The Journal of Neuroscience, February 1, 1997, 17(3):924–931

Overexpression of Ca^{2+}/Calmodulin-Dependent Protein Kinase II in PC12 Cells Alters Cell Growth, Morphology, and Nerve Growth Factor-Induced Differentiation

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To examine the role of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in cell differentiation and neuronal functions, stable transformants of PC12 cells were established that expressed levels of the α-subunit of CaMKII (αCaMKII) equivalent to mammalian neurons. The expression of the transfected αCaMKII gene or the endogenous βCaMKII gene was monitored by RNase protection assays, and αCaMKII protein expression was determined by Western blots. Several PC12-derived clones expressed amounts of αCaMKII mRNA and αCaMKII protein similar to that of hippocampal tissues and several orders of magnitude greater than untransfected PC12 cells. CaMKII catalytic activity was four times higher in extracts from αCaMKII-overexpressing compared with untransfected PC12 cells. All clones overexpressing αCaMKII displayed altered cellular growth and adhesion properties including increased cell-to-substrate adhesion, decreased cell-to-cell adhesion, enhanced contact inhibition, and prolonged survival at confluency. Furthermore, the αCaMKII activity in overexpressing PC12 cells inhibited neurite elongation during NGF-induced differentiation. Inhibition of CaMKII activity *in vivo* with KN-62 caused the morphological phenotypes of αCaMKII-overexpressing cells to partially revert to that of untransfected PC12 cells. These results show that αCaMKII catalytic activity affects growth, morphology, and NGF-induced differentiation of PC12 cells.

Key words: cell Ca^{2+}; cytoskeleton; growth cone; neurite; nerve growth factor; PC12 cells; Ca^{2+}/calmodulin-dependent protein kinase

Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of the most abundant neuronal protein kinases (Kelly, 1991). The activity of CaMKII was first identified in neuronal membrane fractions (Schulman and Greengard, 1978), and the α-subunit of CaMKII was later identified as the major postsynaptic density protein (mPSDp) (Kennedy et al., 1983; Rostas et al., 1983; Kelly et al., 1984; Sahyoun et al., 1985). The mPSDp displays characteristics of a structural protein, such as insolubility in chaotropic agents (Kelly and Cotman, 1976a; Feit et al., 1977), and a propensity to form disulfide bonds with itself and other synaptic proteins (Kelly and Cotman, 1976b). CaMKII is distinct from all known cytoskeletal/structural proteins (Kelly and Cotman, 1976a) and represents 30–40% of total proteins in PSDs, suggesting that αCaMKII could function as a structural/cytoskeletal protein at synapses (Kelly, 1991; Schulman and Hanson, 1993). Among the proteins present in PSDs, tubulin, actin, and fodrin are found in stoichiometric amounts, suggesting that αCaMKII could form complexes with these cytoskeletal proteins (Carlin et al., 1983; Sahyoun et al., 1985, 1986). Purified cytoplasmic CaMKII also appears to interact under *in vitro* conditions with polymers of purified actin (Ohta et al., 1986). However, the mechanisms regulating the accumulation of αCaMKII at synapses, as well as its function in the developing brain, are largely unknown (Kelly, 1991).

CaMKII phosphorylates a broad range of substrates and is expected to have pleiotropic regulatory actions in cellular differentiation and mature neuronal functions. For example, CaMKII in the neuronal nuclear matrix and PSDs are structurally related and phosphorylate a broad range of substrates in these cellular compartments (Sahyoun et al., 1984a,b). The activity of CaMKII also appears to be involved in nuclear membrane breakdown (Baitinger et al., 1990). Expression of a truncated, constitutively active form of CaMKII in mammalian cells leads to arrest of the cell cycle in G2 (Planas-Silva and Means, 1992). In addition, the activity of CaMKII in postsynaptic hippocampal neurons is necessary for the induction of long-term potentiation (Silva et al., 1992; Wang and Kelly, 1995), a cellular model of learning in mammals.

Expression of high levels of αCaMKII in a cell model is important to understand its potential role as an enzyme and/or a structural protein (Sahyoun et al., 1985, 1986). The studies herein have examined the hypothesis that αCaMKII overexpression alters the general cell growth, differentiation, and morphology of neurons. Our goal was to develop a cellular model that displays high levels of CaMKII expression characteristic of many CNS neurons. The PC12 cell model described herein allowed us to examine a number of cellular changes associated with αCaMKII expression and NGF-induced cell differentiation and determine which cellular properties require αCaMKII catalytic activity versus its potential function as a cytoskeletal/structural protein (T. Massé and P. Kelly, unpublished observations). A role for CaMKII in cellular morphology has been reported in neuroblas-

Received May 2, 1996; revised Oct. 31, 1996; accepted Nov. 11, 1996.

This work was supported by grants from the Human Science Frontiers Program (T.M.) and National Institutes of Health (P.T.K.). We thank Kim Huber and Tony Moore for helpful comments.

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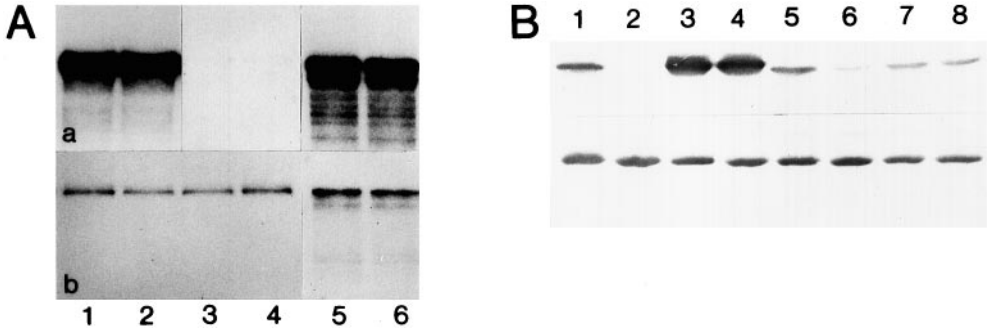

Figure 1. Analysis of αCaMKII mRNA and protein expression in PC12 cells and hippocampus. *A*, RNase protection analysis of αCaMKII (*a*) and βCaMKII (*b*) mRNAs in normal and αCaMKII-overexpressing PC12 cells or hippocampal tissue from adult rat brain. Total cellular RNA from two different PC12 cultures, two independent clones overexpressing αCaMKII, or hippocampi were analyzed. Equivalent amounts of RNA were hybridized with subunit-specific cRNA probes and digested with RNase A + T1 (see Materials and Methods). Autoradiograms show cRNA fragments protected by mRNAs from αCaMKII-overexpressing cells (*lanes 1*, *2*), untransfected cells (*lanes 3*, *4*), and hippocampus (*lanes 5*, *6*). *B*, Western blot analysis of αCaMKII (*top*) and actin (*bottom*) in untransfected PC12 cells (*lane 2*), αCaMKII-overexpressing PC12 cells (*lanes 1*, *4–8*), and rat brain hippocampus (*lane 3*). Equivalent amounts of total cellular protein (50 μg) were analyzed with monoclonal antibodies specific for αCaMKII or actin.

toma NB2a and NG-108 cells (Goshima et al., 1993) or PC12 cells (Tashima et al., 1996) using experimental strategies somewhat like ours. We found that overexpression of αCaMKII in PC12 cells greatly enhances cell survival in confluent cultures, increases cell-to-substrate adhesion, and greatly inhibits neurite outgrowth during NGF-induced cellular differentiation. A preliminary account of these results appeared elsewhere (Massé et al., 1993).

MATERIALS AND METHODS

Cell culture, transfection, and clonal selection. PC12 cells were grown in DMEM (5–10% CO_2) supplemented with 10% horse serum and 5% fetal calf serum (FCS). Differentiation was induced by incubating cells with 50 nM NGF (generously provided by William Mobley, UCSF) in DMEM + 1% FCS.

The cDNA encoding αCaMKII (rat brain) was subcloned into the eukaryote expression vector pRc/cytomegalovirus (CMV, Invitrogen) containing the early CMV promoter and genes for neomycin and ampicillin resistance (pCMV–αCaMKII). PC12 cells were plated on poly-L-ornithine-coated tissue culture plates and transfected 24 hr later with plasmids linearized with *Sca*I (cutting in the ampicillin gene, increasing the likelihood of integrating the full-length plasmid with intact αCaMKII and neomycin resistance genes). Transfections were done by the Ca^{2+} phosphate method (Graham and van der Eb, 1973); precipitates were left on cells for 16 hr, washed with DMEM, and incubated in complete medium for 24 hr before adding the neomycin analog G418. Stable transfectants were selected over 2 weeks in medium containing G418 (0.5–3.0 mg/ml). Clones were isolated and maintained under G418 selection for 2 additional weeks; clones overexpressing αCaMKII (αPC12) were identified by immunofluorescence.

RNase protection. [^{32}P]cRNAs were prepared from pCMV–αCaMKII or pCMV–β-CaMKII (β-CaMKII cDNA was a gift from Dr. Rachael Neve) linearized with *Cla*I or *Hin*fI, respectively. SP6 RNA polymerase (Melton et al., 1984) was used to generate ^{32}P-labeled probes, which were hybridized overnight at 55°C to total cellular RNA prepared from rat hippocampus, untransfected PC12 cells, or αCaMKII-overexpressing PC12 cells. Single-strand cRNAs were digested at room temperature for 1 hr with 40 μg/ml RNase A + 3000 U/ml RNase T1; [^{32}P]cRNA fragments were sized on polyacrylamide/urea sequencing gels.

In vitro *phosphorylation.* Cells were harvested by scraping in PBS, pelleted (1000 × *g*/10 min) at 4°C, resuspended in 150 μl of ice-cold homogenization buffer containing (in mM): 10 Tris-HCl pH 7.5, 1 EGTA, 1 EDTA, 0.5 dithiothreitol (DTT), 0.1 phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin and 20 μg/ml soybean trypsin-inhibitor, and sonicated for 5 sec. Protein concentrations were determined by the micro-BCA method (Pierce). Phosphorylation reactions were initiated by adding 5 or 10 μg of cellular extract protein (in 10 μl) to 40 μl of a solution, such that the following final concentrations were obtained (in mM): 25 HEPES, pH 7.4, 10 $MgCl_2$, 0.5 DTT, 2 $CaCl_2$, 1.5 μM CaM, and 50 μM ATP and 3.5 μCi ^{32}P-γ-ATP (3000 Ci/mmole). Certain reactions contained 20 μM autocamtide-3, a peptide substrate for CaMKII (Hanson and Schulman, 1992). To determine Ca^{2+}-independent CaMKII activity in cell extracts, Ca^{2+}/CaM was replaced with 0.5 mM EGTA or 0.5 mM EGTA + 50 μM of a pseudosubstrate peptide inhibitor of CaMKII [Ala^{286}(281–302)] (Hanson and Schulman, 1992). Phosphorylation reactions (31°C for 45 or 60 sec) were linear with respect to time and protein concentration (5–10 μg) and were terminated by the addition of 4× SDS sample buffer and heated at 85°C for 5 min or spotted on phosphocellulose filters, as described elsewhere (Waxham et al., 1990).

RESULTS

αCaMKII overexpression in PC12 cells

In initial experiments, NB2a and NG-108 cells proved unsuitable as cellular models for the overexpression of αCaMKII. First, we were unable to select clones expressing high levels of αCaMKII (e.g., similar to rat hippocampal neurons). Second, we found that the expression of αCaMKII under the regulation of several promoters (e.g., CMV, RSV, and SV-40) was inhibited when cellular differentiation of these cell lines was induced by 8-bromo-cAMP or retinoic acid (data not shown). In contrast, stable PC12 cell transformants were routinely obtained that expressed high levels of αCaMKII that approximated its expression in hippocampal neurons. In addition, short-term growth (~4 hr) of αCaMKII-overexpressing PC12 cells in NGF produced a transient increase in αmRNA levels (data not shown), which was expected because the CMV promoter contains a serum response element that can be stimulated by NGF.

Independent clones (~100) overexpressing αCaMKII were selected and studied. The level of αCaMKII expression in untransfected versus transformed PC12 (αPC12) cells was determined using RNase protection assays. Total RNA from rat hippocampus and αCaMKII-overexpressing cells showed protected fragments of the expected size for αCaMKII mRNA (Fig. 1*A*,*a*). The levels of αCaMKII mRNA in several αCaMKII-overexpressing cell lines were comparable with levels in rat hippocampus. In contrast, very low α-mRNA signals

Table 1. Substrate phosphorylation (CPMs)

Experimental group		EGTA	Ca^{2+}/CaM	Ca^{2+}-independent activity (%)
Hippocampus	H[a]	2000	21,200	9.4
PC12 Cells	S1	540	5880	9.2
	P1	2040	21,830	9.3
αCaMKII	S1	2640	38,900	6.8
PC12 Cells	P1	5120	56,810	9.0
CaMKII		1240	129,800	1.0

[a]PC12 cell and tissue homogenates (H) were prepared in homogenization buffer (see Materials and Methods) using a Dounce homogenizer; homogenates were centrifuged 10,000 × *g* (10 min/4°C) to prepare crude particulate (P1) and soluble (S1) fractions. Values (average of duplicates) are from a representative experiment; phosphorylation reactions used 5 μg of extract protein (0.1 μg for CaMKII); CaMKII was purified as described elsewhere (Waxham et al., 1990). A background value for each phosphorylation condition was obtained with reactions to which extract protein was not added (background was subtracted from each experimental value).

were detected after RNase protections using RNA prepared from untransfected PC12 cells. Densitometry of autoradiograms showed that untransfected PC12 cells contained ~100- to 250-fold less α-mRNA than αPC12 cells. A probe specific for β-CaMKII mRNA demonstrated that its concentration was similar in untransfected and αPC12 cells, but ~2 times lower than rat hippocampal RNA. Western blot analysis showed high levels of αCaMKII in most G418 resistant clones after transfection, whereas αCaMKII was undetectable in untransfected PC12 cells (Fig. 1*B*). Many PC12 clones expressed levels of αCaMKII comparable with rat hippocampus (i.e., ~2% of total protein) (Erondu and Kennedy, 1985). Although most of the results presented herein were with the two αPC12 clones shown in Figure 1, we observed the same overall phenotype for numerous clones that expressed as little as 25% as much αCaMKII.

The Ca^{2+}/CaM-stimulated CaMKII activity in αPC12 cell extracts was ~50% greater than the activity measured in hippocampal extracts, as determined by the *in vitro* phosphorylation of autocamtide-3 (see Materials and Methods). In addition, αPC12 cells contained 4.1 ± 0.6 (n = 6) times higher Ca^{2+}/CaM-stimulated CaMKII activity compared with untransfected cells. The Ca^{2+}-independent CaMKII activity in cell extracts was determined in assays containing EGTA, with or without a pseudosubstrate inhibitor of CaMKII [Ala^{286}(281–302)]. Although αPC12 cells contained significantly more Ca^{2+}/CaM-dependent CaMKII activity compared with untransfected PC12 cells, the percentage of total CaMKII activity that was Ca^{2+}-independent in αPC12 cells (9.8 ± 0.4%, n = 10) was very similar to the Ca^{2+}-independent activity in extracts from untransfected cells (9.3 ± 1.1%). We also examined the distribution of CaMKII activity in crude soluble (S1) and particulate (P1) fractions prepared from αPC12 and untransfected PC12 cell homogenates (Table 1). These results indicated that αPC12 cells contained ~3.8 ± 1.1 (n = 4) times more Ca^{2+}/CaM-stimulated CaMKII activity compared with untransfected cells. The percentage of total CaMKII activity that was Ca^{2+}-independent in S1 and P1 fractions from αPC12 cells (5.1 ± 1.7%, n = 4) was 50% lower compared with untransfected cells (9.3 ± 0.6%, n = 4). These results indicate that although the percentage of Ca^{2+}-independent CaMKII activity in αPC12 cells is slightly lower than untransfected PC12, the absolute amount of Ca^{2+}-independent activity is higher (three- to fourfold) in αPC12 cells.

Table 2. NGF-induced[a] neurite extension of αCaMKII-overexpressing PC12 cells

Culture condition	No neurites (%)	Short neurites (%)	Long neurites (%)
Untransfected cells (n = 652)	6	22	72
Untransfected cells + KN-62 (n = 401)	7	26	67
αCaMKII-overexpressing cells (n = 543)	68	26	6
αCaMKII-overexpressing cells + KN-62 (n = 728)	24	43	33

[a]Untransfected or αCaMKII-overexpressing PC12 cells were cultured in NGF (50 nM) in the presence or absence of KN-62 (10 μM). Neurites were analyzed by phase-contrast microscopy after 2 d of differentiation in NGF. Neurite extension was assessed by counting cells with short neurites (length ≤ 1 body diameter) or long neurites (length ≥ 1 cell body diameter); data are expressed as the percentage of total cells with long, short, or no neurites.

The *in vitro* phosphorylation of endogenous proteins in cell extracts was analyzed by SDS-PAGE and autoradiography. Scanning densitometry of autoradiograms showed that the Ca^{2+}/CaM-dependent phosphorylation of several proteins was four- to sixfold greater in αPC12 cell extracts compared with untransfected PC12 cells (data not shown). These results confirm that αCaMKII-overexpressing PC12 clones contain similar levels of functional CaMKII activity compared with hippocampal tissue, which makes them a suitable model to study the role of CaMKII in neuronal functions.

αCaMKII overexpression alters cell growth and adhesion properties

A majority of clones overexpressing αCaMKII displayed very similar growth properties. Within 30–60 min after plating, αPC12 cells attached and spread on substratum; all were flat and phase-contrasted within 24 hr. Under similar conditions, untransfected PC12 cells displayed small, round, and phase-bright morphologies, which by 24 hr, remained the predominant phenotype (data not shown). Over the course of 3–5 d in culture, untransfected PC12 cells flattened and grew as tight aggregates, whereas αPC12 cells remained flat and separated from one another, with distinct cell borders (Fig. 2). In contrast, clones selected for overexpression of β-galactosidase (under control of the CMV promoter) displayed morphological properties (± NGF) that were similar to untransfected PC12 cells (data not shown).

When untransfected PC12 cells were plated on plastic bacteriological Petri dishes, <10% of the cells attached within 24 hr, and most cells formed suspension aggregates (data not shown). In contrast, ~50% of αPC12 cells attached to bacteriological plastic within 60 min of plating, and all attached within 24 hr (data not shown). The morphology and growth cones of G418-resistant αPC12 clones expressing very low or undetectable levels of αCaMKII were similar to that of untransfected PC12 cells. These differences in cell morphology indicated that αCaMKII overexpression results in increased cell-to-substrate adhesion and decreased cell-to-cell contact.

During the first 3 d after trypsinization and replating, the initial replication rate of αPC12 cells was ~50% slower than untransfected PC12 cells (Fig. 3). However, during exponential growth (3–6 d after replating), both αPC12 and untransfected PC12 cells displayed similar growth rates. αPC12 cells stopped dividing at lower cell densities. The slower initial replication rate and the

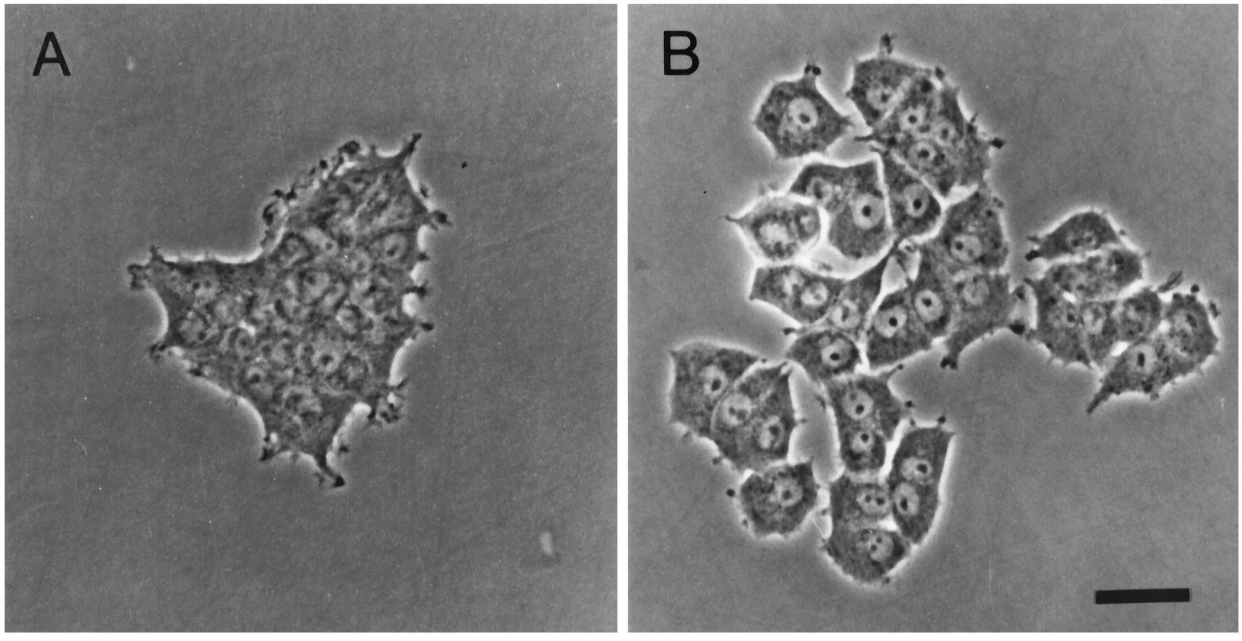

Figure 2. General morphology of normal (*A*) and αCaMKII-overexpressing (*B*) PC12 cells. Phase-contrast micrographs were obtained on living cells plated at low density on untreated tissue culture plastic and grown for 5 d in standard medium containing 15% serum. Scale bar, 100 μm.

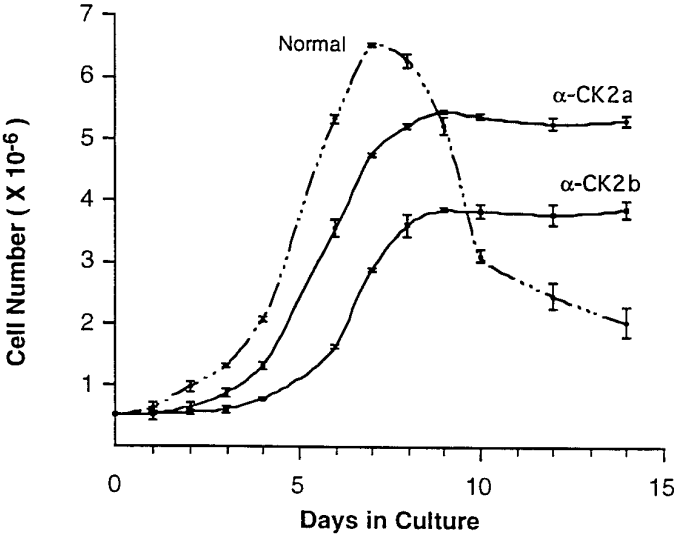

Figure 3. Cellular growth curves of untransfected (*dashes*) and two independent clones of αCaMKII-overexpressing PC12 cells (*a*, *b*). After trypsinization of confluent cultures, equivalent numbers of cells (5×10^5) were plated on tissue culture plastic and grown for the indicated times; half of the medium was replaced with fresh medium every 2 d. Cells were trypsinized, pelleted, and resuspended in PBS plus Trypan blue for counting. The number of total viable cells are given as the mean of triplicate cultures for each condition. Error bars indicate SEM.

inhibition of replication at lower cell density both suggest that contact inhibition is enhanced in αPC12 cells. αPC12 cells displayed a high capacity to survive after reaching confluency compared with untransfected cells (Fig. 3). Untransfected PC12 cells underwent significant cell death 2–3 d after reaching confluency, even though half of the culture medium was replaced with fresh medium every 2 d. In contrast, αPC12 cells showed little cell death for >7-10 d after reaching confluency; in several experiments, confluent cultures of αPC12 cells displayed no apparent cell death for 3–4 weeks (data not shown).

αCaMKII overexpression alters cellular response to NGF

Because PC12 cells overexpressing αCaMKII displayed distinct growth properties under standard culture conditions, we examined their behavior during NGF-induced cell differentiation. All clones overexpressing αCaMKII displayed very similar morphologies during NGF-induced cell differentiation. Within 1–2 hr after adding NGF (50 nM) and decreasing serum from 15 to 1%, αPC12 cells flattened and formed numerous growth cone-like structures, which did not extend from the perimeter of the cell body (Fig. 4). Even after 2–3 d in NGF, αPC12 cells still displayed large growth cones with little or no neurite extension (Figs. 4*A*, 5). Untransfected PC12 cells, or G418 selected clones that did not overexpress αCaMKII, rapidly extended many long neurites in NGF (Fig. 4*B*). At least 4–5 d in NGF was required for a significant number of αPC12 cells to extend neurites longer than 1 cell body diameter (see below). Untransfected PC12 cells grown in NGF for 4 d displayed long and slender neurites that appeared to contact the substrate only once or twice throughout their length (Fig. 4*B*). This apparent weak substrate adhesion of untransfected PC12 neurites was supported by their fragility during media changes or histochemical processing. In contrast, the very short neurites of αPC12 cells, when present, appeared flat, wide at their base, and more strongly adherent to the substratum compared with neurites of untransfected cells (see Fig. 4*A*). Thus, the overexpression of αCaMKII appeared to inhibit neurite elongation, promote growth cone flattening, and increase the adhesion of cells and neurites to substrate.

To determine whether these alterations in cell morphology and inhibition of neurite extension required αCaMKII activity, αPC12 cells were cultured in KN-62, an inhibitor of CaMKII (IC_{50} of ~1 μM when assayed using purified CaMKII) (see Tokumitsu et al., 1990). Cell morphologies were analyzed by phase-contrast microscopy after differentiation in NGF + or − KN-62 (1–10 μM). Neurite extension was assessed by counting cells presenting short neurites (length ≤ 1 body diameter) and cells with long neurites

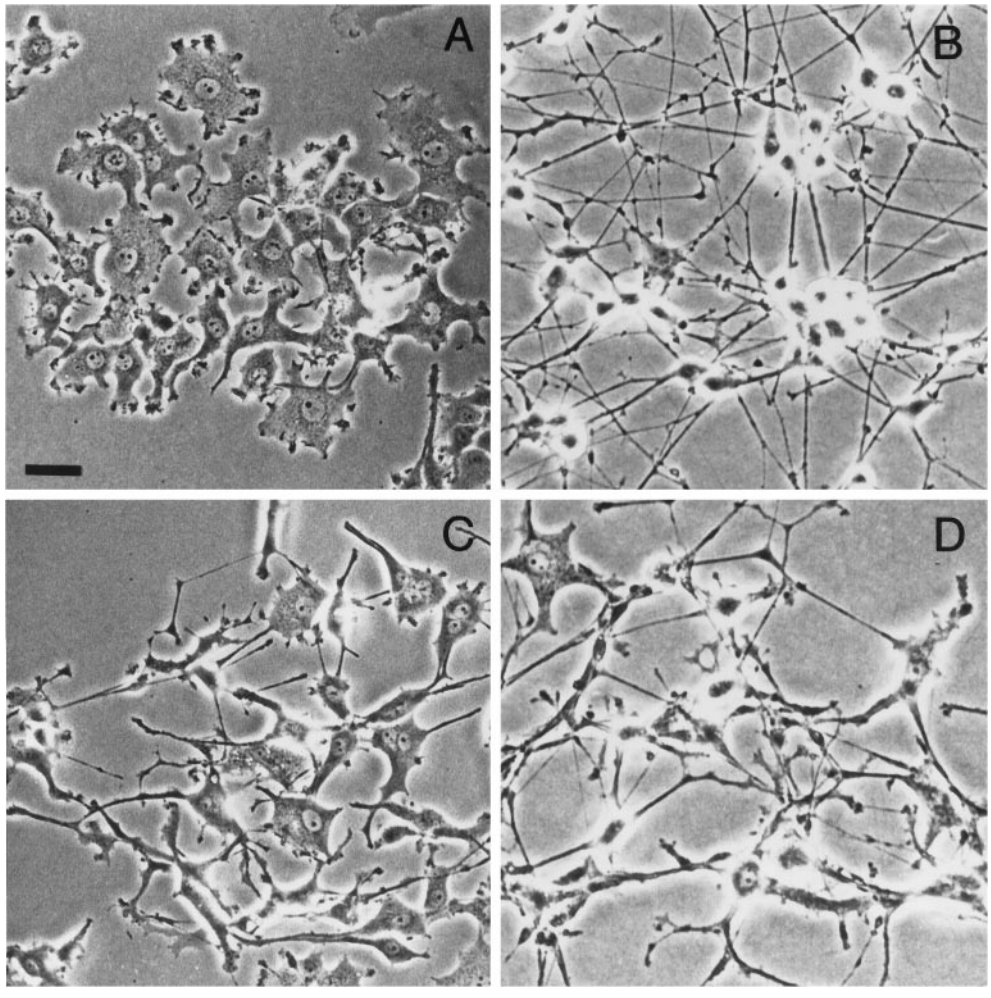

Figure 4. Phase-contrast morphology of αCaMKII-overexpressing (*A*, *C*, *D*) and untransfected (*B*) PC12 cells after NGF differentiation under the following conditions: NGF differentiation for 2 d (A), NGF differentiation for 5 d (*B*), NGF differentiation in KN-62 (10 μM) for 2 d (*C*), and NGF differentiation in KN-62 (10 μM) for 5 d (*D*). Scale bar, 100 μm.

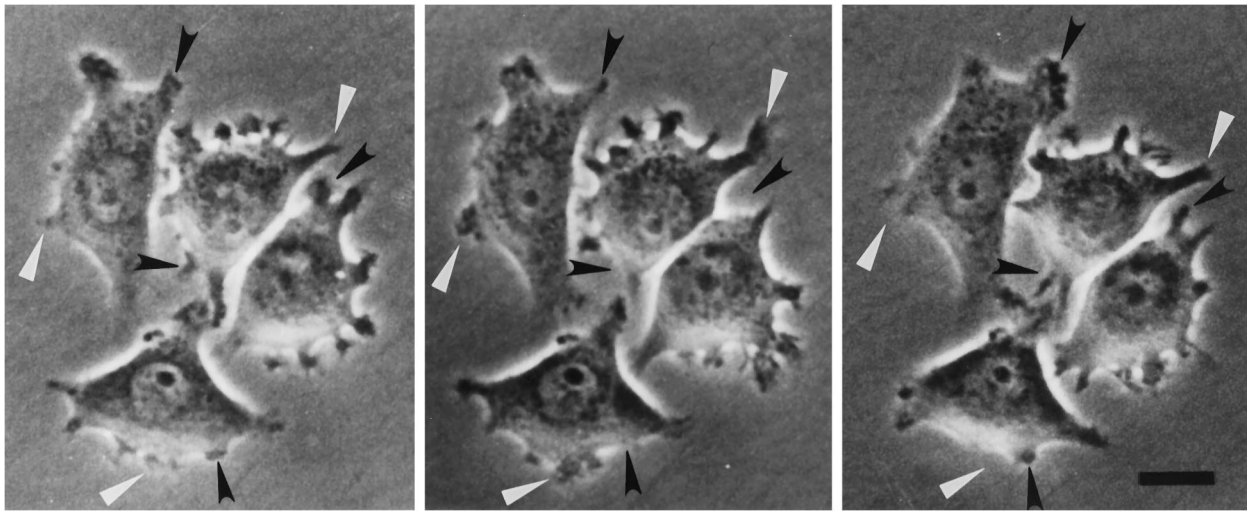

Figure 5. Time-lapse microscopy of growth cone dynamics. Phase-contrast micrographs of αCaMKII-overexpressing PC12 cells after culture in NGF for 2 d. Sequential micrographs of the same cells every 3 hr showed that growth cones are not stable during a 6 hr period (*white arrowheads*); after growth cone retraction/collapse, they reappear at the same location on the cell surface (*black arrowheads*). Scale bar, 50 μm.

(length ≥ 1 cell body diameter). After 2 d in NGF, 72% of untransfected PC12 cells displayed long neurites and 22% short neurites (Table 1). After 2 d in NGF + KN-62 (10 μM), KN-62 had no detectable effect on the percentage of untransfected cells displaying short (26%) versus long neurites (67%). In contrast, αPC12 cells differentiated very little after 2 d in NGF, with only 6% of the cells displaying long neurites and 26% showing short neurites. The remaining cells (68%) displayed large growth cones immediately adjacent to the cell body with no apparent neurites. In contrast, αPC12 cells differentiated in NGF + KN-62 (10 μM)

for 2 d displayed highly differentiated morphologies, with 33% displaying long neurites and 43% short neurites (Table 1, Fig. 4*C*). Because this KN-62 concentration is 10 times higher than its *in vitro* IC_{50} (see above), we examined the effects of lower concentrations of inhibitor. αPC12 cells differentiated in NGF + 1 μM KN-62 displayed morphologies that were very similar to αPC12 cells differentiated in NGF alone (data not shown), whereas cells grown in NGF + 5 μM KN-62 displayed long neurites very similar to cells grown in NGF + 10 μM KN-62. KN-62 was added to cultures only once at the start of each experiment (1–2 hr after NGF addition), and its stability in cultures was not assessed; most experiments used 10 μM KN-62. Phase-contrast microscopy also showed that cell-to-substrate adhesion of αPC12 cells decreased during differentiation in NGF + KN-62. The number of growth cones per cell was not affected by KN-62 in either untransfected or αPC12 cells. Thus, KN-62 significantly altered the morphology and neurite extension of αCaMKII-overexpressing cells grown in NGF such that they appeared similar to untransfected PC12 cells during NGF-induced differentiation.

To further examine the apparent inhibition of neurite outgrowth in αCaMKII-overexpressing PC12 cells, cells were grown in NGF for 2 d and then time-lapse micrographs were taken at 3 hr intervals. Figure 5 shows that growth cones routinely form, grow in size, and then retract in <3 hr without extending neurites (growth cones marked with *black arrowheads*). Growth cones also appear to retract and reappear at the same location on a cell (growth cones marked with *black* and *white arrowheads*). In contrast, neurites of untransfected PC12 cells grown in NGF extended long distances from the cell body (Fig. 4*B*). These neurites do not retract during the course of 1 cell cycle (Waymire et al., 1978). These results indicate that the activity of αCaMKII in overexpressing cells inhibits neurite elongation but not growth cone formation.

DISCUSSION

One goal of these studies was to develop a cell model that displays certain phenotypes of CNS neurons, such as the expression of high levels of the αCaMKII. This goal was achieved using PC12 cells transfected with αCaMKII cDNA under control of the strong CMV promoter. Comparison of many G418-resistant PC12 clones expressing high versus low or undetectable levels of αCaMKII showed that the properties described above were positively correlated with αCaMKII overexpression. αCaMKII overexpression resulted in increased cell-to-substrate adhesion (i.e., larger and flatter cells), decreased cell-to-cell adhesion, and growth saturation at lower cell densities. Furthermore, αCaMKII overexpression inhibited neurite outgrowth during NGF-induced differentiation.

A correlation between αCaMKII overexpression and cell morphology has been reported in neuroblastoma (NB2a) and NG-108 cells (Goshima et al., 1993) or PC12 cells (Tashima et al., 1996) using experimental strategies similar to ours. The major differences between our study and the latter studies are the cell models and differentiation protocols. We observed that transfected NB2a and NG-108 cells did not express very high levels of αCaMKII compared with hippocampal or cortical tissues, which we believe is important to assess the enzymatic and structural roles of αCaMKII in neuronal functions. In addition, we used NGF as a morphogen, whereas the other studies used membrane-permeable cyclic nucleotide analogs (i.e., dibutyryl-cAMP or 8-bromo-cAMP), and cAMP analogs may cause a short-term induction of CRE-containing promoters (e.g., c-fos) followed by a downregulation or refractory period (Gius et al., 1990; Foulkes et al., 1991; Marksitzer et al., 1995) (Massé and Kelly, unpublished observations). It is also possible that long-term activation of cAMP pathways may enhance other phosphorylation pathways, including those mediated by CaMKII. For example, the phosphorylation of inhibitor-1 by PKA will increase the inhibition of type-1 protein phosphatase that dephosphorylates many proteins phosphorylated by CaMKII (Shields et al., 1985). In our studies, the differentiation of αPC12 cells with dibutyryl- or 8-bromo-cAMP decreased the levels of αCaMKII mRNA and protein. In contrast, growth of overexpressing PC12 cells in NGF for 1–5 d did not significantly change their cellular content of αCaMKII or its *in vitro* catalytic activity assayed with cell extracts.

In agreement with Goshima et al. (1993), we found that αCaMKII overexpression enhances cell-to-substrate adhesion. However, our studies with PC12 cells show that increased adhesion does not stimulate neurite outgrowth, suggesting that high levels of αCaMKII activity inhibit other mechanisms involved in growth cone motility. With regard to the properties of NGF- versus cAMP-induced differentiation, we observed that the retarded neurite outgrowth of αPC12 cells was reversed by the CaMKII inhibitor KN-62; this reversal was not observed by Tashima et al. (1996). Because the inhibition of neurite extension in αPC12 cells was reversed with KN-62, it seems unlikely that our results were attributable to selecting G418-resistant PC12 clones that were simply defective in extending neurites.

Although αCaMKII overexpression altered the growth and neurite extension properties of PC12 cells, it did not compromise their ability to respond to NGF; growth cones formed rapidly after adding NGF, and the relative abundance of NGF receptors in PC12 cells as determined immunohistochemically changed very little in αCaMKII-overexpressing cells (data not shown). Neurite elongation appeared to be inhibited by CaMKII catalytic activity. This inhibition seemed to be attributable to growth cone instability of αPC12 cells grown in NGF, because growth cones emerged transiently and repetitively without forming neurites (Massé and Kelly, unpublished observations). αCaMKII overexpression did not dramatically change the percentage of Ca^{2+}-independent CaMKII activity in cells relative to total Ca^{2+}/CaM-stimulated CaM-kinase activity. Although αPC12 cells contained three- to fourfold higher absolute levels of Ca^{2+}-independent CaMKII activity compared with untransfected cells, we believe this activity is unlikely to contribute to the distinct phenotype of αPC12 cells. This is based on the fact that the CaMKII inhibitor KN-62 is competitive with Ca^{2+}/CaM and does not inhibit Ca^{2+}-independent CaMKII activity (Tokumitsu et al., 1990). Our results show that the apparent inhibition of Ca^{2+}/CaM-dependent CaMKII activity with KN-62 resulted in growth cone stabilization and neurite extension and suggest that αCaMKII activity in αPC12 cells inhibits neurite outgrowth by disrupting one or more steps in the mechanism of neurite elongation, possibly through increased phosphorylation of specific protein substrates (Tokui et al., 1990; Lapadula et al., 1991). Mechanisms that regulate neurite extension and growth cone motility most likely require a dynamic balance between Ca^{2+}/CaM-dependent protein kinase and phosphatase activities. Recent observations show that the Ca^{2+}/CaM-dependent protein phosphatase calcineurin functions in neurite outgrowth and directed filopodia motility in dorsal root ganglion neurons (Chang et al., 1995).

Results published recently suggest that KN-62 may have additional actions besides inhibiting CaM kinases. For example,

KN-62 appears to inhibit Ca^{2+} influx in adrenal chromaffin cells (Maurer et al., 1996); however, KN-62 had no observable effect on cell morphology. Additional data suggest that KN-62 blocks voltage-gated Ca^{2+} channels in adrenal chromaffin cells (Marley and Thomson, 1996), whereas it does not affect Ca^{2+} currents in hippocampal CA1 neurons (Wyllie and Nicoll, 1994). It is also important to note that CaM-kinase activity can upregulate Ca^{2+} channel activity (Wang and Best, 1992; Kitamura et al., 1993), which suggests that cells treated with KN-62 may indirectly downregulate Ca^{2+} channel influx attributable to KN-62's inhibition of CaM-kinase.

αCaMKII-overexpressing cells displayed altered growth properties, including decreased replication rates, enhanced contact inhibition, and greatly enhanced survival after reaching confluency, compared with untransfected PC12 cells. This result suggests that αCaMKII may enhance cell-to-cell signaling that regulates cell replication as well as participates in maintaining metabolic homeostasis during quiescence. Because the expression of αCaMKII in postmitotic neurons *in vivo* remains high in the adult brain (Rostas et al., 1983; Burgin et al., 1990), our discovery that αCaMKII overexpression greatly enhances cell survival at confluency suggests that CaMKII contributes to cellular homeostasis and longevity of neurons in the brains of adult and aging humans. The mechanisms underlying such roles for CaMKII in neuronal function probably involve the regulation of gene expression (Sheng et al., 1990; Lerea and McNamara, 1993). However, it is tempting to propose that certain features of the αCaMKII-overexpression phenotype is attributable to a direct regulation of cytoskeletal functions by CaMKII (Iwig et al., 1995) (Massé and Kelly, unpublished observations).

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