Nuclear CaMKII inhibits neuronal differentiation of PC12 cells without affecting MAPK or CREB activation

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Submitted 5 November 2002; accepted in final form 21 January 2003

Kutcher, Louis W., Shirelyn R. Beauman, Eric I. Gruenstein, Marcia A. Kaetzel, and John R. Dedman. Nuclear CaMKII inhibits neuronal differentiation of PC12 cells without affecting MAPK or CREB activation. Am J Physiol Cell Physiol 284: C1334–C1345, 2003. First published Feb 5, 2003; 10.1152/ajpcell.00510.2002.—Ca2+/calmodulin-regulated protein kinase II (CaMKII) mediates many cellular events. The four CaMKII isoforms have numerous splice variants, three of which contain nuclear localization signals. Little is known about the role of nuclear localized CaMKII in neuronal development. To study this process, PC12 cells were transfected to produce CaMKII targeted to either the cytoplasm or the nucleus and then treated with nerve growth factor (NGF). NGF triggers a signaling cascade (MAPK) that results in the differentiation of PC12 cells into a neuronal phenotype, marked by neurite outgrowth. The study found that cells expressing nuclear targeted CaMKII failed to grow neurites, whereas cells expressing cytoplasmic CaMKII readily produced neurites. Inhibition of neuronal differentiation by nuclear CaMKII was independent of MAPK signaling, as sustained Erk phosphorylation was not affected. Phosphorylation of CREB was also unaffected. Thus nuclear CaMKII modifies neuronal differentiation by a mechanism independent of MAPK and CREB activation.

When the level of Ca2+ increases inside a cell, several Ca2+-regulated signal transduction cascades are initiated. One such pathway involves calmodulin (CaM), which activates several downstream targets, including calcium/calmodulin-regulated protein kinase II (CaMKII) (for reviews see Refs. 5 and 39). CaMKII regulates a wide variety of cellular events, from controlling cell cycle progression to regulating cellular differentiation and influencing apoptosis (1).

There are four isoforms of CaMKII, α, β, γ, and δ, each encoded by a separate gene but having very high sequence homology (1, 8, 46, 49). Each CaMKII isoform is composed of a catalytic domain, a regulatory domain, and an association domain. The association domain is responsible for 8–12 individual CaMKII molecules forming a holoenzyme complex that can be composed of one or several different isoforms (1, 24, 40). At the carboxyl end of the regulatory domain, there is a short sequence known as the variable region (1). Outside this variable region, the amino acid sequence homology is 80–90% (46, 49). The functional differences between isoforms provide unique developmental, regional, or subcellular expression patterns. The substrate specificity, kinetics, and calmodulin affinities are extremely similar (1, 3, 4, 25, 46).

Alternative splicing of these four genes gives rise to a large number of variants (for reviews see Refs. 1, 22, 24, 40). The α- and β-isoforms are found in neuronal tissue, whereas the γ- and δ-isoforms are more widely distributed (49); all cells express at least one gene type. The details of tissue distribution and developmental regulation of these splice variants are under investigation. Three of the isoforms, αB, γA, and δB, contain nuclear localization signals (NLS) within the variable region (3, 10, 22). The nuclear localized δB-isoform was first identified in rat hearts (10) and has subsequently been found in the rat cerebellum and, to a lesser extent, in the cerebrum (47). The nuclear targeted αB-isoform is found in astrocytes (46), whereas mRNA for it has been detected in the diencephalon and midbrain of rats but not in the hippocampus or cortex (3). For the αB- and δB-isoforms, nuclear localization can be blocked by phosphorylation of a serine located immediately adjacent to the NLS (23), which decreases binding of CaMKII to a nuclear import receptor. Nuclear targeting may therefore be a dynamic event, changing as a result of several influences, potentially including kinases and phosphatases.

Studies with several cytoplasmic isoforms of CaMKII have shown them to be involved in neurite outgrowth, their exact role depending on both the cell type and the isoform studied. When the δB-isoform (an embryonic mouse type) is expressed in NIH/3T3 cells, it locates in the perinuclear region and induces the spontaneous formation of processes coming from the cell (4, 25). This localization is in contrast to the δC-isoform, which remains in the cytoplasm when expressed in NIH/3T3

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cells and does not induce these extensions (4, 25). A constitutively active mutant of the δC-isofom is capable of inducing neurite outgrowth in P19 cells (8). The role of the α-isofom in neurite outgrowth has been studied extensively. When overexpressed in CAD cells (9), Nb2a cells (15, 41), and NG108–15 cells (15), α-CaMKII causes spontaneous neurite outgrowth. However, when α-CaMKII is overexpressed in pheochromocytoma (PC12) cells, it has been shown to inhibit neurite outgrowth that is normally induced by either nerve growth factor (NGF) (31) or cAMP (48). The latter two studies are of particular interest, because they both employ PC12 cells, and they will be considered in more detail in DISCUSSION.

A further influence on the role of various CaMKII isoforms is the fact that their expression in a cell can change as a result of cellular development. For instance, a central nervous system (CNS) cell line, CAD cells, shows increased CaMKII activity on differentiation, as well as a shift in the predominant isoform expressed, from the cytoplasmic δC (62)-isoform in undifferentiated cells to another cytoplasmic isoform, δD (64), after differentiation (8). In P19 cells undergoing differentiation, expression of the δC isoform is decreased (8). However, this does not happen when PC12 cells are differentiated; rather, the δD- and δA (61)-isoforms are increased, whereas the δC remains constant (8). This has led Donai et al. (8) to suggest that the lineage and maturity of the cell may determine which CaMKII isoforms are expressed.

Because of the importance of the role of selected CaMKII isoforms in neurite outgrowth, the current study was undertaken to explore how the subcellular location of CaMKII influences NGF-induced neuronal differentiation. The similarity in kinetics, substrate specificity, and CaM-binding properties between the different CaMKII isoforms has allowed the α-isofom to be used in many studies of CaMKII function (9, 15, 31, 41, 48). Thus transgenes were constructed to target expression of α-CaMKII to either the nucleus or the cytoplasm, and these transgenes were transfected into rat PC12 cells. The effects of targeted CaMKII proteins on NGF-induced neurite outgrowth were then studied.

PC12 cells respond to NGF by elaborating neurites in response to NGF, whereas lines with cytoplasmic CaMKII responded to NGF similar to control lines expressing CaMKII in the nucleus failed to grow neurites in response to NGF, whereas lines with cytoplasmic CaMKII responded to NGF similar to control cells.

**MATERIALS AND METHODS**

**Cell culture.** Rat PC12 cells (18) were cultured in a 1:1 mix of DMEM-F12 medium supplemented with 10% fetal bovine serum, 15 mM HEPES, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (all from GIBCO-BRL). For transfected cell lines, medium was supplemented with 500 μg/ml G418 antibiotic (BioWhittaker). Cells were grown on tissue culture plastic at 37°C in 5% CO2 and subcultured before reaching confluence. PC12 cells were a gift of Dr. Maria Czyzyk-Krzeska (University of Cincinnati) and were used between passages 7 and 20 of receipt. Transfected cell lines were used between passages 3 and 15 from initial selection. Stocks of all cell lines were kept on liquid N2 and thawed as needed. To assess the overall viability of transfected cell lines, the MTT assay for cell growth was used. Following the protocol of Loo and Rillema (30), cells were plated in one-half of a 96-well plate at 1.5 × 10⁴ cells/well and grown under standard conditions in the presence of G418 antibiotic (if appropriate). On a given assay date, medium was removed from the wells and replaced with medium containing 1.25 mg/ml MTT reagent [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and incubated at 37°C for 2 h. An equal volume of extraction buffer (10% SDS dissolved in a 1:1 mix of H₂O and N,N-dimethylformamide) was then added to each well, and the plates were incubated overnight. The optical density was measured at 570 nm and averaged over the 30 center wells of the 48 treated wells (omitting the outside edge wells). The experiment was repeated three to five times for each growth curve. These experiments showed that cell viability and proliferation rate were not changed in cell lines expressing either nuclear or cytoplasmic CaMKII (data not shown).

**Neuronal differentiation with NGF.** Lines of PC12 cells were plated at 5 × 10⁴ cells/cm² on glass coverslips coated with poly-L-lysine. A thin film of poly-L-lysine was applied in the coverslips in tissue culture dishes and allowed to dry for 2–4 h. Dishes were then washed four times with sterile water and once with complete medium. One day after cells were plated, medium was changed to serum-free medium with 50 ng/ml NGF (Alamone Laboratories) and 500 μg/ml G418 antibiotic for stable cell lines. After the given incubation time, the coverslips were fixed in 10% formalin and mounted on glass slides. To measure neurite outgrowth, six adjacent microscope fields were photographed from each coverslip and scanned (Nikon Coolscan II) as JPEG files along with an image of a stage micrometer. The JPEG file names were encoded so that images could be analyzed blinded as to treatment. Neurites were measured on enlarged photomicrographs with ImagePro software (Media Cybernetics), with spatial calibration taken from the stage micrometer. The criteria used to define a neurite-bearing cell required one process to extend ≥15 μm from the cell body. The length of the longest neurite coming from any given cell was also recorded. For branched neurites, the portion giving the great-
est total length was used. In cases where the beginning point of a neurite could come from one of two cells, the neurite was arbitrarily assigned to one of the cells, and the other cell was scored as nonneurite bearing. Likewise, if two neurites overlapped and the end points could not be established, only one neurite and cell were included in the analysis. Because assigning individual neurites to their appropriate cell body was difficult in large clumps, groups of more than six cells were excluded from analysis. After all measurements for one experiment had been completed, the coding on the JPEG image files was broken, and the data were analyzed using an Excel (Microsoft) spreadsheet. For fluorescent photomicrographs, coverslips were fixed in formalin and then permeabilized in ice-cold acetone for 6–8 min and blocked with preimmune serum for a minimum of 30 min at 37°C. Coverslips were incubated with appropriate primary antibody either overnight at 4°C or for 1 h at room temperature. Coverslips were then washed four times with PBS and incubated with an FITC-conjugated secondary antibody at room temperature for 60 min. Coverslips were again washed four times with PBS and mounted in 90% glycerol on glass slides. Images were taken with Kodak film on a Nikon Optiphot inverted microscope.

Producing stable PC12 cell lines expressing targeted \( \alpha \)-CaMKII. Constructs for targeted CaMKII are shown in Fig. 1. An epitope-specific sequence (the “FLAG tag,” amino acids DYKDDDDK), used to identify the transgenic protein, and a Kozak sequence (nucleotides ACCACC) were added to the amino terminus of \( \alpha \)-CaMKII DNA. Nuclear targeting was achieved by including an SV40 NLS (amino acids PKKRRKVE); this construct is designated NLS-CaMKII. The cytoplasmic targeting was achieved by leaving the NLS out; that construct is designated Cyto-CaMKII. Constructs were cloned into the HindIII/EcoRI sites of the pcDNA3 mammalian expression vector. Transfast (Qiagen) reagents were used to cotransfect wild-type PC12 cells with a plasmid coding for neomycin resistance and either the cytoplasmic or nuclear localized, FLAG-tagged CaMKII. Cell lines were selected for antibiotic resistance with the neomycin analog G418 and then screened for properly targeted CaMKII expression by immunocytochemistry by use of a monoclonal anti-FLAG antibody (Eastman Kodak), as described. A cell line transfected to express neomycin resistance alone was also produced to be used as a mock transfected control. Three independent clones of the Cyto-CaMKII transfected cells (Cyto-A2, Cyto-12, and Cyto-B4), and three clones of NLS-CaMKII transfected cells (NLS-Tc2, NLS-KA1, and NLS-KB5) were chosen for further analysis.

Measuring CaMKII activity. The CaMKII activity assay followed the protocol of Hanson and Schulman (21). This assay measures CaMKII activity on the basis of the incorporation of \( ^{32} \text{P} \) into an artificial CaMKII substrate, autocamtide III. Control or transfected PC12 cell lines were plated at 1–2 \( \times \) 10\(^5\) cells in a 60-mm dish on the day before assay. Cells were rinsed with PBS and lysed 2 min before the beginning of the autocamtide phosphorylation reaction by use of an extraction buffer containing PIPES (20 mM), EGTA (0.5 mM), leupeptin (10 \( \mu \)g/\( \mu \)l), Na-pyrophosphate (10 mM), ammonium molybdate (0.4 mM), Triton X-100 (0.1%), and dithiothreitol (20 \( \mu \)M). Cell lysate (20 \( \mu \)l) was then added to a reaction tube containing ATP (0.05 mM), PIPES (50 mM), BSA (0.2 mg/ml), MgCl\(_2\) (20 mM), and 0.2–0.25 \( \mu \)Ci of \( \gamma^{32} \text{P} \)ATP. To measure CaMKII activity in the lysate, CaCl\(_2\) (1 mM), calmodulin (20 \( \mu \)g/ml), and autocamtide III (15 \( \mu \)M) were included; for Ca\(^{2+}\)-independent activity, the CaCl\(_2\) and CaM were replaced with EGTA (1 mM). The reaction mix was incubated at 30°C for 2 min and then quenched by adding ice-cold trichloroacetic acid (3% final) and storing on ice. Proteins were pelleted, and 20 \( \mu \)l of supernatant were spotted onto phosphocellulose paper. Radioactivity, corresponding to incorporation of \( ^{32} \text{P} \) into autocamtide III by CaMKII, was measured in a scintillation counter. All assays were performed in duplicate and averaged for one experiment. Three to fourteen experiments were performed on each cell line. For CaMKII assays in subcellular fractions, the nuclear and cytoplasmic fractions were separated (see next section), and the CaMKII activity assay was run on samples of each, as above.

Separation of nuclear and cytoplasmic fractions. The protocol of Kroll et al. (29) was used to isolate nuclear and cytoplasmic fractions from PC12 cells. Control or transfected
PC12 cell lines were plated at \( \sim 1 \times 10^6 \) cells in 60-mm tissue culture dishes 1–2 days before fractions were separated. Cells were scraped off three to four dishes, combined, and pelleted. The cells were resuspended in ice-cold PBS and repelleted. The plasma membrane was disrupted by resuspending cells in a 10 mM Tris buffer containing NaCl (10 mM), MgCl\(_2\) (3 mM), and NP-40 (0.5%) and incubating for 1 min on ice before centrifuging. Nuclei were pelleted, and the supernatant (cytoplasmic fraction) was removed. Nuclei were washed once in ice-cold PBS with EGTA (1 mM) and then resuspended in the NP-40-containing buffer and sonicated to disrupt nuclear membrane. Debris was pelleted and the supernatant (nuclear fraction) removed.

**Immunoblots.** Transfected or wild-type cell lines were plated at \( 8 \times 10^5 \) cells in 35-mm dishes the day before protein collection. Plates were rinsed once in PBS and then scraped in the presence of warm lysis buffer. Lysates were heated to 95–100°C for 5 min and triturated through a 22-gauge \( \times \) 0.5-in. needle five to six times to shear the DNA before it was aliquoted and stored at \(-80^\circ\)C. Total protein content of lysates was measured using a Lowry protocol modified to accommodate a 96-well format (see Ref. 43). Equal protein loads (usually 20 \( \mu \)g of total protein) were subjected to SDS-PAGE separation in a 12% gel followed by transfer to nitrocellulose (Schleicher & Schuell). The transfer was confirmed with Ponceau S staining and the position of molecular weight markers identified. Membranes were blocked in TBS containing 5% nonfat dry milk and 0.1% Tween 20 (Bio-Rad Laboratories) for 1–2 h and then incubated with primary antibody overnight at 4°C. Membranes were rinsed three times in blocking solution for 5–15 min each, followed by incubation in the appropriate horseradish peroxidase-conjugated secondary antibody for 1–2 h at room temperature. Blots were developed with a chemiluminescence reagent kit (Amer- 

**Statistics.** Data were averaged using an Excel spreadsheet (Microsoft). Unless noted otherwise, the two-tailed Student’s paired \( t \)-test was performed. A significant difference is defined as \( P < 0.05 \); a highly significant difference is defined as \( P < 0.01 \). Error bars in graphs are \( \pm 1 \) SE.

**RESULTS**

**Subcellular targeting of CaMKII in PC12 cells.** To investigate the role that subcellular localization of CaMKII plays in neuronal differentiation, PC12 cells were transfected with transgenes (Fig. 1A) to produce an epitope-tagged \( \alpha \)-CaMKII targeted to concentrate in either the cytoplasm (Cyto-CaMKII) or the nucleus (NLS-CaMKII). Cytoplasmic localization is conferred by the native \( \alpha \)-CaMKII sequence, whereas nuclear localization was achieved by adding an SV40 NLS. To confirm proper targeting of the protein, transfected cells were fixed on glass coverslips and analyzed by immunofluorescence microscopy with an anti-FLAG primary antibody. This revealed that the protein produced by the Cyto-CaMKII construct retains its cytoplasmic localization, whereas the protein produced by the NLS-CaMKII construct accumulates exclusively in the nucleus (Fig. 1B). To generate stable cell lines expressing these proteins, the appropriate CaMKII plasmid was cotransfected into PC12 cells along with a neomycin resistance plasmid. Colonies were selected with G418 antibiotic and screened for appropriately targeted FLAG-tagged protein production by immunocytochemistry (as in Fig. 1B). Several colonies from each construct were identified for further analysis based on the presence of correctly targeted anti-FLAG staining. The identity of this protein as \( \alpha \)-CaMKII was confirmed by immunoblot analysis using an anti-\( \alpha \)-CaMKII antibody (Fig. 1C). PC12 cells lack the \( \alpha \)-isoform of CaMKII, expressing primarily the \( \gamma \) and \( \delta \)-isoforms (48). The absence of \( \alpha \)-CaMKII in control PC12 cells is shown in lanes 1 (untransfected) and 2 (mock transfected) of Fig. 1C. Lanes 3–5 show cell lines with nuclear targeted \( \alpha \)-CaMKII, which migrates slightly higher than cytoplasmic CaMKII, consistent with its extra eight amino acids coded for by the NLS. Lanes 6–8 show \( \alpha \)-CaMKII in cell lines with cytoplasmic targeting. These data confirm that the transfected PC12 cells express a properly localized, FLAG-tagged \( \alpha \)-CaMKII protein. Three independent clonal cell lines expressing cytoplasmic CaMKII (Cyto-A2, Cyto-I2, and Cyto-B4) and three lines expressing nuclear CaMKII (NLS-TC2, NLS-KB5, and NLS-KA1) were selected for further analysis.

The functional integrity of the \( \alpha \)-CaMKII transgenic protein was assessed by measuring the level of kinase activity in these transfected cell lines by use of an assay system based on the phosphorylation of an idealized CaMKII substrate, autocamtide III. When CaMKII becomes activated by Ca\(^{2+}\)/CaM, members of the holoenzyme complex cross-phosphorylate each other, allowing the CaMKII to remain partially active even after Ca\(^{2+}\) levels fall and CaM dissociates (1, 2, 24, 37). This ability of CaMKII to become Ca\(^{2+}\)-independent is thought to be important in allowing CaMKII to detect oscillations in intracellular Ca\(^{2+}\) concentration (22, 40). Therefore, both the total CaMKII activity and the percentage of that activity that was Ca\(^{2+}\)-independent were measured. Untransfected and mock transfected control cell lines show high levels of kinase activity from endogenous CaMKII isoforms, with 2.8–4% of that activity being Ca\(^{2+}\)-independent (Fig. 2A). CaMKII activity is significantly increased above endogenous levels in cell lines expressing cytoplasmic \( \alpha \)-CaMKII. The increase is from 88.4 \( \pm \) 8.6 pmol/min (untransfected cells) to a maximum of 235 \( \pm \) 46.6 pmol/min (Cyto-A2 line, Fig. 2A), but the percentage of Ca\(^{2+}\)-independent activity is unchanged from controls (Fig. 2A). In two of the nuclear CaMKII-expressing cell lines (NLS-TC2 and NLS-KB5), the
percentage of CaMKII activity that was Ca\(^{2+}\) independent was significantly greater than in control cells (Fig. 2A). However, the NLS-CaMKII cell lines do not show an increase in total CaMKII activity in whole cell lysate above control levels despite the fact that \(\alpha\)-CaMKII protein is being expressed in all of these lines (see Fig. 1C). One hypothesis to explain this discrepancy is that the initial CaMKII activity assay, using a pestle tissue grinder to break the cells apart, did not effectively lyse the nuclei; thus nuclear CaMKII might not have been accessible to the autocamtide III substrate. Alternatively, if the nuclei were lysed, it is possible that the CaMKII released may not have contributed significantly to the total cellular CaMKII pool. Because PC12 cells have significant endogenous \(\gamma\)- and \(\delta\)-CaMKII isoforms (48, 50) the nuclear targeted CaMKII, although potentially high in the microdomain of the nucleus, may have contributed little to the overall CaMKII activity. To address these issues, nuclear and cytoplasmic fractions of PC12 cells were prepared (see MATERIALS AND METHODS), and CaMKII kinase activity was assayed on each fraction separately. To verify isolation of the nuclear fraction, cells were incubated with Hoechst 33342 (to stain nuclear DNA) and visually inspected during the isolation procedure. A suspension of intact cells showed large, round, phase bright cells with well-defined nuclei (Fig. 2B, top). After the first separation step, which disrupted the plasma membrane releasing the cytoplasmic contents, only the nuclei were visible (Fig. 2B, middle). The nuclear fraction was then completely disrupted by brief sonication. An immunoblot probing with an antibody to \(\alpha\)-tubulin, a cytosolic structural protein that has been used as a marker for subcellular fractionation protocols, showed good separation of the two fractions (Fig. 2C). This ensured that the contents of the nuclei were fully released to the assay mix and that the CaMKII activity could be assayed in the microdomain of the nucleus. By use of this protocol, the level of total CaMKII activity (nuclear plus cytoplasmic fractions; data not shown) in all cell lines was consistent with the activity seen in the CaMKII assay run on whole cell lysate.

Fig. 2. CaMKII activity in the cell lines expressing targeted \(\alpha\)-CaMKII. A: CaMKII activity assayed in whole cell lysate from control cells (untransfected or mock transfected), cell lines expressing nuclear CaMKII (NLS-TC2, NLS-KB5, and NLS-KA1), and cell lines expressing cytoplasmic CaMKII (Cyto-A2, Cyto-I2, and Cyto-B4). Total CaMKII activity is graphed against the left axis, whereas Ca\(^{2+}\) -independent activity is graphed against the right axis. Data are averages of 3–8 independent experiments run in duplicate; error bars, SE. B and C: nuclear/cytoplasmic separation. B: photomicrographs of PC12 cells treated with Hoechst 33342 to visualize the nuclei. Photographs at left are phase contrast, those in middle use an epifluorescent microscope with a UV filter, and those at right are a merge of the 1st two. Top: intact PC12 cells scraped off their tissue culture dish. Bottom: isolated nuclei after treatment with an extraction buffer to break apart the plasma membrane. Cytoplasmic fraction was the supernatant of this pellet. Nuclear pellet was then washed and sonicated to disrupt nuclei, producing a nuclear fraction. Scale bar, 30 \(\mu\)m. C: immunoblots to confirm separation of nuclear and cytoplasmic fractions by using an antibody to the cytoplasmic structural protein, \(\alpha\)-tubulin. W, whole cell lysate; C, cytoplasmic fraction; N, nuclear fraction. D: CaMKII activity in subcellular fractions of representative cell lines NLS-TC2 and Cyto-A2 (expressing nuclear and cytoplasmic CaMKII, respectively). Fractions were prepared as in B, and CaMKII activity was assayed in each fraction as in A. Activity is expressed as percent total CaMKII activity (cytoplasmic + nuclear fractions) measured. Data are averages of 4 independent experiments run in duplicate. Error bars, SE.
lysate. For the untransfected control cells, 82% of the total CaMKII activity was found in the cytoplasmic fraction (Fig. 2D). In the cells expressing Cyto-CaMKII, the total CaMKII activity was significantly higher than in untransfected controls, consistent with the activity seen in unfractionated whole cell lysate. This increased activity was distributed similarly to the activity in control cells, with 76% in the cytoplasm and 24% in the nucleus (Fig. 2D). For the NLS-CaMKII cells, the total CaMKII activity was comparable to that of control cells, but the distribution was shifted to the nucleus. Over 40% of the activity was in that fraction, a significantly higher percentage than the activity in the nuclear fraction of untransfected control cells (Fig. 2D). This demonstrates that CaMKII activity can be increased in specific subcellular domains by expressing a targeted α-CaMKII protein.

Effect of targeted CaMKII on neuronal differentiation. NGF initiates differentiation of PC12 cells into a neuron-like phenotype characterized by a decrease in proliferation, an increase in catecholamine synthesis, and the appearance of long, branching neurites (18). To assess the role of targeted CaMKII in this differentiation process, cells expressing CaMKII in either the nucleus or the cytoplasm were treated with 50 ng/ml NGF for 6–7 days, and the extent of neurite formation was measured (see representative photomicrographs in Fig. 3A). The concentration of NGF chosen (50 ng/ml) causes ~50% of control cells to respond with neurite outgrowth; thus either an increase or decrease in neuronal differentiation could be detected. Untransfected and mock transfected PC12 cells were used as controls. To quantify neurite outgrowth, computer-aided image analysis (ImagePro) was used on enlarged photomicrographs to measure the single longest identifiable process coming from a given cell. By tracing along the neurite path, the computer-aided system allowed measurement of actual neurite length, which was taken from the cell body to the tip of the growth cone. Processes of 15 μm or longer were scored as neurites (see MATERIALS AND METHODS for full criteria of inclusion). Thus not only was the percentage of neurite-bearing cells measured but the lengths of neurites were also compared. After 6–7 days of NGF treatment, 52% of untransfected control cells and 63% of mock transfected cells had at least one defined neurite (Fig. 3B). All of the cell lines expressing Cyto-CaMKII had a slight, but not statistically significant, increase in the percentage of neurite-bearing cells (62–66%) compared with untransfected controls (52%). In contrast, the cells expressing NLS-CaMKII had very few neurites. Of the three independent cell lines studied, from 4 to 25% of the cells had any processes of 15 μm or longer (Fig. 3B), which was significantly fewer than controls. Analyzing the length of neurites, when formed, gives a similar picture: Cyto-CaMKII-expressing cells have longer neurites than controls, whereas NLS-CaMKII-expressing cells have significantly shorter neurites. Figure 3C shows histograms of combined neurite length data. Figure 3C, top, shows neurite length of control cell lines (untransfected and mock transfected combined). A total of 2,528 cells were analyzed, of which 1,502 cells (59%) had neurites. The largest percentage of cells (mode) had neurites of 22 ± 1 μm in length, whereas the average length was 41.2 ± 3.5 μm. Many cells had neurites up to, and occasionally longer than, 100 μm. Figure 3C, middle, shows neurites from all cell lines expressing Cyto-CaMKII. A total of 1,025 cells were analyzed, with 682 (66%) being neurite bearing. The mode of neurite length for these lines is slightly longer than controls, at 26–27 μm, whereas the average neurite length, at 48.6 ± 3.7 μm, was statistically increased over control cells (P < 0.001, Fig. 3C). Figure 3C, bottom, shows the combined data for cell lines expressing NLS-CaMKII. A total of 1,982 cells were analyzed, with 298 (15%) having neurites. When cells did grow neurites, these neurites generally just made the cutoff, with a mode of 15 μm and an average length of 30.7 ± 3.7 μm, significantly (P < 0.001) shorter than controls; very few neurites grew longer than 50 μm.

Thus, on the basis of the percentage of neurite-bearing cells, NGF-treated PC12 cells with nuclear CaMKII exhibit less neuronal differentiation than control cells. In addition, when these NLS-CaMKII cells do differentiate, they produce shorter neurites than controls. In contrast, treating cells that express Cyto-CaMKII with NGF did not decrease the percentage of neurite-bearing cells but did allow the formation of longer neurites.

Modulation of the MAPK pathway. Decreased neurite outgrowth in the NLS-CaMKII cells could be caused by a disruption in the integrity of the NGF signal transduction pathways, which are characterized by several parallel signaling cascades (36, 42, 53, 54). One effect of NGF binding to its high-affinity receptor (Trk A) on PC12 cells is the initiation of signaling cascades leading to the sustained (>2 h) phosphorylation of the MAPKs Erk1 and Erk2 (Ref. 36 and Fig. 5). The sustained activation of this pathway has been shown to be necessary for neuronal differentiation and neurite outgrowth in PC12 cells (26, 36). To address the integrity of this MAPK pathway, PC12 cells were cultured overnight in low-serum medium (to reduce background Erk phosphorylation) and then stimulated with NGF for 10–120 min before proteins were extracted. Immunoblots were run, probing for both total and phospho-Erk by 10.220.32.247 on June 24, 2017 http://ajpcell.physiology.org/ Downloaded from
This phosphorylation is accomplished by several intracellular signaling pathways (see reviews in Refs. 12 and 38) including two MAPK cascades in PC12 cells (52) and by PKA after increases in intracellular Ca^{2+} concentration (19). Phosphorylation at Ser^{133} is a necessary step for CREB activation, and activated CREB is important in the maintenance of neuronal cell populations (12). Because NGF can activate CREB through several different signaling pathways, the hypothesis was tested that either the total amount of CREB present or its phosphorylation at Ser^{133} in response to NGF was modified by targeted expression of NLS-CaMKII in PC12 cells. The results are shown in Fig. 3.

Fig. 3. Nerve growth factor (NGF)-induced neuronal differentiation in cell lines expressing targeted a-CaMKII. A: typical representative phase-contrast photomicrographs of control or transfected cell lines treated with NGF in serum-free medium. Untreated cells (column at left) were maintained in complete medium containing 10% fetal bovine serum but no NGF. Scale bar, 30 μm. B: quantification of neurite outgrowth, shown as percent neurite-bearing cells. Photomicrographs from 6 adjacent fields were taken for each cell line in each experiment. The longest individual process from each cell was measured and a cell defined as neurite bearing if it had ≥1 process of ≥15 μm in length. Data are averages of 3–7 independent experiments (indicated at base of each bar). Error bars, SE. C: histograms showing no. of cells with neurites of a given length as a percentage of all cells analyzed in a given group. Top: grouped data from untransfected and mock transfected cells; middle: data grouped from Cyto-A2, Cyto-I2, and Cyto-B4 cell lines; bottom: data from NLS-TC2, NLS-KA1, and NLS-KB5 cell lines. Average is mean of all cells ± SE. Statistical significance is established by 2-way ANOVA on log transformed data.
CREB can also be phosphorylated by CaMKII at Ser\textsuperscript{142} (28, 44), which may decrease CREB transcriptional activity in some contexts (13, 28, 51). A relevant hypothesis, then, is that nuclear CaMKII could phosphorylate CREB at Ser\textsuperscript{142} and thus block neuronal differentiation by inhibiting transcription of some CREB-responsive genes. To test this, proteins from control PC12 cells or PC12 cells with nuclear CaMKII were probed in an immunoblot for this phosphorylation (Fig. 5C). Rat cortical neurons, used as a positive control, show robust CREB Ser\textsuperscript{142} phosphorylation after 15 min of depolarization with KCl (Fig. 5C). Very little basal phosphorylation of CREB at Ser\textsuperscript{142} was observed in untransfected, NLS-CaMKII-transfected, or Cyto-CaMKII-transfected PC12 cells. Application of KCl to these cell lines appears to give a slight increase in CREB Ser\textsuperscript{142} phosphorylation, particularly in the NLS-CaMKII-expressing cells, indicating that this serine can be phosphorylated, given the correct stimulus. However, treating these cell lines with NGF (50 ng/ml for 15 min) failed to change CREB Ser\textsuperscript{142} phosphorylation consistently. Thus, although phosphorylation of Ser\textsuperscript{142} may be a critical step in regulating CREB activity in some contexts, the decreased neurite outgrowth in NLS-CaMKII PC12 cells is probably not due to an inhibitory phosphorylation at this site.

**DISCUSSION**

CaMKII mediates many signal transduction systems and has been found in virtually every cell type examined (for reviews see Refs. 1 and 24). It is particularly abundant in neuronal populations, where it has been implicated in such diverse functions as promoting neuronal survival and regulating long-term potentiation. Although it appears necessary for proper neuronal development, the exact mechanisms of CaMKII’s actions are still being defined.

In the area of neurite outgrowth, results of several studies have been inconclusive. A CNS cell line, CAD cells, which normally differentiate on serum withdrawal, underwent spontaneous neuronal differentiation after being transfected to produce CaMKII (9). In two neuroblastoma cell lines, overexpressing CaMKII led to an increase in both the percentage of cells with neurites and the neurite length (15). However, when CaMKII was overexpressed in PC12 cells, which were then stimulated with either NGF (31) or cAMP (48), neurite formation was inhibited. These latter two studies are similar to the experiments performed here and will be discussed separately.

Perhaps one reason for the incomplete picture arises from differences in the subcellular locations of various CaMKII splice variants. At least three CaMKII splice variants, α\textsubscript{B}, γ\textsubscript{A}, and δ\textsubscript{B}, contain NLSs (3, 10, 46). Some progress has been made in determining tissue distribution of these isoforms (10, 47). Two of the variants, α\textsubscript{B} and δ\textsubscript{B}, have been shown to concentrate in the nucleus of astrocytes (46), whereas the δ\textsubscript{B}-isoform has also been seen in cerebellar granule cells (47). Little is known about the role that these isoforms play in nor-
Normal cellular function. It is known that expression levels of various isoforms can change for a given cell type, depending on the stimuli to which the cells are exposed or the stage in their development (3, 8). Therefore, this study has set about specifically to investigate the role of nuclear targeted vs. cytoplasmic targeted CaMKII in neuronal differentiation with the PC12 cell line as a test system.

PC12 cells were transfected with an α-CaMKII gene, either containing or omitting an NLS. The CaMKII protein produced by these constructs is efficiently expressed in one of two subcellular locations, either the cytoplasm (Cyto-CaMKII) or the nucleus (NLS-CaMKII). Furthermore, the activity of CaMKII was increased in the appropriate compartment relative to either untransfected controls or cell lines with the opposite CaMKII targeting. These experiments demonstrate that functional CaMKII can be targeted to mimic various endogenous isoforms.

Control or transfected cell lines expressing Cyto-CaMKII or NLS-CaMKII were treated with a submaximal concentration of NGF (one that leads to ~50% of control cells differentiating and growing neurites within 6–7 days), and the percentage of neurite-bearing cells and neurite length were measured as an index of neuronal differentiation. In three independent clonal cell lines expressing Cyto-CaMKII, the percentage of neurite-bearing cells was similar to the percentage of neurite-bearing cells in untransfected or mock transfected PC12 cells. In addition, the length of neurites formed was increased compared with the control cell lines. These data indicate that an increase in cytoplasmic CaMKII above the endogenous levels found in PC12 cells does not inhibit neurite outgrowth.

This result is consistent with studies done on several neuroblastoma cell lines. When Nb2A (15, 41), NG108–15 (15), or CAD cells (9) are transfected with an α-CaMKII gene, the extent of neurite outgrowth after appropriate stimulation is markedly increased over untransfected control cells. Masse and Kelly (31), however, produced PC12 cell lines expressing α-CaMKII and showed a decrease in the percentage of neurite-bearing cells after NGF treatment. Several differences were observed in cellular growth properties, and one important difference in technique was noted between that study and this one. Their cell lines transfected with α-CaMKII showed a decreased initial replication rate compared with controls. The CaMKII-transfected cells studied here all proliferated similarly to untransfected controls (data not shown). In addition, Masse and Kelly reported increased cell-to-substrate adhesion in transfected cells compared with controls, something not seen in the cell lines used for the present study (data not shown). Experimentally, the culture medium used by Masse and Kelly to maintain their PC12 cells contained 10% horse serum and 5% fetal calf serum. In the present study, 10% FBS was used and no horse serum. Because serum is an undefined factor......
component known to contain numerous cytokines and growth factors, it seems reasonable that this difference in sera may have influenced the basal state of the cells. Therefore, the interaction of NGF signaling and CaMKII could be occurring in distinct intracellular contexts. These differences seem to include changes in cell-to-substrate adhesion, an important parameter for neurite growth.

Another group that has overexpressed α-CaMKII in the cytoplasm of PC12 cells, Tashima et al. (48), used cAMP to stimulate neurite outgrowth. They also saw a decrease in neurite-bearing cells in lines expressing α-CaMKII (48). However, when a different pathway from NGF is used, cAMP induces neurite outgrowth; the neurites appear much more rapidly and exhibit a different morphology (20, 35, 48). A similar effect to that of Tashima et al. was seen in the Cyto-CaMKII cells in our study. Neurite outgrowth was greatly reduced in Cyto-CaMKII cells following cAMP treatment compared with untransfected control cells (Kutcher LW, unpublished observation).

A novel feature of this study is employing an NLS to target CaMKII to the nucleus. When α-CaMKII is targeted to the nucleus of PC12 cells, a different picture emerges. In contrast to the Cyto-CaMKII-expressing cells, three independent clonal cell lines expressing NLS-CaMKII all showed significantly fewer neurite-bearing cells after NGF stimulation. When neurites did form in the NLS-CaMKII-transfected cells, they were shorter than the neurites formed in either control or Cyto-CaMKII-expressing cells. Thus, on the basis of the percentage of neurite-bearing cells and the length of neurites formed, nuclear targeted CaMKII inhibits NGF-induced neuronal differentiation in PC12 cells.

Several steps in the NGF-signaling cascade were evaluated to investigate the mechanisms leading to this phenomenon. NGF initiates neurite outgrowth in PC12 cells by a complex signaling pathway, with many sites of potential regulation (reviewed in Refs. 6, 27, and 45; see also Ref. 54). Binding of NGF to its high-affinity receptor, Trk A, initiates several intracellular events, including a transient increase in intracellular Ca$^{2+}$ concentration (33, 34) and the sustained phosphorylation of the MAPKs Erk1 and Erk2 (36, 53). This Erk activation is mediated via Ras (36, 54) and Rap1 (54) and is necessary for neurite outgrowth (26). Stimuli other than NGF, such as EGF and activation of CaM (11), can transiently phosphorylate Erk without initiating neurite outgrowth, suggesting that the sustained phosphorylation of Erk is critical to neurite induction. To evaluate the integrity of this MAPK-signaling cascade, both the total amount of Erk and its phosphorylation in response to NGF were evaluated in Cyto-CaMKII and NLS-CaMKII cell lines. These cell lines show robust phosphorylation of Erk after 10 min of NGF, which is maintained at levels similar to controls for 120 min. Therefore, neither immediate nor longer-term Erk phosphorylation is affected by the presence of either NLS-CaMKII or Cyto-CaMKII. Thus the observed differences in neuronal differentiation are not related to this NGF-stimulated pathway, at least to the point of Erk phosphorylation.

NGF-signaling cascades ultimately activate the transcription factor CREB. Several kinases, such as PKA, PKC, RSK2, and CaM kinases, phosphorylate CREB at Ser$^{133}$. This phosphorylation recruits coactivators into a signaling complex capable of driving transcription of genes that have the cAMP response element (CRE) in their promoters (see reviews in Refs. 7, 32, and 38). Xing et al. (52) have determined that NGF leads to the phosphorylation of CREB at Ser$^{133}$ by two distinct mechanisms. The first is via the sustained activation of Erk (discussed above), which activates the kinases RSK1, -2, and -3; blocking this pathway decreases, but does not eliminate, CREB Ser$^{133}$ phosphorylation. A second MAPK pathway, involving p38 MAPK activation of MAPK-activated protein kinase 2, is also initiated by NGF and leads to Ser$^{133}$ phosphorylation (52). In hippocampal cells, the upstream activator of CaMKII, CaM, can translocate to the nucleus, where it correlates with increased CREB phosphorylation (6). It has been suggested that a nuclear targeted isoform of CaMKII, δ3, may be involved in CREB regulation (47). Therefore, the hypothesis was tested that either total CREB or phospho-CREB Ser$^{133}$ was altered in NLS-CaMKII cell lines. Immunoblots probing for these proteins in NLS-CaMKII, Cyto-CaMKII, and control cell lines all show equivalent amounts of CREB, and the cell lines respond to NGF with equivalent levels of Ser$^{133}$ phosphorylation. Thus the inhibited neuronal differentiation in NLS-CaMKII cells does not arise from differences in either total CREB or its ability to be phosphorylated at a site critical for activation, Ser$^{133}$.

CREB can be phosphorylated at sites in addition to Ser$^{133}$. Phosphopeptide mapping shows that Ser$^{142}$ (28, 44) and Ser$^{143}$ (28) can both be phosphorylated by CaMKII. In vitro assays indicate that phosphorylation of Ser$^{142}$ inhibits dimerization of CREB and subsequent binding of the CREB-binding protein (CBP) (51). Because CBP is critical to the activated CREB complex (32, 38), phosphorylation at Ser$^{142}$ by CaMKII would be expected to inhibit CREB activity, which seems to happen in vitro. In cortical neurons stimulated with KCl (28) and in GH3 cells (44), full activity of a CREB/Gal4 reporter gene requires that the Ser$^{142}$ of CREB be mutated to an alanine.

Recent in vivo work, however, indicates that the picture may be more complex; CREB Ser$^{142}$ phosphorylation may actually increase gene transcription in certain contexts (13, 28). Gau et al. (13) produced transgenic mice with a Ser$^{142}$-to-Ala mutation in CREB. These animals had alterations in their circadian clock that correlated with decreased expression of c-Fos (13), a transcription factor controlled by CREB (14).

Because of the emerging role of Ser$^{142}$ phosphorylation in CREB activity, an antibody to phospho-CREB Ser$^{142}$ (28) was used to probe for this phosphorylation in rat cortical neurons, control PC12 cells, and NLS-CaMKII- and Cyto-CaMKII-expressing PC12 cells. Immunoblots of primary cortical neurons treated for 15
min with depolarizing concentrations of KCl show robust phosphorylation of CREB Ser142 similar to that seen by Kornhauser et al. (28). In control or transfected PC12 cells, the basal level of CREB Ser142 phosphorylation was very low, equivalent to untreated cortical neurons. There was no increase in this phosphorylation when the cells were treated with NGF and only a slight increase when they were depolarized with KCl. The data shown here indicate that, in PC12 cells, CREB is not phosphorylated at Ser142 in response to NGF. Furthermore, the presence of a nuclear localized CaMKII is not sufficient to drive that phosphorylation either with or without treatment. In addition, NGF-stimulated c-fos expression was not decreased by either NLS-CaMKII or Cyto-CaMKII (data not shown), indicating that CREB remains fully competent to drive gene expression in these cell lines. Therefore, the decreased neuronal differentiation in NLS-CaMKII-expressing PC12 cells does not seem to be due to an inhibitory phosphorylation of CREB at Ser142.

In conclusion, this study has used transgenes encoding targeted α-CaMKII to elevate CaMKII kinase activity in specific subcellular compartments and has shown that nuclear CaMKII inhibits NGF-induced neuronal differentiation of PC12 cells, whereas cytoplasmically localized CaMKII does not. The inhibition of differentiation is independent of MAPK activation, which is required for neurite outgrowth. The inhibited neurite outgrowth is also independent of the transcription factor CREB, as total CREB remained constant in cells with NLS-CaMKII compared with controls, nor was phosphorylation at Ser133 or Ser142 changed. Thus it would appear that the role of CaMKII in promoting or inhibiting neuronal differentiation depends on the subcellular location of the expressed CaMKII isoform. As more is learned about which neuronal populations express nuclear localized CaMKII isoforms and at what developmental stages these expressions occur, a more complete picture of the role for nuclear CaMKII in neurons will emerge.

We thank Dr. Maria Czyzyl-Krzeska (University of Cincinnati) for the gift of PC12 cells and Drs. Michael Greenberg and Jon Kornhauser (Harvard Medical School) for the anti-phospho-CREB Ser142 antibody. We also thank Dr. Linda Levin (University of Cincinnati) for expert advice with the statistical analysis.

This work was partially funded by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-46433 to J. R. Dedman.

REFERENCES


AJP-Cell Physiol • VOL 284 • JUNE 2003 • www.ajpcell.org


