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Expression of Ca^{2+}/Calmodulin-Dependent Protein Kinase Types II and IV, and Reduced DNA Synthesis Due to the Ca^{2+}/Calmodulin-Dependent Protein Kinase Inhibitor KN-62 (1-[*N*,O-Bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) in Small Cell Lung Carcinoma

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ABSTRACT. Because changes in intracellular Ca^{2+} affect progression through the mitotic cell cycle, we investigated the role of Ca^{2+}-binding proteins in regulating cell cycle progression. Evidence was found demonstrating that the inactivation of Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) inhibits cell cycle progression in small cell lung carcinoma (SCLC) cells. We also demonstrated that SCLC cells express both CaM kinase type II (CaMKII) and CaM kinase type IV (CaMKIV). Five independent SCLC cell lines expressed proteins reactive with antibody to the CaMKII β subunit, but none expressed detectable proteins reactive with antibody to the CaMKII α subunit. All SCLC cells lines tested expressed both the α and β isoforms of CaMKIV. Immunoprecipitation of CaMKII from SCLC cells yielded multiple proteins that autophosphorylated in the presence of Ca^{2+}/calmodulin. Autophosphorylation was inhibited by the CaMKII(281-302) peptide, which corresponds to the CaMKII autoinhibitory domain, and by 1-[*N*,O-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), a specific CaM kinase antagonist. Influx of Ca^{2+} through voltage-gated Ca^{2+} channels stimulated phosphorylation of CaMKII in SCLC cells, and this was inhibited by KN-62. Incubation of SCLC cells with KN-62 potently inhibited DNA synthesis, and slowed progression through S phase. Similar anti-proliferative effects of KN-62 occurred in SK-N-SH human neuroblastoma cells, which express both CaMKII and CaMKIV, and in K562 human chronic myelogenous leukemia cells, which express CaMKII but not CaMKIV. The expression of both CaMKII and CaMKIV by SCLC cells, and the sensitivity of these cells to the anti-proliferative effects of KN-62, suggest a role for CaM kinase in regulating SCLC proliferation. BIOCHEM PHARMACOL 51;5:707–715, 1996.

KEY WORDS. Ca^{2+}/calmodulin-dependent protein kinase; small cell lung carcinoma; KN-62; cell cycle; DNA synthesis; cell proliferation

One of the most well known proliferative signals is an increase in intracellular Ca^{2+}. Fertilization-dependent increases in intracellular Ca^{2+} cause eggs to complete meiosis, and growth factor-dependent increases in intracellular Ca^{2+} induce quiescent somatic cells to enter the cell cycle. Similarly, cell cycle-dependent fluctuations in intracellular Ca^{2+} indicate a regulatory role for this signal in actively proliferating cells [for reviews, see Refs. 1–3]. Intracellular Ca^{2+} regulates cell cycle progression in organisms as diverse as yeast [4], sea urchins [5], and humans [6]. Although changes in intracellular Ca^{2+} must ultimately affect the activity of cell cycle-regulatory proteins, few of the proteins involved in this pathway have been identified.

A likely participant in this pathway is the Ca^{2+}-binding protein calmodulin. This ubiquitous protein has been implicated in regulating both the meiotic and mitotic cell cycles [for review, see Ref. 7]. Exogenous calmodulin can act as a mitogen in mammalian cells cultures [8], and overexpression of calmodulin in transfected mouse cells shortens the cell cycle [9, 10]. Conversely, inhibiting calmodulin with pharmacological agents [11, 12], calmodulin-specific antibodies [13], or antisense constructs [10] inhibits proliferation of mammalian cells.

One way Ca^{2+} and calmodulin could affect proliferation is by altering the activity of CaM kinase†. Several types of CaM

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† *Abbreviations:* CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase type II; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase type IV; KN-62, 1-[*N*,O-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; SCLC, small cell lung carcinoma; and VGCC, voltage-gated Ca^{2+} channel(s).

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kinase have been cloned and characterized, including CaMKII [reviewed in Ref. 14] and CaMKIV, also known as CaM kinase-Gr [15–18]. In mammalian cells, CaMKII is a multimer consisting of 6–12 subunits encoded by at least four genes [14], whereas CaMKIV is a monomer of either the α or β isoform encoded by a single gene [15–18]. Both CaMKII and CaMKIV are expressed in neuronal cells [14–20] and have been reported to regulate processes that may affect proliferation. Activation of CaMKII results in cyclin B degradation, cdc2 inactivation, and completion of meiosis-II in fertilized *Xenopus* eggs [21]. CaMKIV may regulate proliferation by its ability to phosphorylate the cyclic AMP response element-binding protein and stimulate Ca^{2+}-dependent gene transcription [22, 23]. Although these CaM kinase-mediated events may affect cell proliferation, little is known about the role of CaM kinase in regulating progression through the mitotic cell cycle.

We have examined the role of CaM kinase in controlling the proliferation of SCLC cells. We found that SCLC cells express both CaMKII and CaMKIV, consistent with the expression of neuronal characteristics by SCLC cells [reviewed in Ref. 24]. Incubation of exponentially proliferating SCLC cells with KN-62, a specific CaM kinase antagonist [22, 25–32], potently inhibited DNA synthesis and reduced S phase progression. These findings suggest that CaM kinase plays a role in regulating the proliferation of SCLC cells. Our demonstration that SCLC cells express CaM kinase, and that inactivation of this kinase inhibits SCLC proliferation, indicates that CaM kinase is a potential target for chemotherapeutic intervention in SCLC and other cancers expressing this enzyme.

MATERIALS AND METHODS

Reagents

The isoquinolinesulfonamide KN-62 was purchased from Seikagaku America, Inc. (Rockville, MD) and Research Biochemicals International (Natick, MA). Stock solutions were prepared by dissolving 10 mM KN-62 in anhydrous Me_2SO (Sigma Chemical Co., St. Louis, MO). In all experiments, control cells were incubated with the same amount of Me_2SO used to dissolve KN-62 in cultures of experimental cells. Rabbit antiserum No. 2354 to human CaMKIV was a gift from Dr. Carol Ann Ohmstede, Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC. The production and characterization of this antiserum were described previously [18]. Mouse monoclonal antibodies CBα-2 to the CaMKII α subunit, and CBβ-1 to the CaMKII β subunit, were purchased from Gibco BRL (Gaithersburg, MD). Autocamtide-2 and CaMKII(281–302) were purchased from Research Biochemicals International. Inorganic ^{32}P in the form of H_3PO_4 was purchased from ICN Biochemicals Inc. (Irving, CA). Enhanced chemiluminescence reagents, [^{32}P]ATP, and [^{3}H]thymidine were purchased from Amersham (Arlington Heights, IL). Other reagents were obtained from sources listed in the text.

Cell Culture

The SCLC cell line SCC-9 was established from a biopsy specimen and has been characterized extensively [24, 33, 34]. The SCLC cell lines NCI-H69, NCI-H128, NCI-H146, and NCI-H345 were obtained from the American Type Culture Collection (Rockville, MD). The SK-N-SH human neuroblastoma cell line (provided by Dr. Robert Aronstam, Guthrie Research Institute) was maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), nonessential amino acids (Fisher Scientific, Pittsburgh, PA), L-glutamine (0.58 mg/mL), glucose (4.5 mg/mL), penicillin (20 U/mL), and streptomycin sulfate (20 μg/mL). All other cell lines, including the K562 human chronic myelogenous leukemia cell line (provided by Dr. John Noti, Guthrie Research Institute), were maintained in RPMI 1640 medium containing 10% calf bovine serum (Hyclone Laboratories), glutamine (0.3 mg/mL), penicillin (20 U/mL), and streptomycin sulfate (20 μg/mL). Cells were maintained at 37° in a humidified atmosphere of 5% CO_2/95% air at densities that promoted exponential growth.

[^{3}H]Thymidine Uptake Studies

Assays were performed as previously described [33]. Cells were plated in 96-well microtiter plates at a density of 2×10^4 cells/well and incubated for 1–3 days at 37° in 5% CO_2/95% air before addition of drugs and [^{3}H]thymidine (74 nM, 84 Ci/mmol). After incubating at 37° in 5% CO_2/95% air, the medium in wells containing adherent cell lines was replaced with phosphate-buffered saline containing 0.25% trypsin plus 0.02% EDTA, and the cells were incubated for an additional 10 min. After washing and lysis with distilled water, the cells were collected on filters using an automatic cell harvester (Skatron, Sterling, VA), and subjected to liquid scintillation counting using an LS-6000IC β-counter (Beckman Instruments, Fullerton, CA).

Cell Cycle Analysis

Cells were stained with propidium iodide as previously described [33]. Fluorescence of propidium iodide was measured at 488 nm with a fluorescence-activated cell sorter (Becton–Dickinson Immunocytometry Systems, Mountain View, CA). Data were analyzed using the Modfit program (Verity Software House, Topsham, ME).

Immunoprecipitation

All buffers contained protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin) and were kept ice-cold. Cell lysates were prepared by incubating cells for 10–40 min with periodic agitation in lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, 0.5% NP-40, pH 7.4). After centrifugation (16,000 *g*, 5–10 min), supernates were incubated (1.5 hr, 4°) with 5 μg/mL of the appropriate antibody and 10 μg/mL Protein A-Sepharose CL4B in dilution buffer (10 mM Tris-HCl, 130 mM NaCl, 0.05% Triton X-100, 0.1% BSA, pH 8.0). Immunoprecipitates were washed sequentially in dilution buffer, TSA solution (10

mM Tris-HCl, 140 mM NaCl, pH 8.0) and 50 mM Tris–HCl (pH 8.0), and used in kinase assays.

Kinase Assays

To assay CaM kinase *in vitro* activity, immunoprecipitates were incubated (15 min, 25°) in 30 μL of 25 mM Tris-HCl, pH 7.4, containing 10 μM KN-62, 1 mg/mL CaMKII(281–302), or no additive. Samples were mixed with 90 μL of EGTA-kinase buffer (50 mM Tris-HCL, 15 mM $MgCl_2$, 5 mM EGTA, 5 μM ATP, 0.1 mCi/mL [^{32}P]ATP, pH 7.4) or CaM-kinase buffer (50 mM Tris-HCL, 15 mM $MgCl_2$, 2 mM $CaCl_2$, 0.3 μM calmodulin, 5 μM ATP, 0.1 mCi/mL [^{32}P]ATP, pH 7.4) with or without 50 μM autocamtide-2 and incubated for 5 min at 25°. Phosphorylated autocamtide-2 was isolated in Spinzyme separation units (Pierce, Rockford, IL) and quantitated by Cerenkov counting. To visualize phosphorylated CaMKII subunits, kinase reactions were mixed with sample buffer, boiled, and subjected to SDS-PAGE and autoradiography.

To assay CaM kinase *in vivo* activity, SCC-9 cells were incubated for 3 hr (37°, 5% CO_2/95% air) in 2.75 mL phosphate-free RPMI-1640 medium containing heat-inactivated calf bovine serum (1%) and inorganic ^{32}P (0.8 mCi/mL) in the presence or absence of 10 μM KN-62. Cells were incubated for 5 min after the addition of 7.25 mL Krebs buffer containing 4.7 mM KCl and 118 mM KCl ("low K^+ buffer") or 90 mM KCl and 32.8 mM NaCl ("high K^+ buffer"), as previously described [24]. Immunoprecipitates were prepared in the presence of protease and phosphatase inhibitors using the CBβ-1 antibody, as described above. To visualize phosphorylated CaM kinase subunits, immunoprecipitates were mixed with sample buffer, boiled, and subjected to SDS-PAGE and autoradiography.

To measure the activities of protein kinase A and protein kinase C, cell lysates were prepared in the presence of protease and phosphatase inhibitors and assayed for kinase activity using commercially available kits (Gibco, Gaithersburg, MD). Specific phosphorylation of kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was used to assay protein kinase A activity, and specific phosphorylation of a peptide corresponding to a sequence in myelin basic protein was used to assay protein kinase C activity, according to instructions supplied by the manufacturer (Gibco).

Western Blotting

Cell lysates were prepared in the presence of phosphatase and protease inhibitors, as described above. After centrifugation of the lysates (16,000 *g*, 10 min), supernates were diluted with lysis buffer to equal protein concentrations, boiled with sample buffer, and subjected to SDS-PAGE. Nitrocellulose with electrophoretically transferred proteins was incubated overnight in blocking buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, 10% dried milk powder, pH 7.6), and placed in a 25-channel miniblotter (Immunetics Inc., Cambridge, MA). Each protein preparation on the nitrocellulose was incubated (1.5 hr, 4°) with various dilutions of the CaMKII and CaMKIV antibodies. After washing and incubating the nitrocellulose (1 hr, 4°) with horseradish peroxidase-labeled anti-mouse or anti-rabbit immunoglobulins diluted 1:4000, bound antibody was visualized by enhanced chemiluminescence and quantified by densitometry.

RESULTS

Expression of CaMKII and CaMKIV in SCLC Cell Lines

We examined the expression of CaM kinase in SCLC cell lines by western blotting of whole-cell lysates. The CBβ-1 antibody to the β subunit of CaMKII reacted with a 60-kDa protein in all SCLC cell lines tested (Fig. 1). This is similar to the molecular mass of the CaMKII β subunit expressed in other cell types [for review, see Refs. 14 and 35]. Proteins reactive with antibody to the α subunit of CaMKII were not detected in any of the SCLC cell lines tested (Fig. 1). All SCLC cell lines expressed proteins reactive with an antiserum to CaMKIV (Fig. 1). These proteins corresponded in molecular mass to the 65-kDa α isoform and the 67-kDa β isoform of CaMKIV expressed by other cell types [18, 36]. These results indicate that SCLC cells express the CaMKII β subunit and CaMKIV α and β isoforms.

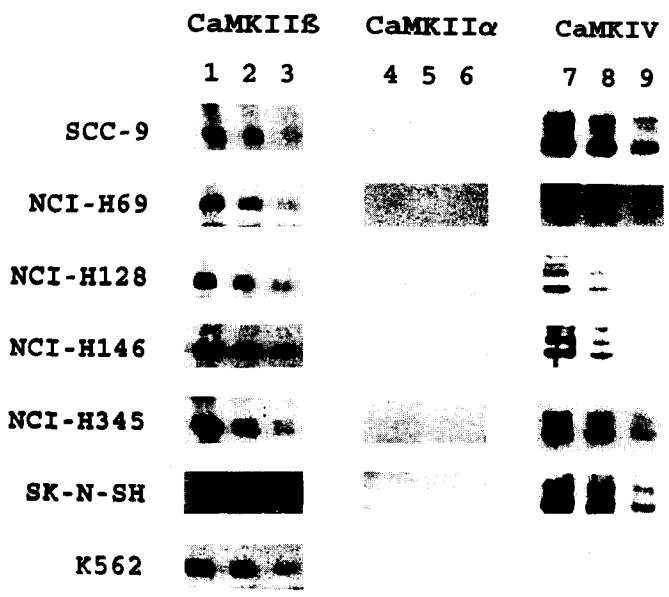

FIG. 1. Expression of CaMKII and CaMKIV by SCLC cells. Whole-cell lysates prepared from SCLC cell lines, SK-N-SH human neuroblastoma cells, and K562 human chronic myelogenous leukemia cells were immunoblotted using monoclonal antibody to the CaMKII β subunit (lanes 1–3), monoclonal antibody to the CaMKII α subunit (lanes 4–6), and rabbit antiserum to the CaMKIV α and β isoforms (lanes 7–9). Concentrations of the CaMKII antibodies used in the western blot were 5 μg/mL (lanes 1 and 4), 2.5 μg/mL (lanes 2 and 5) and 1.25 μg/mL (lanes 3 and 6). The dilutions of the CaMKIV antiserum used in the western blot were 1:250 (lane 7), 1:500 (lane 8), and 1:1000 (lane 9). Proteins with mol. wt of 50–65 kDa are shown in lanes 1–3, 45–60 kDa in lanes 4–6, and 60–70 kDa in lanes 7–9. Two other independent experiments produced similar results.

Expression of CaMKII and CaMKIV varied among other types of cells. Like SCLC cells, SK-N-SH human neuroblastoma cells expressed the CaMKII β subunit and CaMKIV α and β isoforms, but not the CaMKII α subunit (Fig. 1). This agrees with previous reports of CaMKII and CaMKIV expression in SK-N-SH cells [18, 37]. We detected the CaMKII β subunit in the K562 human chronic myelogenous leukemia cell line (Fig. 1), which is a colonal cell line of hematopoietic cells that differentiate into progenitors of the erythrocytic, granulocytic, and monocytic series [38]. This agrees with a previous report that K562 cells express CaMKII [39]. We did not detect either the CaMKIV α or β isoform in K562 cells, consistent with previous reports that CaMKIV is expressed in T cells but not in hematopoietic cells of other lineages [18, 36].

In neuronal cells, binding of Ca^{2+}/calmodulin to CaMKII results in autophosphorylation and activation [for review, see Refs. 14 and 35]. To determine whether CaMKII in SCLC cells is similarly activated, we tested the ability of Ca^{2+}/calmodulin to induce CaMKII autophosphorylation. We found that CaMKII immunoprecipitated from the SCC-9 SCLC cell line consisted of multiple proteins that autophosphorylated upon exposure to Ca^{2+}/calmodulin but not to EGTA-kinase buffer (Fig. 2). This phosphorylation was inhibited by the drug KN-62, which specifically inhibits Ca^{2+}/calmodulin-dependent autophosphorylation and activation of CaM kinase in a variety of tissues [22, 25–32]. SCLC CaMKII autophosphorylation was also inhibited by the CaMKII(281–302) peptide, which corresponds in sequence to the CaMKII autoinhibitory domain [40] (Fig. 2). CaMKII immunoprecipitated from SCC-9 cells was also able to phosphorylate autocamtide-2, which is a peptide containing the sequence of the CaMKII α-subunit autophosphorylation site [41] (Fig. 3). Phosphorylation of autocamtide-2 was inhibited by KN-62 and CaMKII(281–302) (Fig. 3). These findings indicate that CaMKII immunoprecipitated from SCLC cells has functional activity.

Having found that CaMKII is activated *in vitro* by Ca^{2+}/calmodulin, we next investigated the *in vivo* activity of CaMKII. To do this, we took advantage of the fact that the SCC-9 SCLC cell line expresses functional VGCC [24]. In

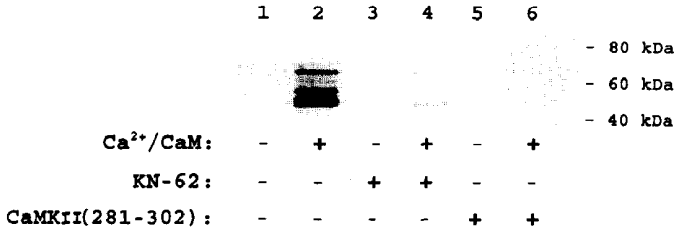

FIG. 2. Ca^{2+}/calmodulin-dependent autophosphorylation of CaMKII immunoprecipitated from the SCC-9 SCLC cell line. Lanes 1, 3, and 5: CaMKII immunoprecipitates incubated in the presence of EGTA-kinase buffer; lanes 2, 4, and 6: CaMKII immunoprecipitates incubated in the presence of CaM-kinase buffer. Immunoprecipitates were preincubated with 10 μM KN-62 (lanes 3 and 4) or with 1 mg/mL CaMKII(281–302) (lanes 5 and 6). Two other independent experiments produced similar results.

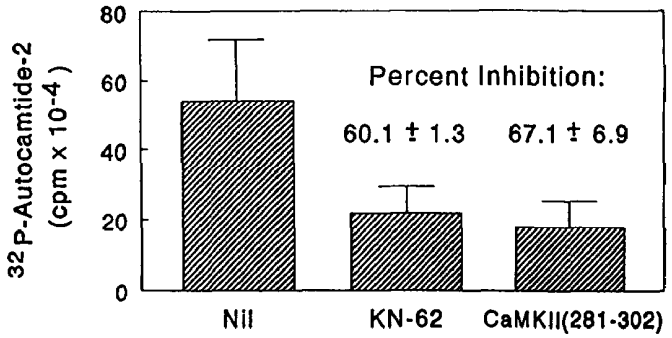

FIG. 3. Ca^{2+}/calmodulin-dependent phosphorylation of autocamtide-2 by SCLC CaMKII. Ca^{2+}/calmodulin-dependent phosphorylation of autocamtide-2 by CaMKII immunoprecipitated from the SCC-9 SCLC cell line (Nil) was also measured after preincubating the immunoprecipitates with 10 μM KN-62 or 1 mg/mL CaMKII(281–302) peptide. Ca^{2+}/calmodulin-dependent phosphorylation was determined by subtracting the amount of autocamtide-2 phosphorylation in the presence of EGTA-kinase buffer from that in the presence of Ca^{2+}/calmodulin-kinase buffer. Results are the means (±1 SEM) of three independent experiments.

agreement with previous reports that calcium influx through VGCC causes CaMKII autophosphorylation [42–44], we found that depolarization-dependent activation of VGCC in SCC-9 cells caused CaMKII autophosphorylation (Fig. 4). Preincubation with KN-62 inhibited this autophosphorylation (Fig. 4). These results demonstrate that SCLC CaMKII is active both *in vivo* and *in vitro*. In contrast to these findings, we could not detect Ca^{2+}/calmodulin-dependent autophosphorylation or activation or immunoprecipitated CaMKIV under conditions that induced maximal autophosphorylation of immunoprecipitated CaMKII.

Concentrations of KN-62 up to 100 μM do not affect the activities of protein kinase A, protein kinase C, Ca^{2+}/calmodulin-dependent phosphodiesterase, or myosin light chain kinase in a variety of cell types [28, 29, 32]. To test the specificity of KN-62 for CaM kinase in SCLC cells, we measured the activation of protein kinases A and C in SCC-9 cells following a 50-min preincubation with 10 μM KN-62. Incubation with 100 μM dibutryl cAMP for 20 min increased protein kinase A activity by 207.7 ± 8.2 vs 222.3 ± 23.3% in the absence or presence of KN-62, respectively (N = 3). SCC-9 cells express M_3 muscarinic acetylcholine receptors [24], and activation of these receptors with 10 μM carbachol for 10 min increased protein kinase C activity by 20.8 ± 8.9 vs 17.1 ± 8.6% in the absence or presence of KN-62, respectively (N = 3). Additionally, we found that the histone H1 kinase activity of cdk2 was not altered by incubating immunoprecipitated cdk2 with 10 μM KN-62 for 15 min (conditions that maximally inhibit immunoprecipitated CaMKII activity). These results indicate that KN-62 specifically inhibits CaM kinase, and does not directly affect the activities of these other protein kinases in SCLC cells.

Inhibition of DNA Synthesis and Reduced Cell Proliferation Due to KN-62

To determine whether CaM kinase regulates cell cycle progression in SCLC cells, we measured the effects of inactivating

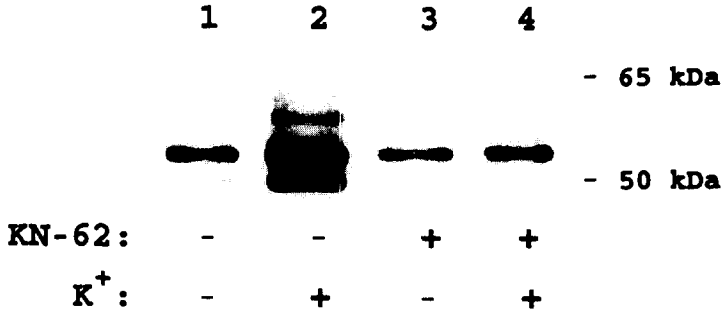

FIG. 4. Phosphorylation of CaMKII due to depolarization-dependent activation of voltage-gated Ca^{2+} channels in SCLC cells. CaMKII was immunoprecipitated from ^{32}P-labeled SCC-9 cells that were incubated with a normal K^+ concentration (lanes 1 and 3) or a high K^+ concentration to activate voltage-gated Ca^{2+} channels (lanes 2 and 4). Before K^+-dependent depolarization, cells were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 μM KN-62. A duplicate independent experiment produced similar results.

CaM kinase on DNA synthesis in the cells. Incubation with KN-62 potently inhibited DNA synthesis in the majority of the SCLC cell lines tested (Fig. 5A). Inhibition of DNA synthesis due to CaM kinase inactivation also occurred in SK-N-SH cells and in K562 cells (Fig. 5B). Inhibition of DNA synthesis in SCC-9 cells occurred almost immediately upon exposure to KN-62 (Fig. 5C). Incubation of SCC-9 cells with KN-62 for several days did not decrease the viability of the cells, but reduced cell proliferation (Fig. 5D).

The finding that KN-62 decreased DNA synthesis almost immediately (Fig. 5C) indicates that inactivation of CaM kinase causes the cells to arrest late in G_1 phase or in S phase. If the cells arrested in G_2 or M phase, DNA synthesis would be unaffected for several hours after exposure to KN-62 as the cells progressed normally through G_1 and S phases into a drug-induced arrest in G_2 or M phase. To determine the phase of the cell cycle which is affected by inactivating CaM kinase, we performed cell cycle analysis on KN-62-treated SCC-9 cells. Within 4 hr of exposure to KN-62, SCC-9 cells accumulated in S phase, with a concomitant reduction in the number of cells in G_2/M phase (Fig. 6). Accumulation of KN-62-treated cells in G_1 phase was not detected at any time during a 24-hr

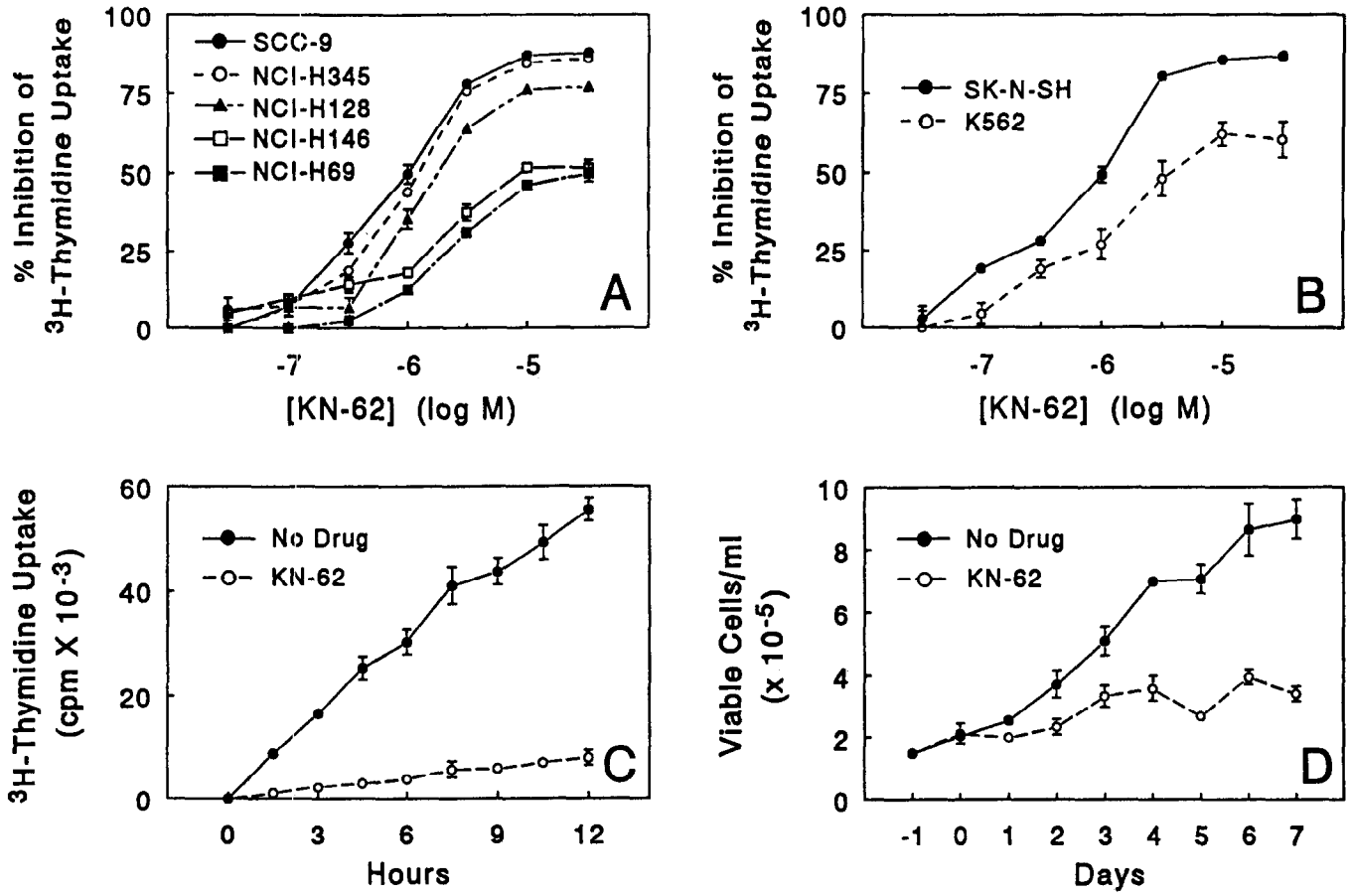

FIG. 5. Inhibition of DNA synthesis due to inactivation of CaM kinase in exponentially proliferating cells. The effects of KN-62 on [^{3}H]-thymidine uptake by SCLC cell lines (A) and other types of cells (B) were measured over a 3-h period. Results are the means (±1 SEM) of 12–20 determinations from 3–5 independent experiments. [^{3}H]-Thymidine uptake (C) and cell proliferation (D) by the SCC-9 SCLC cell line was determined after exposing the cells to 10 μM KN-62 at time 0. Results are the means (±1 SEM) of 3–6 determinations from one experiment. Two other independent experiments produced similar results. All cells were incubated in 10% serum to induce exponential proliferation.

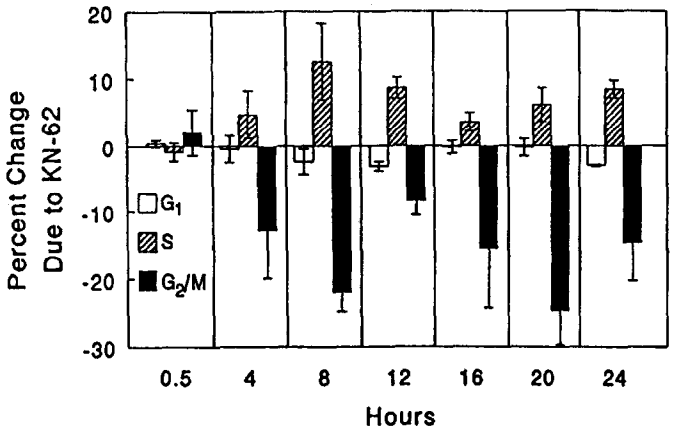

FIG. 6. Reduced S phase progression due to CaM kinase inactivation in SCLC cells. The percent of exponentially proliferating SCC-9 cells in each phase of the cell cycle was determined at various times after incubation in the presence or absence of 10 μM KN-62. The percentage of untreated cells in G_1, S, and G_2/M phase was 65.4 ± 0.2, 27.9 ± 0.2, and 6.6 ± 0.2, respectively, and this did not change significantly during the experiment. Four hours after KN-62 exposure, the percentage of KN-62-treated cells in S phase increased significantly, ($P \leq 0.05$, Student's *t*-test) and the percentage of KN-62-treated cells in G_2/M phase decreased significantly. ($P \leq 0.05$, Student's *t* test). The percent changes due to KN-62 in S phase cells at the different time points after 4 hr were not significantly different from one another, and merely reflect inter-time point variation. Similarly, the percent changes due to KN-62 in G_2/M phase cells after 4 hr were not significantly different from one another. Results are the means (±1 SEM) of 9–12 determinations from 3–4 independent experiments.

exposure to the drug. These findings indicate that inactivation of CaM kinase reduces progression of SCLC cells through the S phase.

Effects of Reduced Ca^{2+} Influx on DNA Synthesis

Previous studies from our laboratory and others indicate that KN-62 inhibits VGCC activity in SCLC cells [45] and other cell types [30, 31]. If reduced Ca^{2+} influx is responsible for the anti-proliferative effects of KN-62, then inhibiting Ca^{2+} influx by other means should mimic the effects of KN-62 and inhibit DNA synthesis. We previously found that addition of 2.5 mM EGTA to the culture medium lowers extracellular free Ca^{2+} to levels that prohibit receptor-mediated and depolarization-dependent Ca^{2+} influx in SCC-9 cells [24]. Inhibition of Ca^{2+} influx using this same method did not mimic the effect of KN-62 on DNA synthesis (Fig. 7). This indicates that inhibition of Ca^{2+} influx is probably not the cause of reduced DNA synthesis in KN-62-treated cells.

DISCUSSION

We have demonstrated that SCLC cells express CaMKII and CaMKIV. Both the α and β isoforms of CaMKIV are expressed by SCLC cells, similar to CaMKIV expressed in SK-N-SH cells and T cells [18, 36]. All SCLC cell lines tested also expressed a 60-kDa protein that reacted with the CBβ-1 antibody to the CaMKII β subunit. The CaMKII β subunit has been detected previously only in neuronal cells [reviewed in Ref. 35]. Because SCLC cells express many neuronal characteristics [reviewed in Ref. 24], it is plausible that SCLC cells express the neuronal CaMKII β subunit. However, it is also

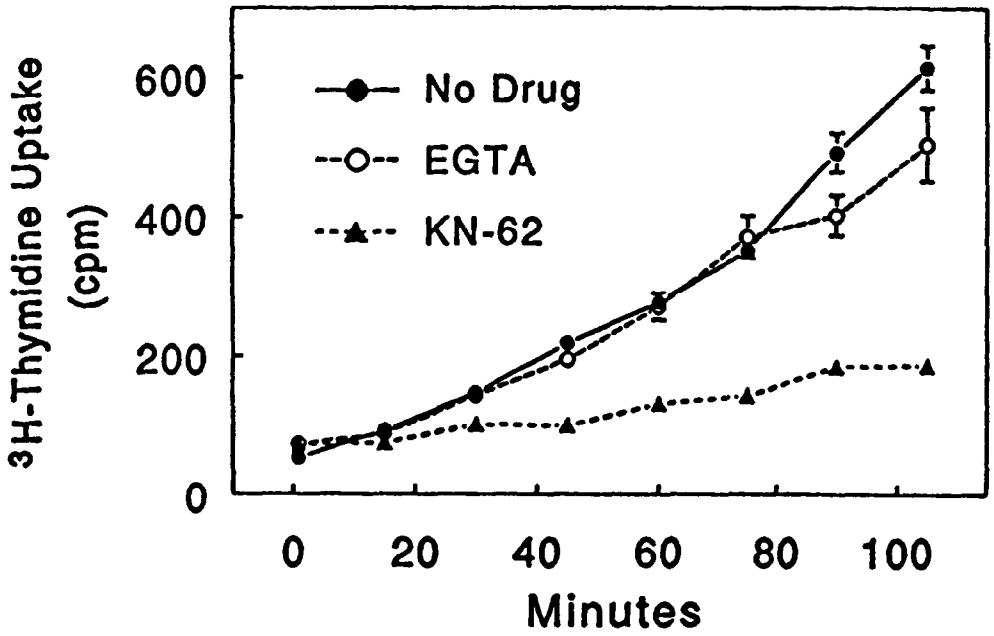

FIG. 7. Effect of reduced Ca^{2+} influx on DNA synthesis in SCLC cells. [^{3}H]-Thymidine uptake by the SCC-9 SCLC cell line was measured after exposing the cells to no drug, 2.5 mM EGTA, or 10 μM KN-62 at time 0. Results are the means (±1 SEM) of 8 determinations from one experiment. Another independent experiment produced similar results.

possible that in SCLC cells, the CBβ-1 antibody recognizes a different CaMKII subunit that is related to the neuronal β subunit, similar to the CaMKII subunit recognized by CBβ-1 in K562 cells (Fig. 1) and in sea urchin eggs [5].

To investigate the functional activity of CaM kinase in SCLC cells, we tested the ability of Ca^{2+}/calmodulin to activate CaMKII immunoprecipitated from SCLC cells. We found that SCLC CaMKII consists of multiple proteins that undergo Ca^{2+}/calmodulin-dependent autophosphorylation, similar to CaMKII in neuronal cells [for reviews, see Refs. 14 and 35]. It is possible that several of these immunoprecipitated proteins are different phosphorylated forms of the CaMKII β subunit. We are currently investigating this possibility, as well as the expression of other CaMKII subunits in SCLC cells.

Selective inhibitors inactivate CaMKII in SCLC cells. The Ca^{2+}/calmodulin-dependent activation of SCLC CaMKII is inhibited by the peptide CaMKII(281–302), which corresponds in sequence to the CaMKII autoinhibitory domain [40]. This peptide specifically inhibits a variety of CaMKII-mediated processes in other cell types [for review, see Ref. 14]. Activation of CaMKII immunoprecipitated from SCLC cells is also inhibited by the drug KN-62, which blocks calmodulin binding to CaMKII, both *in vitro* and *in vivo* [25–32]. The activity of SCLC CaMKII was inhibited maximally by 10 μM KN-62, which agrees with the efficacy of KN-62 in other cell types [28, 29, 32].

Somewhat surprisingly, we were unable to detect Ca^{2+}/calmodulin-dependent autophosphorylation of immunoprecipitated CaMKIV using conditions that induce maximal autophosphorylation of immunoprecipitated CaMKII. This may reflect the different autophosphorylation sites and activation kinetics of CaMKIV compared with CaMKII [46]. Alternatively, the CaMKII and CaMKIV antibodies we used may react differently with native protein and immunoprecipitate different amounts of the respective CaM kinases. Consistent with this, we found that the CaMKIV antiserum reacted better with denatured protein in CaM kinase western blots than did the CaMKII antibody. A third possibility is that CaMKIV is not functional in SCLC cells. We are currently investigating these possibilities.

To determine the role of CaM kinase in cell proliferation, we measured the effects of CaM kinase inactivation in exponentially proliferating SCLC cells. We found that incubation of SCLC cells with KN-62 inhibited both DNA synthesis and S phase progression. Although KN-62 has generally been regarded as a specific antagonist for CaMKII [25–32], a recent study demonstrates that KN-62 also inactivates CaMKIV [22]. Because SCLC cells express both CaMKII and CaMKIV, we cannot conclude that the effect of KN-62 on DNA synthesis in SCLC cells is mediated by either CaMKII or CaMKIV. However, our studies with K562 cells suggest that CaMKIV is not a required participant in this event. We found that KN-62 inhibits DNA synthesis in K562 cells, which were found previously to express CaMKII and to have reduced cell numbers following prolonged exposure to KN-62 [39]. Our demonstration that K562 cells do not express detectable levels of CaMKIV suggests that CaMKII or another KN-62-sensitive CaM kinase mediates the inhibitory effect of KN-62 on DNA synthesis in these cells.

Our finding that CaM kinase inactivation slows S phase progression is consistent with previous studies showing that S phase progression is inhibited by calmodulin antagonists [11] and by calmodulin-specific antibodies [13]. Our results suggest that the inhibitory effects of these calmodulin antagonists and antibodies on S phase progression are due to CaM kinase inactivation.

Incubation with KN-62 inhibits Ca^{2+} influx through VGCC, but does not affect Ca^{2+} mobilization from intracellular stores in SCLC cells [45]. This agrees with previous reports regarding the effects of KN-62 on Ca^{2+} channel activity in other cell types [30, 31]. Several of our findings argue against the possibility that inhibition of DNA synthesis by KN-62 is due to VGCC inactivation and reduced Ca^{2+} influx. First, VGCC are active only when SCLC cells are depolarized [24, 45]. The SCLC cells we used in the present study were not depolarized, so VGCC were inactive. Thus, the effects of KN-62 could not be due to inactivation of VGCC because these channels were already inactivated. Second, incubation with KN-62 does not alter the basal intracellular free Ca^{2+} concentration in SCLC cells [45]. Thus, the KN-62-mediated inhibition of DNA synthesis could not be due to changes in the intracellular free Ca^{2+} concentration because this is unaffected by KN-62. Finally, inhibiting Ca^{2+} influx by chelating extracellular free Ca^{2+} did not mimic the inhibitory effects of KN-62 on DNA synthesis (Fig. 7). Thus, decreased DNA synthesis in KN-62-treated cells is probably not due to reduced Ca^{2+} influx.

Our findings may have therapeutic implications for lung cancer. Drugs that act as calmodulin antagonists have been found previously to inhibit proliferation of lung cancer cell lines [47, 48] and other cell types [49]. Several of these drugs are used clinically and have a variety of effects, including inhibition of tumor proliferation [for review, see Ref. 50]. Our results suggest that the anti-proliferative effect of these drugs is due to inactivation of CaM kinase. The potent inhibition of DNA synthesis in SCLC cells following CaM kinase inactivation indicates that CaM kinase is an important regulator of SCLC proliferation, and thus a potential target for chemotherapeutic drugs.

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