treatments; that is, NS or 10 ng/ml BDNF or 30K. Survival was evaluated 72 h later as the percentage of remaining fluorescent cells in the culture dish with respect to those present at the beginning of the treatment. Fig. 6*B* shows that RNAi, but not RNAic, blocked the survival effect induced by BDNF or by 30K medium. This effect on cell survival with the RNAi construct demonstrates that endogenous CaMKIV plays a role in regulating MN survival in both experimental paradigms, neurotrophic factor- or membrane depolarization-induced chicken MN survival.

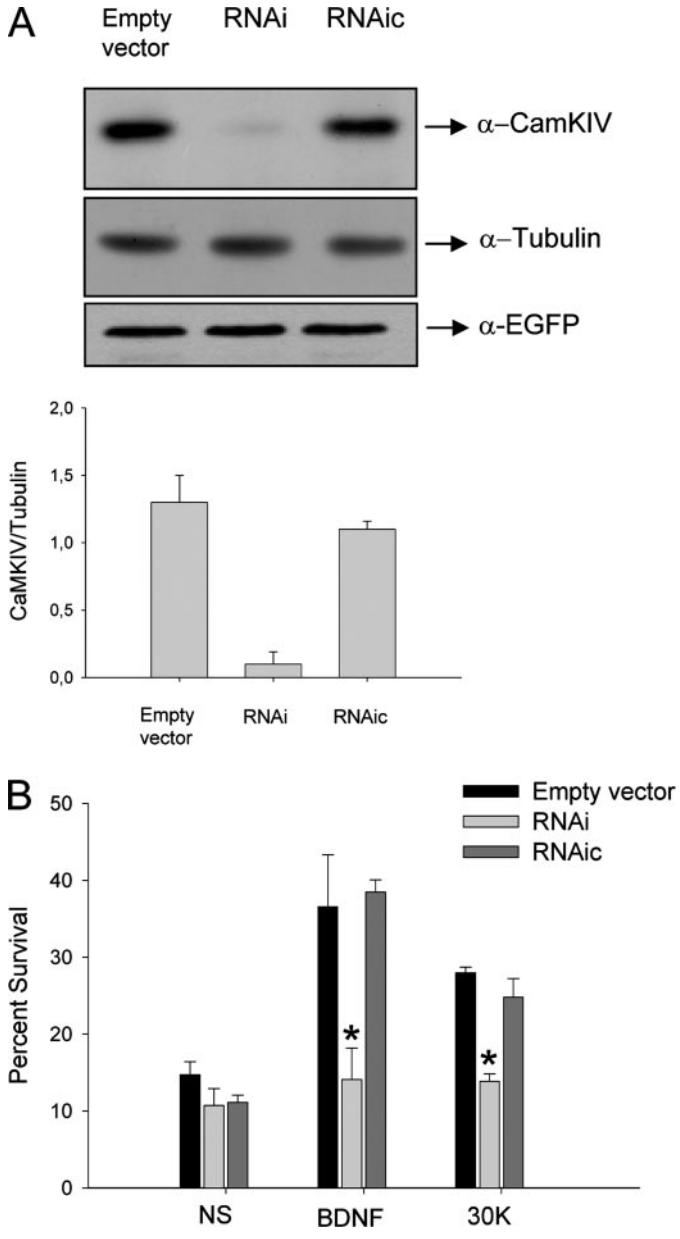

FIGURE 6. **Endogenous CaMKIV is necessary for MN survival *in vitro*.** *A*, PC12 cells were transiently transfected with pcDNA3-FLAG-CaMKIV. After 4 h cells were infected with lentivirus containing a sequence encoding RNAi against CaMKIV (*RNAi*) or an RNAi against an unspecific RNA sequence (*RNAic*) or the empty vector. Protein extracts were probed with the anti-CaMKIV antibody by Western blot analysis. Membranes were reprobed with an antibody against α-tubulin, used as a loading control, or an anti-EGFP antibody used as expression control. The graph represents the expression of CaMKIV *versus* tubulin and corresponds to the quantification of three independent experiments. *B*, purified MNs were co-transfected using Lipofectamine with pEGFP- and pSUPER.retro.puro-containing sequences encoding RNAi or RNAic or the empty vector. After 24 h cultures were washed, and medium was changed to different conditions; that is, NS or 10 ng/ml BDNF or 30 mM KCl (30K). Survival was evaluated after 72 h as the percentage of remaining fluorescent cells with respect to the fluorescent cells present at the beginning of the treatment. Values are the mean ± S.E. of three wells from a representative experiment that was repeated at least twice with comparable results. *Asterisks* indicate significant differences between RNAi and empty vector-transfected cultures in BDNF or 30K medium using the Student *t* test (*, $p < 0.001$).

Constitutively Active Form of Chicken CaMKIV Induces PKB Phosphorylation—It is well known that the activation of the PI 3-kinase/PKB pathway induces neuronal survival in various neuronal populations (33), including spinal cord MNs (34, 35). Ca^{2+}/CaM has a role in both the activation of the intracellular pathway PI 3-kinase and MN survival induced by GDNF or BDNF (2). GDNF stimulation induces a moderate increase of intracellular Ca^{2+} concentration, and this increase is involved in MN survival through CaM activation, association to PI 3-kinase, and PKB activation (2). In this context we wanted to analyze whether the survival-promoting effect of CaMKIV was also mediated by the activation of the PI 3-kinase/PKB pathway. PC12 cells were transiently co-transfected with pEGFP and the pcDNA3-FLAG-$CaMKIV_{CA}$ or a constitutively active form of PI 3-kinase (PI 3-K_{CA}) or the empty vector (Fig. 7*A*). After 48 h cells were washed and then stimulated for 5 min with different culture conditions that is, non-supplemented or 100 ng/ml NGF or 70 mM KCl (K) with or without the PI 3-kinase inhibitor LY294002 (50 μM). Cell lysates were analyzed by Western blot using specific antibodies against phospho-PKB (Ser^{473}) or phospho-ERK (Thr^{202} and Tyr^{204}) to check the phosphorylation of both proteins as representative steps of activated PI 3-kinase/PKB and ERK mitogen-activated protein kinase pathways, respectively. Cells transfected with $CaMKIV_{CA}$ or PI 3-K_{CA} showed an increased level of PKB phosphorylation in Ser^{473} (Fig. 7*A*, *lanes 6* and *8*, respectively) and Thr^{308} (data not shown) compared with the cultures transfected with the empty vector (Fig. 7*A*, *lane 1*). In both conditions PKB phosphorylation was inhibited by the presence of the PI 3-kinase inhibitor LY294002, indicating that PI 3-kinase activation mediates this process (Fig. 7*A*, *lanes 7* and *9*). However, ERK phosphorylation was not increased in the same extracts, suggesting that neither $CaMKIV_{CA}$ nor PI 3-K_{CA} is able to activate the ERK mitogen-activated protein kinase pathway in these experimental conditions. Control cultures treated with NGF or high potassium medium induced an increase of PKB phosphorylation (*lanes 2* and *4*) that was blocked by LY294002 (*lanes 3* and *5*). In both cases the same treatment induced ERK phosphorylation that was not inhibited by LY294002, as expected (Fig. 7*A*). All these results suggest that in PC12 cells, neurotrophic factors and high potassium medium exert their biological effects, activating both the PI 3-kinase and ERK mitogen-activated protein kinase pathway. Nevertheless, $CaMKIV_{CA}$ induces PKB phosphorylation, but not ERK phosphorylation, indicating the involvement of the PI 3-kinase pathway but not the ERK mitogen-activated protein kinase pathway in its survival promoting effect.

To determine whether the kinase activity of CaMKIV induces PKB phosphorylation, we cloned a $CaMKIV_{CA}$ kinase dead form (pcDNA3-FLAG-$CaMKIV_{CA}$-KD), which has an amino acid mutation in the ATP binding domain (K60E). To evaluate its kinase activity, it was overexpressed in HEK293T cells, and protein extracts were immunoprecipitated using an anti-FLAG-Sepharose. CaMKIV activity was assayed in those immunoprecipitates using recombinant CREB as a substrate (Fig. 7*B*). As shown in Fig. 7*B*, in the presence of Ca^{2+}, CREB phosphorylation was significantly lower in $CaMKIV_{CA}$-KD (5.4 ± 1.7%) immunoprecipitates when compared with $CaMKIV_{CA}$, indicating that kinase activity was blocked in the mutated form. On the other hand, PC12 cells were transfected either with pcDNA3-FLAG-$CaMKIV_{CA}$ or pcDNA3-FLAG-$CaMKIV_{CA}$-KD or the empty vector, and PKB phosphorylation was analyzed. Fig. 7*B* shows that $CaMKIV_{CA}$ or

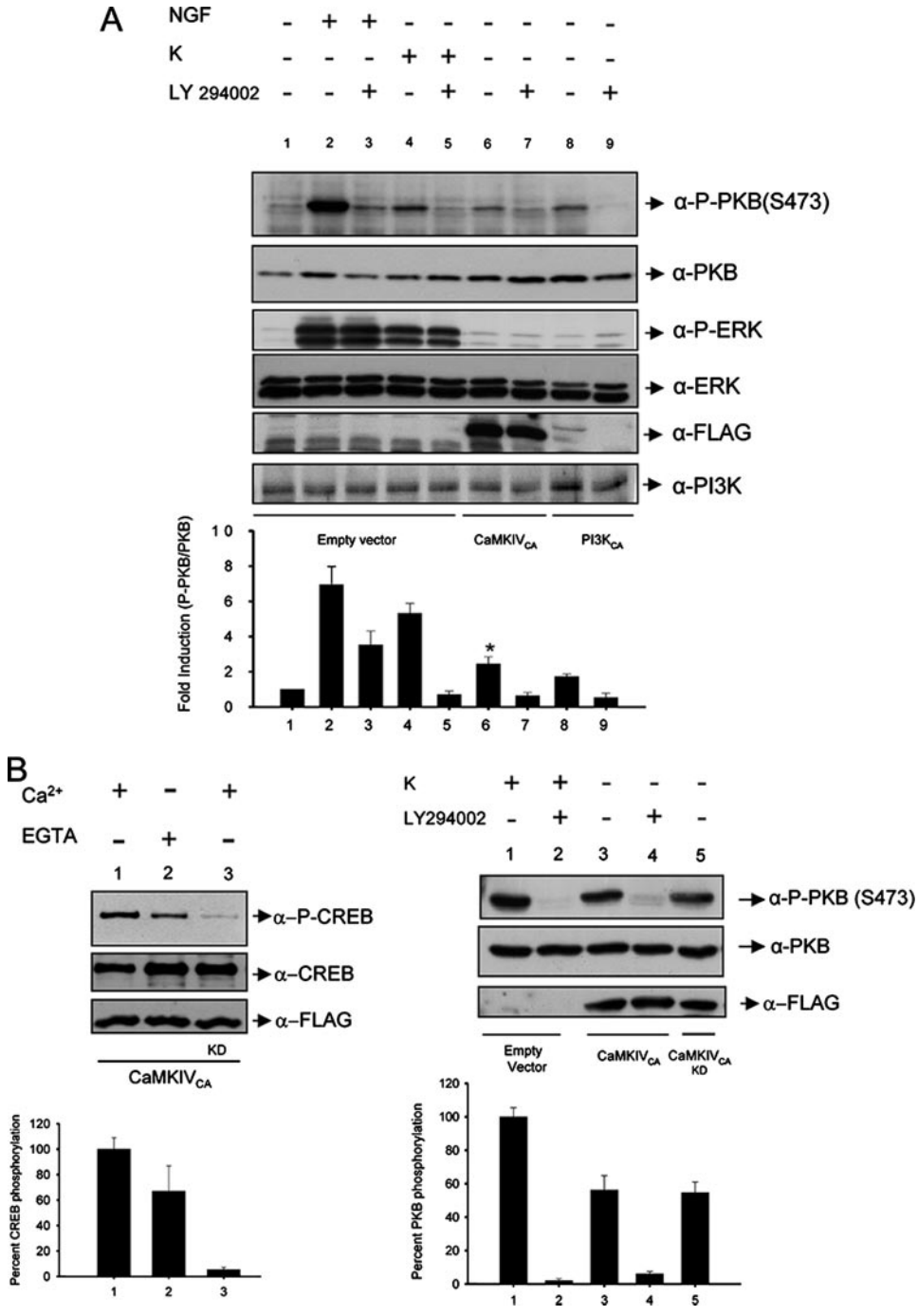

FIGURE 7. **CaMKIV$_{CA}$ and CaMKIV$_{CA}$-KD induce PKB phosphorylation.** *A*, PC12 cells were transiently co-transfected with pEGFP and PI 3-K$_{CA}$ or pcDNA3-FLAG-CaMKIV$_{CA}$ or the empty vector. After 48 h cells were washed and stimulated for 5 min with different culture conditions; that is, 100 ng/ml NGF or 70 mM KCl (*K*) with or without 50 μM LY294002 (*LY*) or non-stimulated. Total cell lysates were analyzed by Western blot using anti-phospho-PKB antibody (α-*P-PKB* (S473)) or an anti-phospho-ERK antibody (α-*P-ERK*). Membranes were stripped and reprobed with an anti-pan-PKB (α-*PKB*), anti-pan-ERK (α-*ERK*), anti-FLAG (α-*FLAG*), or anti-PI 3-kinase (α-*PI3K*) antibodies. The graph represents measures of phospho-PKB *versus* total PKB from three independent experiments. *Asterisks* indicate significant differences when compared CaMKIV$_{CA}$-transfected cells with non-stimulated empty vector-transfected cells using Student's *t* test (*, $p < 0.05$). *B*, HEK293T cells were transfected with pcDNA3-FLAG-CaMKIV$_{CA}$-KD immunoprecipitated with an anti-FLAG-Sepharose, and activity was determined using CREB as a substrate in the presence or the absence (EGTA) of Ca^{2+}. The graph represents the percentage of CREB phosphorylation in the different conditions with respect to CaMKIV$_{CA}$-transfected cells. Values are the mean ± S.E. of three independent biological replicates. PC12 cells were transfected and then stimulated with 70 mM KCl with or without LY294002 or non-stimulated. Total cell lysates were analyzed by Western blot using anti-phospho-PKB antibody (α-*P-PKB (S473)*). The graph represents the percentage of PKB phosphorylation measured in the different conditions with respect to empty vector-transfected cells treated with KCl. Values are the mean ± S.E. of three independent biological replicates.

CaMKIV$_{CA}$-KD transfection promoted PKB phosphorylation. This result indicates that the kinase activity of CaMKIV does not induce PKB phosphorylation.

We also analyzed the effect of this kinase dead form on MN survival. To this end MN cultures were co-transfected with pEGFP and either pcDNA3-FLAG-CaMKIV$_{CA}$-KD or pcDNA3-FLAG-CaMKIV$_{CA}$ or the empty vector; 24 h later cultures were washed and treated with the different conditions (NS, 10 ng/ml BDNF or 30K). Cell death (percentage of blebbing cells) was analyzed 24 h after treatment. Cultures transfected with CaMKIV$_{CA}$-KD showed a percentage of cell death similar to those transfected with the CaMKIV$_{CA}$ (Fig. 8*A*), indicating that the MN survival-promoting effect mediated by CaMKIV is independent of its kinase activity. Finally, to establish the role of PI 3-kinase in MN survival mediated by CaMKIV, cells were co-transfected with the same plasmids as described above. Twenty-four hours later cells were washed and treated with NS or 10 ng/ml BDNF in the presence or absence of the PI 3-kinase inhibitor LY294002 (50 μM). Cell survival was evaluated 48 h later as the percentage of the fluorescent cells remaining in the culture well with respect to the fluorescent cells at treatment initiation. As shown in Fig. 8*B*, the presence of LY294002 prevents the survival effect induced by CaMKIV$_{CA}$ or CaMKIV$_{CA}$-KD, indicating that PI 3-kinase activation mediates MN survival induced by CaMKIV.

CaMKIV Associates with the 85-kDa Regulatory Subunit of PI 3-Kinase—We demonstrated that CaMKIV$_{CA}$ transfection induces PKBphosphorylationinaPI3-kinase-dependent (Fig. 7*A*) and CaMKIV kinase activity-independent (Fig. 7*B*) manner. To further analyze the physiological regulation of PI 3-kinase/PKB pathway by CaMKIV, we evaluated the interaction between PI 3-kinase and CaMKIV using a co-immunoprecipitation strategy.

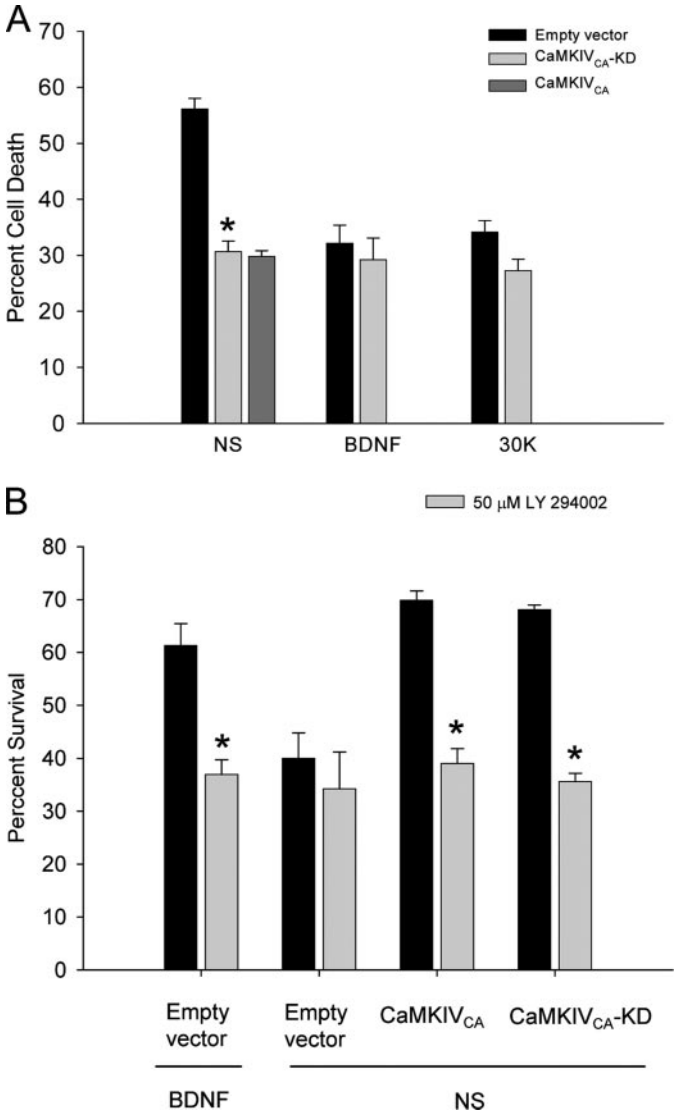

FIGURE 8. **Effect of PI 3-kinase inhibitors on CaMKIV$_{CA}$-induced MN survival.** MNs were co-transfected with pEGFP and pcDNA3-FLAG-CaMKIV$_{CA}$, pcDNA3-FLAG-CaMKIV$_{CA}$-KD, or the empty vector. Twenty-four hours after transfection, cultures were washed, and medium was changed with the different conditions. *A*, NS, 10 ng/ml BDNF, or 30 mM KCl (*30K*). Cell death was expressed as the percentage of blebbing cells with respect to the total EGFP-positive cells present in the culture dish 24 h after treatment. Values are the mean ± S.E. of three wells from a representative experiment that was repeated at least two more times. *Asterisks* indicate significant differences between pcDNA3-FLAG-CaMKIV$_{CA}$-KD and the empty vector cultures in NS medium using Student's *t* test (*, $p < 0.001$). *B*, NS or 10 ng/ml BDNF in the presence or absence of 50 μM LY294002. Cell survival was evaluated 48 h later and is expressed as the percentage of the fluorescent cells remaining in the culture well with respect to the fluorescent cells at treatment initiation. Values are the mean ± S.E. of three wells from a representative experiment that was repeated at least two more times. *Asterisks* indicate significant differences in cell survival between cultures treated with or without LY294002 using Student's *t* test (*, $p < 0.005$).

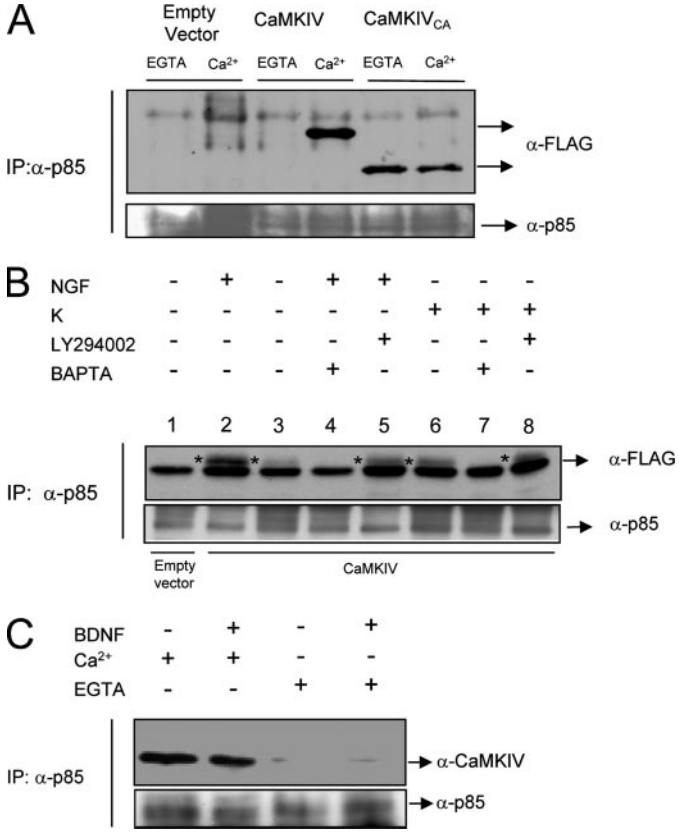

FIGURE 9. **CaMKIV co-immunoprecipitates with p85 regulatory subunit of PI 3-kinase.** *A*, HEK293T cells were transfected with pcDNA3-FLAG-CaMKIV or pcDNA3-FLAG-CaMKIV$_{CA}$. Two days later cells were lysed and immunoprecipitated (*IP*) with an anti-p85 antibody (α-*p85*) in the presence or absence (*EGTA*) of Ca^{2+}. Immunocomplexes were analyzed by Western blot using an anti-FLAG antibody (α-*FLAG*). *B*, PC12 cells were co-transfected with pEGFP and the empty vector (*lane 1*) or pcDNA3-FLAG-CaMKIV (*lanes 2–8*). Cultures were stimulated with the following conditions: 100 ng/ml NGF (*NGF*) and 70 mM KCl (*K*) with or without 50 μM BAPTA or 50 μM LY294002. Cells lysates were then immunoprecipitated with the anti-p85 antibody (α-*p85*). Immunocomplexes were analyzed by Western blot with an anti-FLAG antibody (α-*FLAG*). Efficiency of p85 immunoprecipitation in the different conditions was checked by reprobing the membranes using the α-p85. *C*, MNs were lysed, and equal amounts of protein were immunoprecipitated with anti-p85 in the presence or absence (EGTA) of Ca^{2+}. Immunocomplexes were analyzed by Western blot using an anti-CaMKIV antibody. Efficiency of p85 immunoprecipitation in the different conditions was checked by reprobing the membranes with anti-p85. *A*, *B*, and *C* experiments were repeated three times using different biological replicates. The results were the same than those showed in the figure.

HEK293T cells were transfected with pcDNA3-FLAG-CaMKIV or pcDNA3-FLAG-CaMKIV$_{CA}$ or the empty vector. Two days later cultures were lysed and immunoprecipitated in the presence or absence (EGTA) of Ca^{2+} using a specific monoclonal antibody against the 85-kDa regulatory subunit of the PI 3-kinase (p85). Immunoprecipitates were resolved in SDS-PAGE and analyzed by Western blot using an anti-FLAG or anti-p85 antibodies. As shown in Fig. 9*A*, wild type CaMKIV only immunoprecipitates in the presence of Ca^{2+}; nevertheless, CaMKIV$_{CA}$ binds to p85 in a Ca^{2+}-independent manner. This result indicates that both CaMKIV forms, wild type and truncated constitutively active, associate to p85, suggesting that p85 binding site of CaMKIV is not located in the CBD.

To study the regulation of p85 and CaMKIV association, PC12 cells were co-transfected with pEGFP and the empty vector (Fig. 9*B*, *lane 1*) or pcDNA3-FLAG-CaMKIV (Fig. 9*B*, *lanes 2–8*). Two days later cultures were washed and stimulated with different culture conditions; that is, non-supplemented or 100 ng/ml NGF or 70 mM KCl with or without 50 μM BAPTA-AM (intracellular calcium chelator) or 50 μM LY294002. Cells were then lysed and immunoprecipitated with anti-p85 antibody. Western blot analysis of PC12 immunoprecipitates probed with an anti-FLAG antibody showed that CaMKIV co-immu-

noprecipitates with p85 (Fig. 9*B*, *lane 3*). When cultures were treated with NGF (Fig. 9*B*, *lane 2*) or high potassium (Fig. 9*B*, *lane 6*), the level of co-immunoprecipitation increased with respect to the non-stimulated cells (*lane 3*). In both cases (*lane 5* for NGF; *lane 8* for high potassium) LY204002 did not prevent the association induced by these treatments, indicating that this association is independent of PI 3-kinase activation. However, when intracellular Ca^{2+} was chelated with BAPTA-AM, the co-immunoprecipitation was abolished, demonstrating the Ca^{2+} dependence of the association (*lane 4* for NGF; *lane 7* for high potassium).

Finally, we evaluated the endogenous interaction between CaMKIV and p85 in chicken MN. Cells were cultured 48 h in the presence of neurotrophic factors then washed and serum- and neurotrophic factors-starved during 12 h. Cultures were either stimulated with 100 ng/ml BDNF or left untreated, then lysed and immunoprecipitated with the anti-p85 antibody in the presence or absence (EGTA) of Ca^{2+}. Western blot analysis with an anti-CaMKIV antibody showed that CaMKIV co-immunoprecipitates with p85 in the presence of Ca^{2+} but not in the presence of EGTA (Fig. 9*C*). Together these results suggest that CaMKIV and p85 association is mainly regulated by the intracellular Ca^{2+} levels.

DISCUSSION

In the present work we cloned *G. gallus* CaMKIV and analyzed its intracellular role in MN survival. Our results show that chicken CaMKIV is shorter compared with other species but contains the domains that characterize this family of proteins. CaMKIV shows nuclear localization and is responsible for Ca^{2+}-dependent gene transcription through the phosphorylation of several transcription factors, including CREB (36). However, previous results have shown that CaMKIV is present in the cytoplasm as well as the nucleus, indicating that this kinase has a physiological function other than phosphorylation of transcription factors. In fact, CaMKIV phosphorylates oncoprotein 18 and regulates microtubule dynamics in response to external signals that involve Ca^{2+} (37). The results presented here are in accordance with this possible role of CaMKIV in regulating cytoplasmic events associated with cell differentiation and survival.

The constitutively active form of CaMKIV induces MN survival in the absence of neurotrophic factors. However, reduction of endogenous CaMKIV by RNAi significantly decreases BDNF-induced MN survival. Our results indicate that CaMKIV mediates MN survival, as has been previously described for other neuronal populations (13, 14). We suggest that CaMKIV mediates this survival effect through its association to p85 but not by direct activation of PKB given that transfection of the kinase dead form of $CaMKIV_{CA}$ did not block either PKB phosphorylation or MN survival. CaMKIV associates to p85 in a Ca^{2+}-dependent manner, suggesting that intracellular Ca^{2+} regulates this association and affects neuronal survival. Neurotrophic factor treatment induces intracellular Ca^{2+} increase and neuronal survival (1, 2). Our results suggest that these intracellular Ca^{2+} changes together with CaM activation induce Ca^{2+}/CaM binding to CaMKIV. CaMKIV suffers a conformational change, associates to p85, and promotes PKB phosphorylation. When intracellular Ca^{2+} is chelated or CaM activation is antagonized, PKB phosphorylation and cell survival are blocked (2, 5) as a consequence of CaMKIV not associating to p85.

We also demonstrate that CaMKIV RNAi blocks membrane depolarization-induced cell survival. However, our previous results in chicken spinal cord MNs showed that CaM, but not PI 3-kinase activation (4), regulates the membrane depolarization survival effect, suggesting the involvement of another protein(s) regulated by CaMKIV. One candidate to be activated by membrane depolarization can be PKB. It has been reported that Ca^{2+}/CaM or CaMKK directly regulates PKB binding to plasma membrane (38) or PKB activation (6), respectively, suggesting that the increase of Ca^{2+} after membrane depolarization regulates PKB without affecting PI 3-kinase activity. Furthermore, membrane depolarization signaling mechanisms for cell survival may act through the regulation of several proteins at the same time. For example, in spiral ganglion neurons, depolarization uses at least three distinct Ca^{2+}-dependent signaling pathways that act in parallel and in distinct intracellular compartments to promote cell survival (39). From our present and previous (4) results, we can conclude that CaMKIV regulates survival in MNs through PI 3-kinase activation in the neurotrophic factor model. However, in the membrane depolarization paradigm, CaMKIV may be involved in cell survival through the regulation of other proteins that could be located in the same and/or distinct cellular compartments that remain uncharacterized. Thus, in this work we show that CaMKIV reverses MN survival induced by neurotrophic factors or membrane depolarization, indicating the convergence of both stimuli in CaMKIV to induce neuronal survival. Appropriate levels of neurotrophic factors and neuronal activity are two essential requirements for developing neurons to survive and differentiate. These requirements can be reconstructed *in vitro* by adding neurotrophic factors or depolarizing concentrations of potassium in the culture medium (40). Both treatments induce the activation of survival pathways, but it is not clear whether these signaling mechanisms are shared by both stimuli. Although the activation of the PI 3-kinase/PKB pathway is well known as a mediator of survival induced by neurotrophic factors (33, 34), the involvement of this pathway in mediating high potassium survival effect is not clear. As we mentioned above in the NG108 neuroblastoma cell line, Yano *et al.* (6) found that Ca^{2+} increase promotes cell survival by directly activating PKB with CaMKK in a PI 3-kinase-independent manner. However, in primary cultures of MNs, the constitutively active form of CaMKK did not promote cell survival, suggesting that this kinase is not upstream of the CaMKIV effects and is not involved in the intracellular pathways that regulate survival. Recently, Johnson and D'Mello (41) also concluded that the neuroprotective effect of high potassium in cerebellar granule neurons is mediated by PKB activation, in this case through the activation of PAK-1, the downstream effector of Rac and Cdc42. However, in sympathetic neurons depolarization and neurotrophic factors converge on the activation of PI 3-kinase and synergistically promote neuronal survival (42).

Two different CaMKIV null mice have been generated by two independent laboratories. Both describe deficits in CREB

phosphorylation and cerebellar defects, affecting either late-phase of long-term depression (43) or the number and size (44) of Purkinje cells. These studies confirm the importance of this kinase during cerebellar function and development. The analysis of MN function and number in these mice had not yet been reported. However, because we describe here an important role of CaMKIV in mediating the survival of these cells, we would expect a deficiency in the motor system that is not described in the phenotype of CaMKIV knock-out mice. It would be interesting to explore the possibility that another protein(s) has a redundant role to compensate the lack of CaMKIV in these mice. CaMKIV role in mediating MN survival does not depend of the kinase activity. However, Ca^{2+}/CaM-activated CaMKIV is required for this survival effect, suggesting that proteins with a potential redundant role must have a structural homology instead of a kinase activity equivalent to CaMKIV. On the other hand, mRNA expression during mouse nervous system development is chronologically consistent with periods of extensive cellular differentiation, proliferation, and neuronal survival (15, 42). The results from these studies suggest an important role of CaMKIV during embryonic nervous system development and provide a basis for further investigation of its involvement in other neuronal population development.

Acknowledgments—We thank Isabel Sànchez and Roser Pané for technical support and all the members of the Cell Signaling and Apoptosis Group for unconditional support. We specially thank Dr. Elaine M. Lilly for editing and proofreading the manuscript.

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J. Biol. Chem. 2008, 283:4133-4144.
doi: 10.1074/jbc.M705477200 originally published online December 13, 2007
