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Overexpression of Ca^{2+}/Calmodulin-dependent Protein Kinase II in Neuro2a and NG108-15 Neuroblastoma Cell Lines Promotes Neurite Outgrowth and Growth Cone Motility

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To elucidate the functional role of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) in neuronal cells, we studied the phenotypic effects of overexpression of the CaM kinase II wild-type α subunit and a mutant enzyme α isoform (Ala-286), in which formation of the Ca^{2+}-independent form by autophosphorylation is markedly suppressed by replacement of Thr-286 with Ala, using Neuro2a (Nb2a) and NG108-15 neuroblastoma cell lines. The cDNAs inserted into the EcoRI site of pEF321 expression vector were introduced into Nb2a and NG108-15 cells with pEF321-neo (neo). Stable clones were obtained by G418 selection. The specific activities of CaM kinase II in α and Ala-286 transfectants were two to four times higher than those in non-transfectants and in cells transfected with neo alone. Indirect immunofluorescence using a monoclonal antibody specific to the CaM kinase II α isoform revealed that CaM kinase II was mainly localized in the perikaryal and dendritic cytoplasm of the α and Ala-286 transfectants. Immediately after plating, Nb2a and NG108-15 cells transfected with neo, α and Ala-286 cDNAs appeared round. Several hours after plating, α transfectants showed cell flattening and initiation of neurite outgrowth, and thereafter extended numerous long and branching neurites. Numerous filopodia protruded from flat growth cones, some of which were accompanied by extensive veil formation. Non- and neo transfectants remained round. In Ala-286 transfectants, however, the phenotypic changes were remarkably less than in α transfectants. In the NG108-15 α transfectant, 1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine, a selective inhibitor of CaM kinase II, suppressed neurite outgrowth to the level of the neo transfectant. These findings provide evidence that CaM kinase II plays a role in regulating neurite outgrowth and growth cone motility in the neuronal cells. The autophosphorylation of CaM kinase II is an essential event for this kinase to exert cellular functions efficiently.

[Key words: Ca^{2+}/calmodulin-dependent protein kinase II, neuroblastoma, neurite outgrowth, growth cone, overexpression, autophosphorylation]

Changes in cell shape and motility result from directed alteration of cytoskeletal dynamics, polymerization–depolymerization of filaments, and changes in their positions (Bray and Chapman, 1985; Letourneau et al., 1986). Calcium ions play an important role in these processes through regulating polymerization–depolymerization of specialized cytoskeletal proteins (Yamauchi and Fujisawa, 1983b, 1988; Bähler and Greengard, 1987; Lankford and Letourneau, 1989; Polak et al., 1991). In developing neurons, it has been demonstrated that motile growth cones have higher free Ca^{2+} levels than growth cones that spontaneously stop growing (Anglister et al., 1982; Conner, 1986; Cohan et al., 1987; Mattson and Kater, 1987; Goldberg, 1988). In the growth cone membrane, there are voltage-dependent Ca^{2+} channels and Ca^{2+} removal and/or Ca^{2+} channel blockers suppress both neurite outgrowth and growth cone movement (Anglister et al., 1982; Mattson and Kater, 1987; Goldberg, 1988). However, relationships between the intracellular Ca^{2+} concentrations and neurite outgrowth are controversial; various Ca^{2+}-mobilizing agents inversely suppress neurite outgrowth and growth cone motility in cultured *Helisoma* neurons (Cohan et al., 1987; Mattson and Kater, 1987; Polak et al., 1991) and in chick dorsal root ganglia (Lankford and Letourneau, 1989). To reconcile these apparently conflicting observations, an idea of a narrow concentration range of cytosolic free Ca^{2+} for optimal neurite elongation has been presented (Cohan et al., 1987; Mattson and Kater, 1987). Although all of these observations indicate a pivotal role for Ca^{2+} in controlling neuronal morphogenesis, the underlying molecular mechanisms remain to be determined.

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is abundant in neuronal tissues (Yamauchi and Fujisawa, 1980, 1983a; Kennedy and Greengard, 1981; Bennett et al., 1983; Goldenring et al., 1983). Because of its broad substrate specificity, CaM kinase II has been implicated in a variety of cellular events that utilize Ca^{2+} as a second messenger, such as excitation/secretion coupling (Llinás et al., 1985), neurotransmitter synthesis (Yamauchi and Fujisawa, 1983a), and cytoskeletal function (Goldenring et al., 1983: Yamauchi and Fujisawa, 1983b, 1988; Le Vine et al., 1986; see reviews, Nairn et al., 1985; Schulman, 1988; Colbran et al., 1989). The distinctive property of CaM kinase II is that autophosphorylation of its Thr-286 switches it to a Ca^{2+}/calmodulin-independent state, which may prolong the activity of the enzyme after intracellular Ca^{2+} has been restored to resting levels (Fong et al., 1989; Hanson et al., 1989; Soderling, 1990; Hagiwara et al., 1991; Ohsako et al., 1991). Despite many reports concerning the structure/function properties of CaM kinase II, the cellular function and physiological significance of the autonomy following autophosphorylation remain obscure.

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The expression of CaM kinase II is temporally correlated with development of the nervous system (Kelly et al., 1987; Rostas et al., 1988; Vallano and Beaman-Hall, 1989). In NG108-15 neuroblastoma cell lines, dibutyryl cAMP–induced differentiation of the cells increases the expression of CaM kinase II (Vallano and Beaman-Hall, 1989). However, the role of the expression of CaM kinase II in the phenotypic changes in the process of neuronal differentiation is still unknown.

To elucidate the role of CaM kinase II in neuronal cells, we introduced a cDNA of the wild-type CaM kinase II α isoform (α) and of a mutant α isoform (Ala-286), in which formation of Ca^{2+}-independent form following autophosphorylation is markedly suppressed by replacement of Thr-286 with Ala (Fong et al., 1989; Hanson et al., 1989; Ohsako et al., 1991), into two types of neuroblastoma, Nb2a and NG108-15, thus generating cell lines stably producing elevated levels of these kinases. We comparatively studied the phenotype of these transfectants, and here provide evidence that CaM kinase II plays a role in regulating neurite outgrowth and growth cone motility in these cells, and that autophosphorylation is an essential event for CaM kinase II to exert cellular functions efficiently.

Materials and Methods

Materials. Mouse neuroblastoma Neuro2a (Nb2a) cells (Narotzky and Bondareff, 1974) were obtained from American Type Culture Collection (ATCC CCL131), and mouse neuroblastoma × rat glioma hybrid (NG108-15) cells (Minna et al., 1972; Amano et al., 1974) were provided by Dr. T. Amano, Mitsubishi Kasei Institute for Life Science. Characterized fetal bovine serum (FBS) was obtained from GIBCO and the United Biotechnological Corp. Hypoxanthine, aminopterine, G418, and *p*-phenylendiamine were obtained from Sigma. The random primer extension labeling system and γ-^{32}P-ATP were purchased from DuPont/New England Nuclear. ^{125}I-labeled anti-mouse IgG $(Fab')_2$ and fluorescein-conjugated sheep anti-mouse IgG (FITC) were from Amersham International. 1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) was purchased from Seikagaku Corporation, Tokyo. Calmodulin was purified from the rat testis (Dedman et al., 1977). The monoclonal antibody specific to the α isoform of CaM kinase II was prepared as previously described (Ohsako et al., 1990). The CaM kinase II substrate peptide syntide 2 (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Gly-Leu-Pro-Gly-Lys-Lys) was synthesized using an Applied Biosystem peptide synthesizer and was purified by preparative reverse-phase HPLC on a C_{18} column as previously described (Hashimoto and Soderling, 1987). pEF321 expression vector was provided by Dr. S. Sugano, Institute of Medical Science, The University of Tokyo.

Cell culture. Nb2a and NG108-15 cells were cultured in 26 cm^2 plastic flasks containing 5 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 7% CO_2 and 93% air in the logarithmic stage of growth, and media were changed every 2–3 d as needed. NG108-15 cells were cultured in medium containing 0.1 mM hypoxanthine, 1 μM aminopterine, and 16 μM *d*-thymidine. Cell viability was approximately 99% as assessed by dye exclusion using trypan blue.

Expression of cDNA in neuroblastoma cell lines. The α and Ala-286 cDNAs were inserted into the EcoRI site of pEF321 expression vector in the sense direction under the control of human elongation factor 1-α (Ohsako et al., 1991). Transfections into Nb2a and NG108-15 cells were performed by the calcium phosphate precipitation technique as described previously (Ohsako et al., 1991). Cells transfected with pEF321-neo plasmid alone (neo) and α cDNA or Ala-286 cDNA combined with pEF321-neo plasmid were selected in the medium containing 0.4 mg/ml G418. After 3–5 weeks of growth in the medium, G418-resistant stable clones were isolated and maintained in the presence of G418. Transfection with neo alone caused no morphological changes in Nb2a and NG108-15 cells.

Assay of CaM kinase II. Cloned cells were cultured in 10 cm dishes to confluence and then harvested and sonicated in 200 μl/dish of 40 mM Tris-HCl, pH 7.6, containing 10% glycerol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μg/ml each of the antibiotic protease inhibitors antipain, leupeptin, and pepstatin A. After centrifugation at 18,500 × *g* for 30 min, the supernatant was collected. CaM kinase II activities in the supernatant were assayed by Ca^{2+}-dependent phosphorylation of syntide 2 for 1 min at 30°C (Ohsako et al., 1990). For the determination of Ca^{2+}-independent activity generated by autophosphorylation, CaM kinase II in the supernatant was autophosphorylated with nonradioactive ATP for 10 min at 0°C, and then aliquots were removed from the reaction mixtures. The Ca^{2+}-independent activities of the kinase were measured under the same conditions except that 1 mM EGTA was added in place of Ca^{2+}.

Northern blot analysis. Total RNA was extracted from the transfected cells (10 cm dish) using hot acid phenol–SDS method as previously described (Yamauchi et al., 1989). The RNA was electrophoresed on a 1% agarose formaldehyde gel and transferred to Gene Screen Plus. The filter was hybridized at 42°C in 50% formamide, 1 M NaCl, 10% dextran sulfate, 1% SDS, 0.15 mg/ml of denatured salmon sperm DNA, and the ^{32}P-labeled EcoRI/PvuII fragment (nucleotides −123 to 1068) of α cDNA prepared in a random primer extension labeling system. After 24 hr of hybridization, the filter was washed in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) at room temperature for 30 min and then twice in 0.2× SSC containing 1% SDS at 65°C for 30 min, and exposed to X-ray film at −80°C.

Western blot analysis. Western blot analysis was carried out as described previously (Yamauchi et al., 1989). The crude supernatant of transfected cells was separated by 10% polyacrylamide–SDS gels (about 40 μg of protein/lane) and electrophoretically transferred to a nitrocellulose filter. The filter was preincubated with 5% non-fat dry skim milk in Tris-buffered saline, pH 7.6 (TBS), and then incubated for 2 hr with the anti-CaM kinase II antibody (Ohsako et al., 1990). After washing with TBS containing 0.1% Tween 20, the filter was incubated with ^{125}I-labeled anti-mouse IgG $(Fab')_2$ (2 μCi/ml) for 1 hr. Radioactive bands were detected by autoradiography.

Immunofluorescence. Nb2a or NG108-15 cells were mildly removed from the surface of dishes by treatment with calcium-magnesium–free DMEM for 10 min and collected by centrifugation for 5 min at 500 × *g*. Cells were resuspended in the normal culture medium and plated on a collagen (0.1%)-coated coverslip in normal DMEM medium at a density of 2–5 × 10^4/cm^2. Cells grown on the slips were washed with phosphate-buffered saline (PBS; 20 mM Na_2HPO_4-NaH_2PO_4, pH 7.4, 0.9% NaCl), fixed in cold ethanol (−20°C) for 5 min and then in cold 80% ethanol, 20% acetone (−20°C) for 5 min. The coverslips were dried for 10 min and incubated in the anti-CaM kinase II antibody solution (pH 7.4) for 1 hr at 37°C. After rinsing the antibody with PBS, the coverslips were incubated in FITC-conjugated anti-mouse IgG solution (1:30 dilution) for 1 hr at 37°C. After rinsing, the coverslips were mounted on glass slides using glycerin PBS solution containing *p*-phenylenediamine (1 mg/ml) to reduce photobleaching of the fluorochrome. CaM kinase II was localized using a Nikon fluorescence microscope. Images were photographed using a Kodak TMAX 400.

Assessments of neurite outgrowth and growth cone morphology. At an appropriate period of time after plating on 60 mm tissue culture grade culture dishes, cells were examined and photographed on a Nikon Diaphot inverted microscope with phase-contrast optics using a Kodak TMAX 100. KN-62, when used, was added to the culture media immediately after plating. Multiple random views were selected. Three parameters of neurite outgrowth were assessed: neurite formation, neurite length, and growth cone structures. Neurite formation was quantified by scoring the number of cells possessing neurites and expressing them as a percentage of the totals. Neurites were defined as process extensions greater than one cell body in length (Heidemann et al., 1985). Neurite length for a given cell was quantified as the average radial distance from the neurite tips to the soma. Growth cone motility was assessed by the number of filopodia per growth cone, as these parameters are known to reflect the underlying activity of the growth cone cytoskeleton (Bray and Gilbert, 1981). Cell clumps containing more than five cells were not counted in the results. Dunnett's multiple comparison test was used for all statistical comparisons, and the values were expressed as means ± SEM.

Results

Expression of α and Ala-286 CaM kinase II in Nb2a and NG108-15 cells

Each of the cell lines stably overexpressing CaM kinase II was assayed for Ca^{2+}- and calmodulin-dependent protein kinase activity using the crude supernatant of cell homogenate. All the cell lines generated by transfection with α and Ala-286 cDNAs showed a two- to fourfold increase in CaM kinase II activity

Table 1. Specific activity of CaM kinase II in transfected cells

Clone	CaM kinase II activity (nmol/min/mg protein)
Nb2a	
Nontransfectant	1.90 ± 0.07
neo.1	1.61 ± 0.30
neo.2	1.52 ± 0.36
α.1	4.12 ± 0.15**
α.2	4.27 ± 0.32**
Ala-286.1	4.49 ± 0.54**
Ala-286.2	3.55 ± 0.21**
Ala-286.3	4.18 ± 0.14**
NG108-15	
Nontransfectant	1.16 ± 0.20
neo.1	1.57 ± 0.28
neo.2	1.37 ± 0.16
neo.5	1.00 ± 0.11
α.5	5.77 ± 0.21**
α.13	2.00 ± 0.20*
α.22	2.18 ± 0.26*
Ala-286.1	5.90 ± 0.20**
Ala-286.7	3.64 ± 0.18**
Ala-286.15	3.12 ± 0.15**

CaM kinase II activity of the crude supernatant of transfectants was determined as Ca^{2+}-dependent phosphorylation of syntide 2. Each value represents mean ± SEM of three estimations. *, $p < 0.05$, **, $p < 0.01$, compared with corresponding nontransfectant.

Table 2. Comparison of Ca^{2+}-independent activities of α and Ala-286 kinases generated by autophosphorylation in transfected Nb2a and NG108-15 cells

Clone	Ca^{2+}-independent activity (%)
Nb2a	
neo.2	4.9 ± 3.1
α.2	49.7 ± 0.9*
Ala-286.1	1.7 ± 1.2**
NG108-15	
neo.1	2.4 ± 0.8
neo.2	5.9 ± 2.5
α.5	10.5 ± 0.2†
α.22	23.2 ± 8.9††
Ala-286.1	1.4 ± 0.9‡
Ala-286.7	0.4 ± 0.1‡

The kinases in the supernatant were autophosphorylated with nonradioactive ATP for 10 min at 0°C, and then the Ca^{2+}-independent activities of the kinases were measured in the absence of Ca^{2+} and calmodulin as described under Materials and Methods. Each value represents the percentage of Ca^{2+}-dependent CaM kinase II activity without autophosphorylation. Values are the means ± SEM of three estimations.

* $p < 0.01$, compared with neo.2 clone.

** $p < 0.01$, compared with α.2 clone.

† $p < 0.05$, compared with neo.1 clone.

†† $p < 0.01$, compared with neo.1 and neo.2 clones.

‡ $p < 0.01$, compared with α.5 and α.22 clones.

when compared with control non-transfected cells or cells transfected with neo alone (Table 1). Non- and neo transfectants showed low activity that probably represents an endogenous protein kinase activity.

The α and Ala-286 kinases in crude extracts of Nb2a and NG108-15 cells had comparable total activities when assayed in the presence of Ca^{2+}. However, the levels of Ca^{2+}-independent activities of Ala-286 kinase were significantly lower than those of α kinases, when these kinases were autophosphorylated and then assayed in the absence of Ca^{2+} (Table 2). This is consistent with our previous results obtained with the enzymes overexpressed in Chinese hamster ovary (CHO) cells (Ohsako et al., 1991), although the value of relative Ca^{2+}-independent activity of the kinases in CHO cells appeared to be higher than that estimated in this study.

In Northern blot analysis, the α mRNA was detectable in cells transfected with α and Ala-286 cDNAs, but not in non- and neo transfectants under our experimental conditions (Fig. 1). The size of the α mRNA was approximately 18 S and was calculated to be 1.8 kb in length, consistent with the size of the introduced cDNA. Immunoblotting demonstrated increased levels of CaM kinase II in the transfected cell lines (Fig. 2). These results indicate that introduction of α and Ala-286 cDNAs resulted in expression of the representative mRNAs and proteins in the transfected cells. There seemed to be a rough correlation between the CaM kinase II enzyme activity, the amount of hybridizable RNA and the amount of immunoreactive protein.

Immunofluorescence detection of α and Ala-286 CaM kinase II in transfected Nb2a and NG108-15 cells

Ten minutes after plating, Nb2a and NG108-15 transfected with neo alone, α, and Ala-286 cDNAs were round in appearance (data not shown). From 1 to 24 hr after plating, α and Ala-286 transfectants showed cell flattening and initiated neurite outgrowth, while control nontransfectants and neo transfectants remained round (Figs. 3, 4). No morphological differences between non-transfectants and neo transfectants were observed. CaM kinase II immunoreactivity was clearly detectable in the perikaryal and dendritic cytoplasm of α and Ala-286 transfectants. Immunoreactivity was absent from the nuclei, although dotted nuclear staining was seen in some cells (Figs. 3, 4). The subcellular localization of CaM kinase II expressed in the neuroblastoma cells was almost comparable to that in rat brain neurons (Ouimet et al., 1984). The non- and neo transfectants showed only diffuse background staining (Figs. 3*D*, 4*C*). The phenotypic effects of overexpression of CaM kinase II were similarly observed in all of the independent clones (Table 3). A significant positive correlation was seen between the CaM kinase II activities and percentage of cells with neurites ($r = 0.983$, $n = 5$, $p < 0.01$) or neurite length ($r = 0.975$, $n = 5$, $p < 0.01$) of independent Nb2a neo and α transfectants. However, the number of morphological changes such as cell flattening and neurite outgrowth observed in the Ala-286 transfectant appeared to be far less than those in the α transfectant (Fig. 3*B,C,E,F*). These differences in the phenotype of α and Ala-286 transfectants were similarly observed in all of the independent clones (Table 3). In both Nb2a and NG108-15 cells, the growth rate and cell attachment to the substratum were not altered in α and Ala-286 transfectants when compared with control neo transfectants.

Neurite outgrowth in NG108-15 cells transfected with α and Ala-286 cDNAs

Figure 5 shows typical time-lapse photographs of NG108-15 cells transfected with control neo, α, and Ala-286 cDNAs. Thirty minutes after plating, all transfectants appeared round. Thereafter, however, we found a large difference in neurite initiation

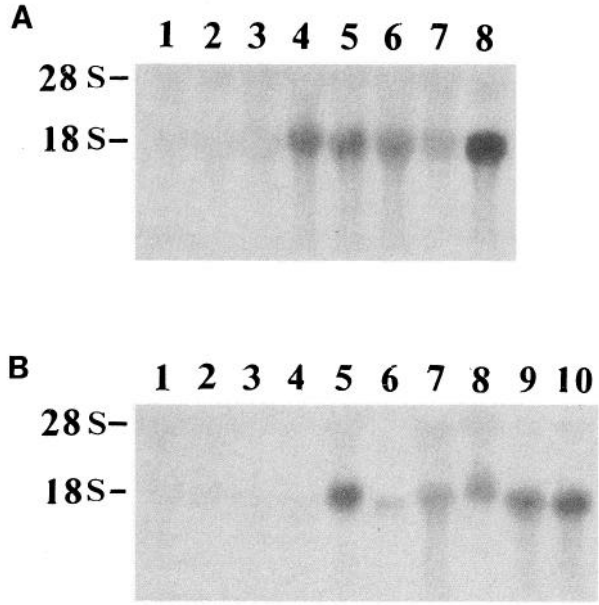

Figure 1. Northern blot analysis of transfected Nb2a and NG108-15 cells. RNA (15 μg) from the transfected cells was hybridized with ^{32}P-labeled probe specific for α cDNA (nucleotides −123 to 1068). *A*, Nb2a: *lane 1*, nontransfectant; *lanes 2* and *3*, neo clones; *lanes 4* and *5*, α clones; *lanes 6–8*, Ala-286 clones. *B*, NG108-15: *lane 1*, nontransfectant; *lanes 2–4*, neo clones; *lanes 5–7*, α clones; *lanes 8–10*, Ala-286 clones.

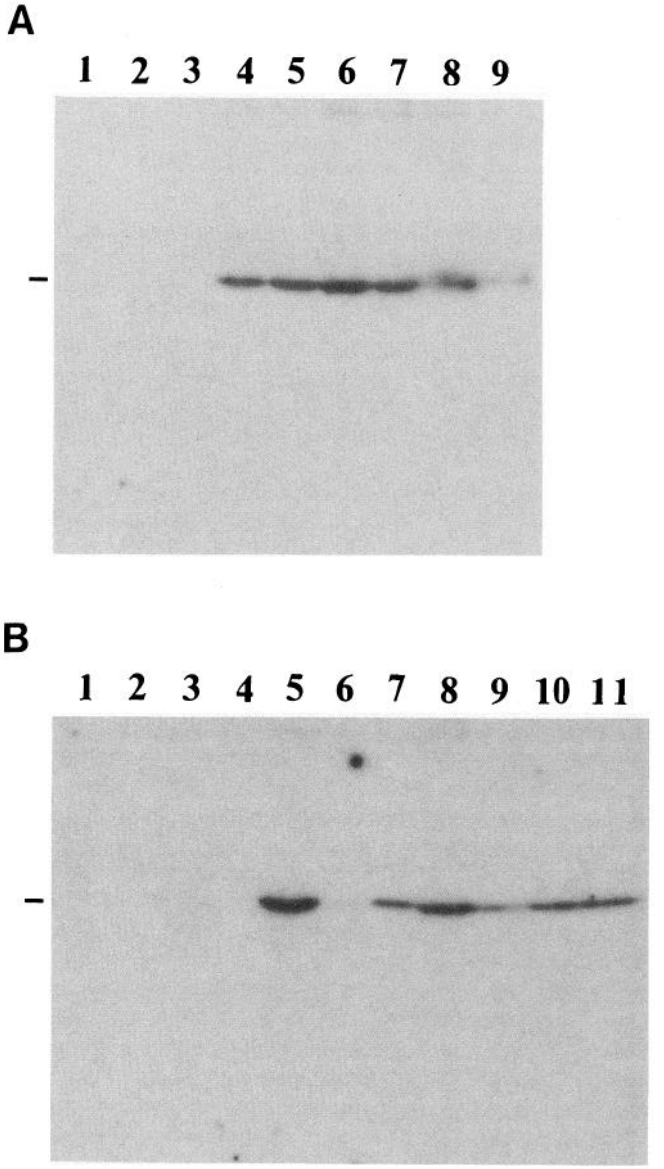

Figure 2. Western blot analysis of transfected Nb2a and NG108-15 cells. The crude supernatants (about 50 μg of protein) of transfected cells were separated on SDS–polyacrylamide slab gels and immunoblotted. *A*, Nb2a: *lane 1*, nontransfectant; *lanes 2* and *3*, neo clones; *lanes 4* and *5*, α clones; *lanes 6–8*, Ala-286 clones; *lane 9*, brain CaM kinase II. *B*, NG108-15: *lane 1*, nontransfectant; *lanes 2–4*, neo clones; *lanes 5–7*, α clones; *lanes 8–10*, Ala-286 clones; *lane 11*, brain CaM kinase II. The position of CaMKIIα polypeptide is marked at *left* in *A* and *B*.

and extension among them. These photographs show that the transfectant (α.5 clone) extended numerous long neurites that were often branched (Figs. 5*B*, 6*C*). The changes in phenotype of these transfectants were similarly observed in all the independent clones (α.13 and α.22) as shown in Table 3. On the other hand, neo transfectants (neo.1, neo.2, and neo.5) rarely extended neurites; when present, they were usually short. Again, a significant positive correlation ($r = 0.965$, $n = 7$, $p < 0.01$) was seen between the CaM kinase II activities and the neurite length of independent NG108-15 neo and α transfectants, though positive correlation between CaM kinase II activities and percentage of cells with neurites was not statistically significant ($r = 0.55$, $n = 7$). The specific activities of CaM kinase II in the NG108-15 α and Ala-286 clones were almost comparable. However, the extent of neurite formation and outgrowth in Ala-286 transfectants was far less than that in α transfectants. In contrast to Nb2a, the neurite length of Ala-286 transfectants was not statistically different from that of α transfectants (Table 3). The neurite length does not appear to be as consistently affected by the formation of Ca^{2+}-independent form of CaM kinase II as the percentage of cells with neurites in NG108-15.

Growth cone morphology of Nb2a and NG108-15 cells transfected with α and Ala-286 cDNAs

The promoted neurite outgrowth in α and Ala-286 transfectants was accompanied by morphological changes in the growth cone. In NG108-15 (α.5 clone) and Ala-286 (Ala.286.1 clone) transfectants, growth cones were flat with numerous protruding filopodia (Fig. 6*C*), and some growth cones had extensive veil formation (Fig. 6*D*). The changes in the growth cone morphology were similarly observed in all of the independent clones (Table 4). On the other hand, in control neo transfectants (neo.1), growth cones were narrow, club shaped (Fig. 6*A*), and possessed few filopodia (Fig. 6*B*). Transfection with neo alone did not affect growth cone morphology. The number of filopodia per growth cone roughly correlated with the specific activities of CaM kinase II in independent neo and α transfectants ($r = 0.834$, $n = 7$, $p < 0.05$). This parameter in the Ala-286 transfectant was substantially smaller than that in α transfectant, which was consistent with the changes of neurite outgrowth (Table 3). Similar morphological changes in growth cone were observed with Nb2a (data not shown).

Effect of KN-62 on neurite outgrowth in NG108-15 cells transfected with α cDNA

KN-62, a specific CaM kinase II inhibitor, concentration-dependently suppressed neurite length at 5 hr after plating in the

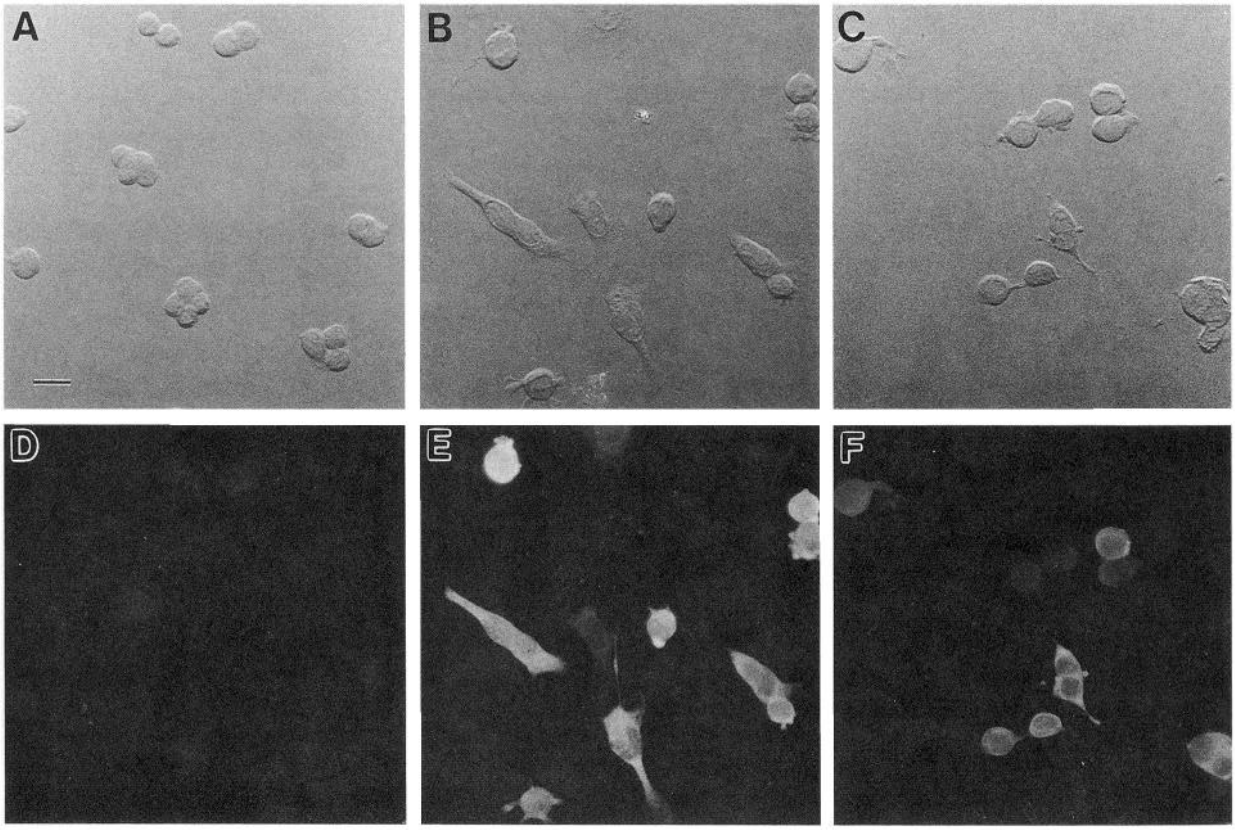

Figure 3. Immunofluorescence detection of CaM kinase II α isoform in Nb2a transfected with neo alone, α, and Ala-286 cDNAs. The transfectants neo.1 (*A, D*), α.1 (*B, E*), and Ala-286.1 (*C, F*) were plated on collagen-coated coverslips. Cells were fixed and examined 4 hr after plating. The corresponding fields show Nomarski images (*A–C*) and immunofluorescence staining with a monoclonal antibody to CaM kinase II α isoform (*D–F*). Note that cells flatten and initiate neurites in α and Ala-286 cDNA transfectants, whereas cells transfected with neo alone remain round. Scale bar, 10 μm.

α.5 clone to the level of neo.2 clone (Fig. 7). Treatment with KN-62 also decreased, in a concentration-dependent manner, the percentage of cells with neurites, and the number of filopodia per growth cone in the α.5 clone (data not shown). Cell viability and cell attachment to the substratum were not affected by KN-62 under the experimental conditions.

Discussion

In the present study, we established a series of neuroblastoma cell lines, Nb2a and NG108-15, that stably overexpressed CaM kinase II α and its mutant Ala-286. In the cells overexpressing CaM kinase II, neurite extension, neurite branching, and growth

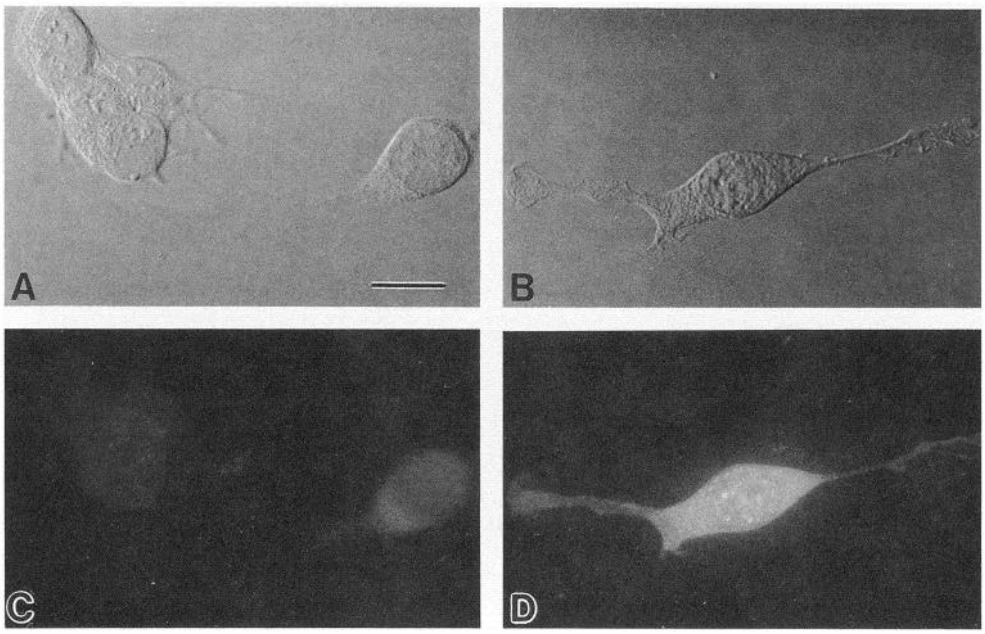

Figure 4. Immunofluorescence detection of CaM kinase II in NG108-15 cells transfected with neo alone and α cDNA. The cells transfected with neo alone (neo.1 clone) (*A, C*) and α cDNA (α.5 clone) (*B, D*) were fixed and photographed 24 hr after plating. Corresponding fields show Nomarski images (*A, B*) and immunofluorescence staining with a monoclonal antibody to the CaM kinase II α isoform (*C, D*). Note the increased immunoreactivity in perikaryal and dendritic cytoplasm of the α transfectant. Scale bar, 10 μm.

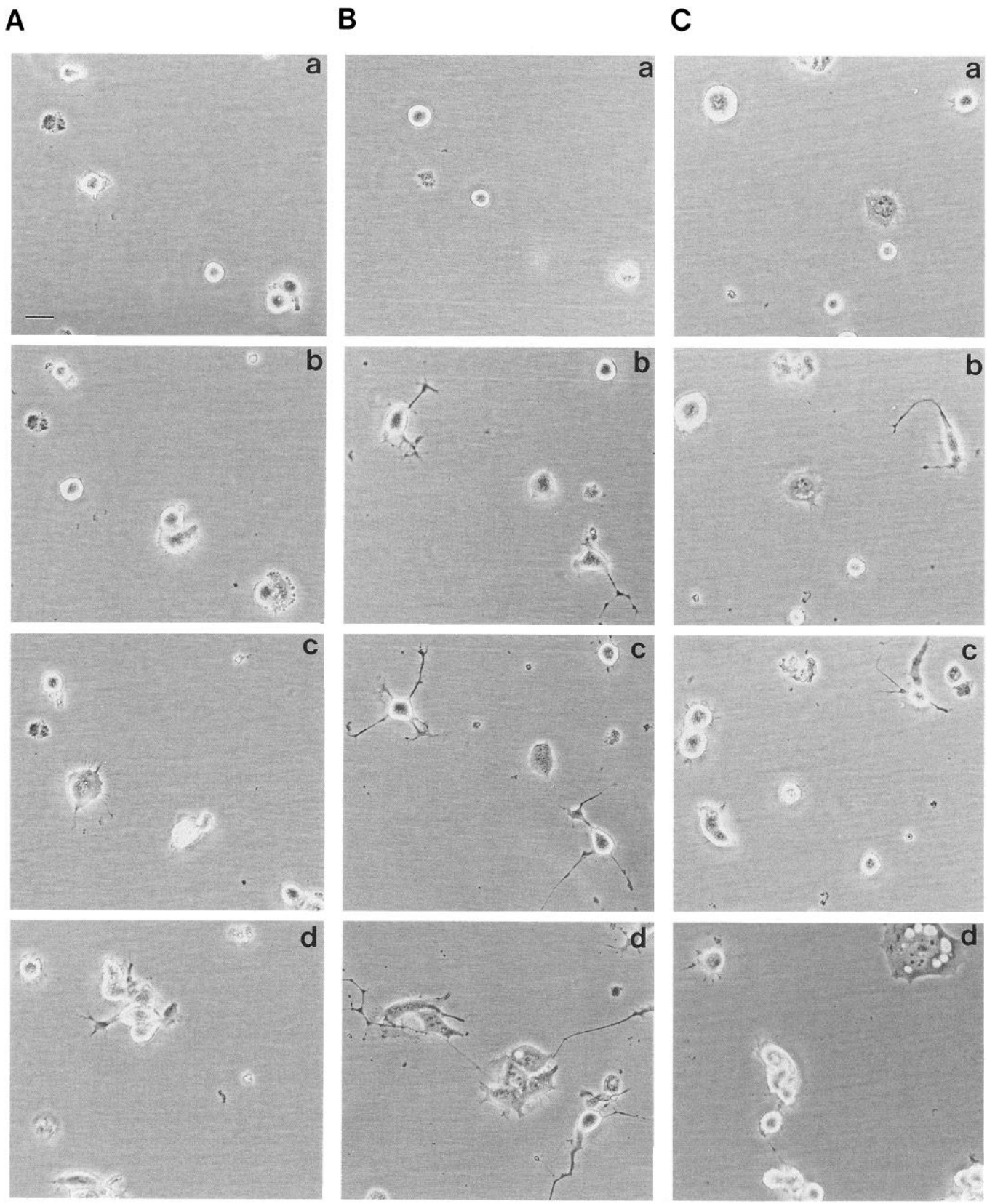

Figure 5. Morphological comparison of NG108-15 cells transfected with neo alone, α, and Ala-286 cDNAs. Shown are phase-contrast micrographs of the neo.1 (*A*), α.5 (*B*), and Ala-286.1 (*C*) clones at 30 min (*a*) and 4 (*b*), 8 (*c*), and 24 (*d*) hr after plating. Scale bar, 10 μm.

Table 3. Neurite outgrowth in transfected Nb2a and NG108-15 cells

Clone	Cell with neurite (%)	Neurite length (μm)
Nb2a		
Non-transfectant	2.7 ± 0.8 (3)	4.7 ± 0.50 (25)
neo.1	2.0 ± 0.3 (3)	4.4 ± 0.36 (26)
neo.2	1.9 ± 0.2 (3)	5.0 ± 0.56 (27)
α.1	11.6 ± 1.9 (3)**	8.1 ± 1.39 (27)*
α.2	15.4 ± 3.1 (3)**	9.4 ± 1.03 (27)**
Ala-286.1	7.2 ± 1.6 (3)†	5.7 ± 0.56 (27)‡
Ala-286.2	5.9 ± 1.7 (3)†	5.3 ± 0.42 (26)‡‡
Ala-286.3	3.7 ± 0.9 (3)††	5.3 ± 0.47 (21)‡
NG108-15		
Non-transfectant	6.5 ± 0.4 (4)	11.4 ± 1.4 (50)
neo.1	8.7 ± 0.5 (3)	8.4 ± 1.2 (32)
neo.2	10.4 ± 1.8 (3)	9.3 ± 1.1 (25)
neo.5	5.2 ± 0.8 (3)	7.2 ± 0.5 (40)
α.5	21.5 ± 1.5 (3)**	33.3 ± 4.2 (35)**
α.13	19.9 ± 3.7 (3)**	14.9 ± 1.6 (40)
α.22	29.5 ± 1.5 (3)**	18.7 ± 1.3 (40)*
Ala-286.1	13.7 ± 1.1 (3)*§	30.3 ± 7.8 (18)**
Ala-286.7	9.0 ± 1.1 (3)§§	16.2 ± 1.2 (40)
Ala-286.15	8.5 ± 0.6 (3)§§	11.2 ± 0.9 (31)

Each value represents mean ± SEM. The number of estimations is given in parentheses. Cell counts and measurement of neurite length were made 4 hr after plating. In NG108-15 cells, neurite length was evaluated 8 hr after plating.

* $p < 0.05$, ** $p < 0.01$, compared with corresponding control non-transfectant.

† $p < 0.05$, compared with α.2 clone.

†† $p < 0.05$, compared with α.1 and α.2 clones.

‡ $p < 0.05$, compared with α.2 clone.

‡‡ $p < 0.01$, compared with α.1 and α.2 clones.

§ $p < 0.05$, compared with α.5 and α.22 clones.

§§ $p < 0.01$, compared with α.5, α.13 and α.22 clones.

Table 4. Growth cone filopodia in transfected NG108-15 cells

Clone	Filopodia/ growth cone
Non-transfectant	4.4 ± 0.40
neo.1	4.9 ± 0.38
neo.2	4.9 ± 0.61
neo.5	4.2 ± 0.41
α.5	9.2 ± 0.50*
α.13	8.0 ± 0.80*
α.22	7.2 ± 0.62*
Ala-286.1	7.7 ± 0.44*
Ala-286.7	5.5 ± 0.25†
Ala-286.15	4.8 ± 0.35††

Each value represents the mean ± SEM of determinations on 21 growth cones. Filopodial counts were made 24 hr after plating.

* $p < 0.01$, compared with control non-transfectant.

† $p < 0.01$, compared with α.5 and α.13 clones.

†† $p < 0.01$, compared with α.5, α.13, and α.22 clones.

cone motility were remarkably promoted when compared with the morphology in control neo transfectants. This provides evidence that the expression of CaM kinase II is causally related to some of the phenotypic changes associated with neuronal differentiation. This is consistent with the finding that CaM kinase II expression in neuronal cells correlates temporally with their differentiation (Kelly et al., 1987; Rostas et al., 1988; Vallano and Beaman-Hall, 1989). However, there were no differences between the growth rates in the control and CaM kinase II transfectants. This suggests that the expression of CaM kinase II per se could not be sufficient for cell differentiation, because differentiated neurons characteristically stop dividing.

Morphological changes in the transfectants were seen in all of the independent clones overexpressing CaM kinase II but in neither the control non- nor neo transfectants, indicating the specific effects of overexpressing CaM kinase II on the phenotype of the cells. This is also supported by our findings that specific activities of wild-type α kinase in the transfectants were correlated with the extent of neurite elongation and growth cone motility, and that these changes were similarly observed in the two neuroblastoma cell lines, Nb2a and NG108-15. Furthermore, the overexpression of CaM kinase II might not have nonspecific cellular effects on the transfectants: CaM kinase II is endogenously expressed in NG108-15 cells, and the activity of the kinase increases by two- to fourfold in differentiated cells (Vallano and Beaman-Hall, 1989). Involvement of Ca^{2+}/calmodulin-dependent protein kinases in neurite extension is in line with previous findings that Ca^{2+}-mobilizing agents promote growth cone motility and neurite outgrowth in neuroblastoma cell lines (Schubert et al., 1978; Anglister et al., 1982; Sobue and Kanda, 1989) and in *Aplysia* neurons (Goldberg, 1988).

An isoquinoline sulfonamide derivative, KN-62, at 0.75 and 1.5 μM concentration-dependently inhibited neurite outgrowth

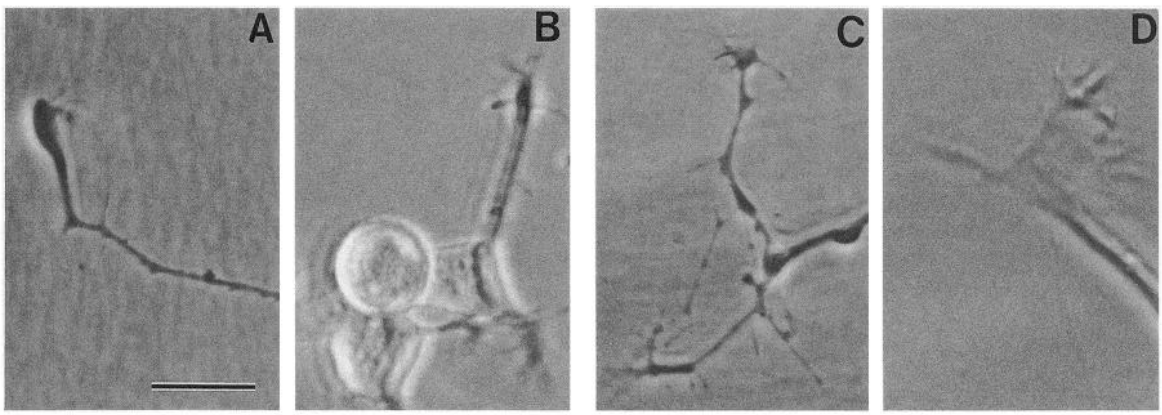

Figure 6. Growth cone morphology of NG108-15 cells transfected with neo alone and α cDNA. The transfected cells neo.1 (*A, B*) and α.5 (*C, D*) were photographed 24 hr after plating. Scale bar, 10 μm.

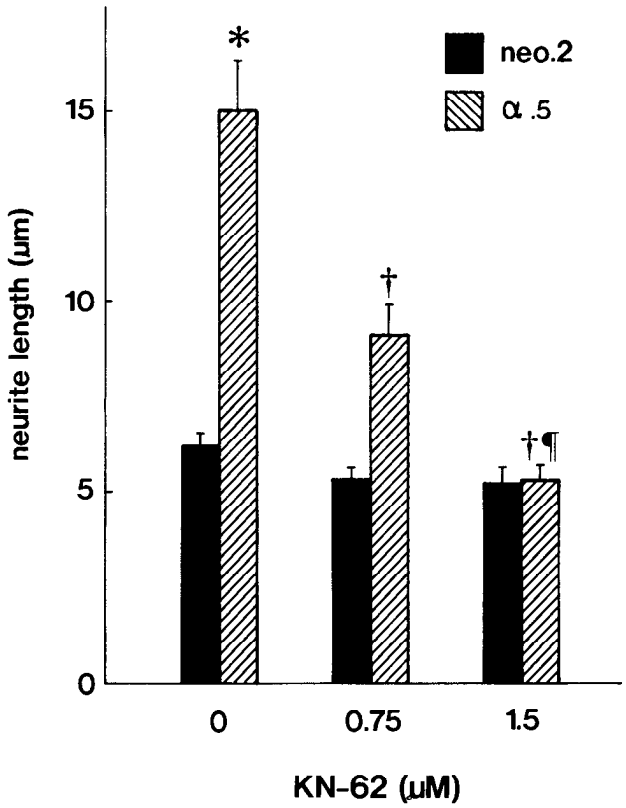

Figure 7. Effect of KN-62 on neurite length in NG108-15 cells transfected with neo alone and α cDNAs. Transfected cells neo.2 and α.5 were plated in the presence or absence of KN-62. Five hours after plating, cells were photographed and neurite length was quantified. Each *bar* represents the mean of 30 separate estimations ± SEM. *, $p < 0.01$, compared with neo.2 clone in the absence of KN-62. †, $p < 0.01$, compared with α.5 in the absence of KN-62. ¶, $p < 0.05$, compared with α.5 at 0.75 μM KN-62.

in the α transfectant. Tokumitsu et al. (1990) demonstrated that KN-62 at micromolar concentrations inhibits more than 80% of CaM kinase II activity without affecting myosin light chain kinase, protein kinase A, and protein kinase C activities. Furthermore, a binding study using KN-62 Sepharose affinity chromatography revealed that KN-62 binds directly to CaM kinase II but not to calmodulin. Thus, the inhibitory effect of KN-62 on neurite outgrowth further demonstrates that the phenotypic changes in the transfectants are due to specific activity of CaM kinase II. The effective concentration of KN-62 in our study was similar to that inhibiting *in vivo* activities of CaM kinase II in PC12 cells (Tokumitsu et al., 1990).

The phenotypic changes in Ala-286 transfectants were significantly less than those in α transfectants. These two kinases had comparable total activities when assayed *in vitro* in the presence of Ca^{2+}. However, the Ca^{2+}-independent activity following autophosphorylation in Ala-286 kinase was significantly lower compared to that in α kinase. This is consistent with the fact that mutation of Thr-286 to Ala suppresses autophosphorylation and blocks formation of Ca^{2+}-independent form of this enzyme (Fong et al., 1989; Hanson et al., 1989; Hagiwara et al., 1991; Ohsako et al., 1991). Hence, the difference between the phenotypes of α and Ala-286 clones indicates that autophosphorylation of CaM kinase II is a prerequisite to the cellular function of this kinase. This is in agreement with kinetics of autophosphorylation of the cytoskeletal form of CaM kinase II. At the cellular level of calmodulin, the activation of autophosphorylation is a cooperative function of Ca^{2+}, which results in enhancement of the sensitivity of the enzyme to small changes in intracellular Ca^{2+} levels (Le Vine et al., 1986).

It is noteworthy that the phenotypic effects of overexpressing CaM kinase II were observed in cells within several hours after plating, without exogenous Ca^{2+} mobilizing agents. In neutrophils, there is a rapid and transient cytosolic Ca^{2+} elevation reaching peaks of 800 nM upon attachment of the cells to a substratum (Jaconi et al., 1991). At the intracellular concentrations of calmodulin, essentially complete activation of CaM kinase II occurs at physiological levels of Ca^{2+} with an EC_{50} of 180 nM (Le Vine et al., 1986). Thus, flattening and neurite initiation after plating in the transfectants could be triggered by Ca^{2+} elevation upon attachment of the cells to the substratum, which may in turn activate CaM kinase II (Fig. 3). High levels of cytosolic free Ca^{2+} concentrations are also noticeable in motile growth cones. In cultured neurons from embryonic rat brain or snail *Helisoma,* active growth cones have mean Ca^{2+} levels estimated in the range of 200–1000 nM, whereas nonmotile growth cones have Ca^{2+} levels in the range of 30–70 nM (Connor, 1986). Thus, CaM kinase II localized in motile growth cones could be activated enough to exert its cellular functions in the transfectants. Given the facts that functional Ca^{2+} channels are abundant in growth cones (Anglister et al., 1982) and that growth cone motility is suppressed by Ca^{2+} channel blockers (Mattson and Kater, 1987), activation of the CaM kinase II triggered by Ca^{2+} influx at the growth cone may be at least in part responsible for the changes of growth cone motility and neurite extension in the transfectants.

The mechanisms by which transfection of the neuroblastoma cell lines with CaM kinase II enhances neurite outgrowth and growth cone motility are unknown. It is likely that phosphorylation of some of the cytoskeletal proteins may participate in these phenotypic changes, because many of these proteins, including microtubule-associated protein 2 and synapsin I, are good substrates for CaM kinase II (Yamauchi and Fujisawa, 1983b, 1988; Bähler and Greengard, 1987). The identification of the endogenous substrates involving the effects of overexpression of CaM kinase II is a subject for ongoing studies.

In summary, overexpression of CaM kinase II in the neuroblastoma cell lines, Nb2a and NG108-15, promotes growth cone motility and neurite outgrowth. The conversion to Ca^{2+}-independent state following autophosphorylation of CaM kinase II appears to be essential for the phenotypic changes to occur. These findings suggest that CaM kinase II plays an important role in changes of neuronal cell shape and motility which may be associated with neuronal differentiation.

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