Pergamon

Biochemical Pharmacology, Vol. 50, No. 12, pp. 1979–1985, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02096-9

INHIBITION OF VOLTAGE-GATED Ca^{2+} CHANNEL ACTIVITY IN SMALL CELL LUNG CARCINOMA BY THE Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE INHIBITOR KN-62 (1-[*N,O*-BIS(5-ISOQUINOLINESULFONYL)-*N*-METHYL-L-TYROSYL]-4-PHENYLPIPERAZINE)

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(*Received* 16 *March* 1995; *accepted* 21 *July* 1995)

Abstract—Although small cell lung carcinoma (SCLC) cells express both voltage-gated Ca^{2+} channels (VGCC) and second messenger-operated Ca^{2+} channels (SMOCC), little is known about the factors that regulate the activity of these channels in SCLC cells. Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) type II has been implicated recently in regulating Ca^{2+} channel activity in other cell types. Because of this, we investigated the effects of the specific CaM kinase antagonist 1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tryosyl]-4-phenylpiperazine (KN-62) on Ca^{2+} channel activity in SCLC cells. Incubation with 10 μM KN-62 for 20 min inhibited depolarization-dependent $^{45}Ca^{2+}$ influx by 96.1 ± 2.1% in four independent SCLC cell lines, and by 42.2 ± 6.8% in the NCI-H146 SCLC cell line. Similar inhibitory effects of KN-62 were observed when Fura-2 was used to measure depolarization-dependent Ca^{2+} influx. These results indicate that KN-62 potently inhibits VGCC activity in SCLC cells. In contrast, KN-62 (10 μM, 20 min) did not inhibit significantly Ca^{2+} mobilization induced by muscarinic acetylcholine receptor (mAChR) activation in SCLC cells. This indicates that SMOCC are less susceptible than VGCC to inhibition by KN-62 in SCLC cells. Because mAChR activation also inhibits VGCC activity in SCLC cells, we examined the effects of KN-62 on the mAChR-mediated inhibition of VGCC activity. To do this, we measured depolarization-dependent $^{45}Ca^{2+}$ influx in SCLC cells incubated with submaximal concentrations of KN-62 and the mAChR agonist carbachol. Treatment of cells with both drugs resulted in almost twice as much inhibition of VGCC activity as in cells treated with only one of the drugs. This indicates that inactivation of CaM kinase with KN-62 does not suppress the ability of mAChR agonists to inhibit VGCC activity.

Key words: Ca^{2+}/calmodulin-dependent protein kinase; small cell lung carcinoma; KN-62; cell cycle; cell proliferation; DNA synthesis

SCLC† is a neuroendocrine tumor that expresses VGCC. The development of Lambert-Eaton myasthenic syndrome in patients with SCLC is believed to arise from an autoimmune response to the VGCC expressed by the tumor cells [1–5]. This finding has generated much interest in characterizing the expression and activity of VGCC in SCLC cells. The expression of P-type, N-type, and L-type VGCC in various SCLC cell lines, including the SCC-9 cell line used in this study, has been documented using the polymerase chain reaction [6–8]. The activity of VGCC in SCLC cells has been studied using $^{45}Ca^{2+}$ influx assays [1, 2, 9], fluorescent Ca^{2+}-binding dyes [5, 9], and patch clamp techniques [8, 10, 11]. Although VGCC in SCLC cells continue to be well characterized, little is known about the factors that regulate the activity of these channels in the cells.

The activity of VGCC in SCLC cells seems to be regulated by pathways similar to those found in neuronal cells. We previously reported that stimulation of the M_3 mAChR inhibits VGCC activity in SCLC cells [9]. This is similar to the inactivation of VGCC following M_1 mAChR stimulation in rat sympathetic neurons [12]. Although protein kinase C activity increases following stimulation of M_1 and M_3 mAChR [reviewed in Ref. 13], previous studies indicate that activation of protein kinase C does not inhibit VGCC in either SCLC cells [9] or in some types of neurons [12]. This indicates that second messengers other than protein kinase C regulate VGCC in these cells.

SCLC cells also express SMOCC, and Ca^{2+} flux through these channels is induced by activating G protein-coupled receptors like the M_3 mAChR [9]. The majority of Ca^{2+} mobilized by activating G protein-coupled receptors comes from internal stores and is dependent on inositol trisphosphate [14]. However, a component of this mobilization depends on extracellular Ca^{2+} [9], indicating that Ca^{2+} channels on the plasma membrane play a role in receptor-mediated Ca^{2+} mobilization. Like the regulation of VGCC, the factors that modulate the activity of SMOCC in SCLC cells are not characterized completely.

The CaM kinases are likely candidates to regulate Ca^{2+} channel activity. Several CaM kinases have been

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† Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; 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; mAChR, muscarinic acetylcholine receptor; SCLC, small cell lung carcinoma; SMOCC; second messenger-operated Ca^{2+} channels; and VGCC, voltage-gated Ca^{2+} channels.

cloned and characterized, including CaMKII [reviewed in Ref. 15] and, more recently, CaMKIV [16–19]. Several studies indicate that CaM kinase can regulate VGCC activity. The activity of VGCC in several cell types is inhibited by the CaM kinase-specific antagonist KN-62 [20, 21], which inactivates both CaMKII [15, 22] and CaMKIV [23]. Inactivation of CaMKII with other inhibitors reduces L-type VGCC activity in cardiac myocytes [24, 25] and T-type VGCC activity in adrenal glomerulosa cells [26]. The ability of CaMKII to phosphorylate L-type [27] and N-type [28] VGCC provides a possible mechanism by which CaMKII could regulate VGCC activity. CaMKII also regulates the activity of other types of Ca^{2+} channels, such as the ryanodine-sensitive Ca^{2+} channel [29]. The phosphorylation of the ryanodine-sensitive Ca^{2+} channel by CaMKII may alter the activity of this channel [30].

We recently demonstrated that SCLC cells express both CaMKII and CaMKIV*. Because KN-62 inactivates these CaM kinases in SCLC cells,* we investigated the effects of KN-62 on Ca^{2+} channel activity in these cells. We found that KN-62 inhibited VGCC activity in five independent SCLC cell lines. In contrast, KN-62 did not inhibit significantly Ca^{2+} mobilization induced by mAChR activation. These results indicate that VGCC are more sensitive than SMOCC to the inhibitory effects of KN-62 in SCLC cells. Thus, CaM kinase may play a more important role in regulating VGCC activity in these cells. We also investigated the role of CaM kinase in the mAChR-mediated inhibition of VGCC activity in SCLC cells. Surprisingly, we found that inactivation of CaM kinase with KN-62 did not alter the ability of mAChR agonists to inhibit VGCC activity in SCLC cells. These findings indicate that inactivation of CaM kinase does not suppress mAChR-mediated inhibition of VGCC activity in SCLC cells.

MATERIALS AND METHODS

Reagents

The CaMKII and CaMKIV inhibitor KN-62 was purchased from Seikagaku America, Inc. (Rockville, MD). A stock solution of 10 mM KN-62 was prepared in anhydrous Me_2SO (Sigma Chemical Co., St. Louis, MO), and diluted in culture medium for assays. Control cells in all experiments were incubated with the same amount of Me_2SO used to dissolve KN-62 in cultures of experimental cells. Fura-2 AM and pluronic were obtained from Molecular Probes, Inc. (Eugene, OR), and $^{45}Ca^{2+}$ was obtained 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 [2, 9, 31, 32]. The SCLC cell lines NCI-H69, NCI-H128, NCI-H146, and NCI-H345 were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured 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.

$^{45}Ca^{2+}$ influx assays

Measurement of $^{45}Ca^{2+}$ influx was performed as previously described [9]. Cells were exposed for 1 min at 37° to Krebs buffer containing 1.2 mM $MgCl_2$, 24.9 mM $NaHCO_3$, 10 mM glucose, 1.2 mM $^{45}Ca^{2+}$ (sp. act. 11.6 mCi/mmol) and either 118 mM NaCl and 4.7 mM KCl (basal buffer) or 32.8 mM NaCl and 90 mM KCl (depolarizing buffer) and the appropriate concentration of drug. Cells were washed twice by centrifugation (250 *g*, 1 min) in basal buffer containing nonradioactive Ca^{2+}, lysed in distilled water, and subjected to liquid scintillation counting using an LS-6000IC β-counter (Beckman Instruments, Fullerton, CA).

Measurement of $[Ca^{2+}]_i$

Cells were incubated in RPMI 1640 medium containing 4 μM Fura-2 AM, 0.1% pluronic, and 2% calf bovine serum for 30 min at 37°. Cell suspensions were diluted 1:1 with RPMI 1640 medium containing 2% calf bovine serum and incubated another 30 min. After washing, cells were allowed to attach to coverslips (Biophysica Technologies, Inc., Sparks, MD) coated with poly-L-lysine (30,000–70,000 mol. wt, Sigma). The coverslips were placed in tissue chambers designed for use with an Olympus IMT2 inverted microscope (Hitech Instruments, Edgemont, PA). The ratios of Fura-2 fluorescence detected at 505 nm using excitation wavelengths of 340 and 380 nm were measured using a CMX Scanning Cation MicroIlluminator (Spex Industries, Edison, NJ). Ratios were converted to $[Ca^{2+}]_i$ values by permeabilizing SCLC cells with 50 μM digitonin, exposing them to different concentrations of extracellular Ca^{2+}, and solving the equations described by Grynkiewicz *et al.* [33].

RESULTS

We previously reported that the SCC-9 SCLC cell line exhibits depolarization-dependent Ca^{2+} influx [9]. Depolarization-dependent Ca^{2+} influx in this and other SCLC cell lines is inhibited by Co^{2+} and Mn^{2+}, dihydropyridines and ω-conotoxin GVIA, and is not affected by the external Na^+ concentration [2, 8, 9]. This indicates that depolarization-dependent Ca^{2+} influx reflects VGCC activation in SCLC cells.

We examined the effects of the CaM kinase-specific antagonist KN-62 on depolarization-dependent Ca^{2+} influx in SCC-9 cells. Incubation of SCC-9 cells with KN-62 inhibited depolarization-dependent Ca^{2+} influx (Fig. 1). The half-maximal inhibitory concentration of KN-62 was 0.40 ± 0.06 μM (Fig. 1A). The inhibitory effect of KN-62 was time dependent, reaching maximum inhibition after a 12-min incubation with the drug (Fig. 1B).

Incubation with KN-62 also inhibited depolarization-dependent Ca^{2+} influx in other SCLC cell lines. The maximum level of depolarization-dependent Ca^{2+} influx varied among different SCLC cell lines (Fig. 2A). The NCI-H146 cell line exhibited the greatest Ca^{2+} influx upon depolarization, whereas the NCI-H69 cell line exhibited only a very small depolarization-dependent Ca^{2+} influx (Fig. 2A). Inactivation of CaM kinase with KN-62 inhibited depolarization-dependent Ca^{2+} influx in all cell

* Williams CL, Phelps SH and Porter RA, Manuscript submitted for publication.

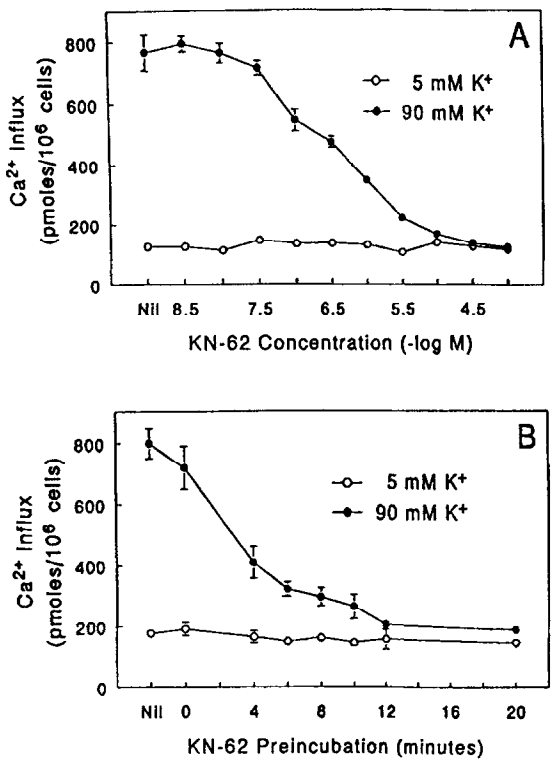

Fig. 1. Inhibition of depolarization-dependent $^{45}Ca^{2+}$ influx by KN-62 in the SCC-9 SCLC cell line. Influx of $^{45}Ca^{2+}$ in the presence of 5 or 90 mM K^+ was measured using SCC-9 cells that were untreated (Nil) or incubated with various concentrations of KN-62 for 20 minutes (A) or with 10 μM KN-62 for varying times (B). Results shown are the means (±SEM) of 9 determinations from 3 independent experiments.

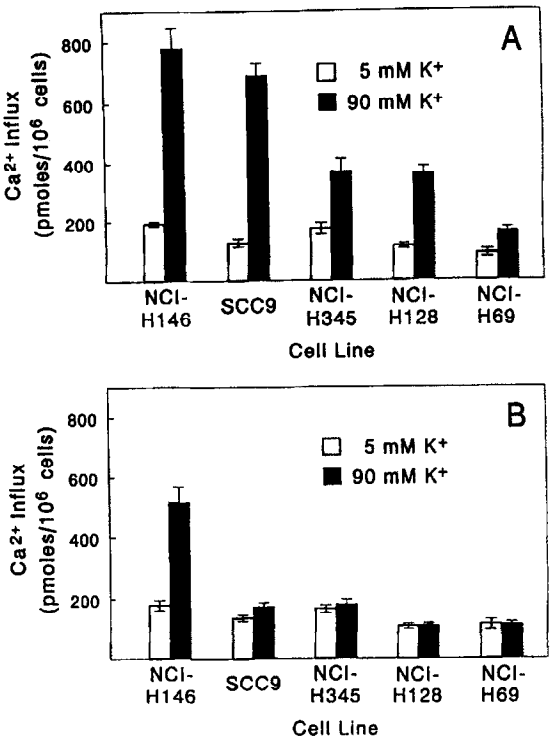

Fig. 2. Inhibition of depolarization-dependent $^{45}Ca^{2+}$ influx by KN-62 in different SCLC cell lines. Influx of $^{45}Ca^{2+}$ in the presence of 5 or 90 mM K^+ was measured using different SCLC cell lines that were untreated (A) or preincubated with 10 μM KN-62 for 20 min (B). Results are the means (± SEM) of 9–12 determinations from 3–4 independent experiments.

lines tested (Fig. 2B). In four of the five cell lines tested, depolarization-dependent Ca^{2+} influx was reduced by more than 90% following a 20-min incubation wtih 10 μM KN-62. In contrast, depolarization-dependent Ca^{2+} influx in the NCI-H146 cell line was reduced by only 42.2 ± 6.8% following a 20-min incubation with 10 μM KN-62 (Fig. 2B). Incubation with KN-62 did not alter basal Ca^{2+} influx in any of the cell lines tested.

Studies using Fura-2 provided further evidence that KN-62 inhibited depolarization-dependent Ca^{2+} influx. In untreated SCC-9 cells, depolarization caused a transient peak in $[Ca^{2+}]_i$ that declined rapidly (Fig. 3A). The increase in $[Ca^{2+}]_i$ was inhibited by chelating extracellular free Ca^{2+} with EGTA (Fig. 3B), indicating that the depolarization-dependent increase in $[Ca^{2+}]_i$ is due to Ca^{2+} influx. Treatment of SCC-9 cells with 10 μM KN-62 did not alter the resting level of $[Ca^{2+}]_i$, but reduced the depolarization-dependent rise in $[Ca^{2+}]_i$ (Fig. 4).

Having found that KN-62 inhibited depolarization-dependent Ca^{2+} influx if the drug was applied before depolarization, we next determined the effects of applying KN-62 after depolarization. To do this, we depolarized SCC-9 cells and 2 min later exposed the cells to 10 μM KN-62 (Fig. 5B). Surprisingly, incubation of these previously depolarized cells with 10 μM KN-62 for 5 min did not inhibit Ca^{2+} mobilization significantly (–3.2 ± 4.5% change in $[Ca^{2+}]_i$ compared with control cells, N = 4) (Fig. 5). These previously depolarized cells were sensitive to other agents that inhibit Ca^{2+} influx, such as EGTA which chelates extracellular Ca^{2+} (Fig. 5C). In contrast to the effects of applying KN-62 after depolarization, incubation of SCC-9 cells with 10 μM KN-62 for 5 min before depolarization inhibited Ca^{2+} influx by 62.2 ± 2.8% compared with control cells (N = 3) (Figs. 1B and 4B). This indicated that KN-62 inhibits depolarization-dependent Ca^{2+} influx more effectively when the drug is applied before, rather than after, depolarization.

To determine the effects of KN-62 on Ca^{2+} mobilization induced by mAChR activation, we incubated SCC-9 cells with no drug or 10 μM KN-62 for 20 min, and then measured Ca^{2+} mobilization induced by the mAChR agonist carbachol. Activation of the M_3 mAChR in SCC-9 cells induced a sustained elevation in $[Ca^{2+}]_i$ (Fig. 6). Two minutes after exposure to carbachol, $[Ca^{2+}]_i$ increased 198.6 ± 27.1% over basal level in control cells, and by 163.4 ± 20.5% over basal level in cells preincubated with KN-62 (N = 5). These values were not significantly different as measured by Student's *t*-test, indicating that KN-62 does not inhibit significantly Ca^{2+} mobilization induced by mAChR activation.

We also examined the role of CaM kinase in the reduced activity of VGCC that occurs following mAChR activation in SCLC cells [9]. If CaM kinase is required for mAChR-mediated inhibition of VGCC, inactivation of CaM kinase should suppress the ability of mAChR agonists to inhibit VGCC activity. To examine this, we tested the ability of the mAChR agonist carbachol to inhibit depolarization-dependent Ca^{2+} influx in SCC-9 cells that were preincubated in the absence or presence

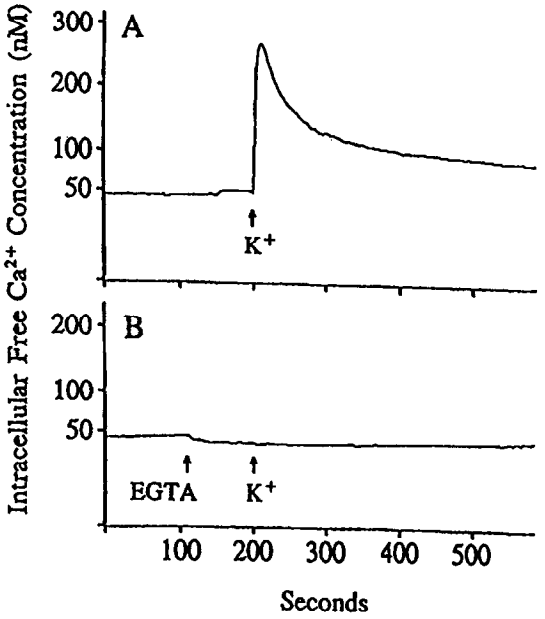

Fig. 3. Inhibition of depolarization-dependent Ca^{2+} influx in SCC-9 cells by chelating extracellular Ca^{2+} with EGTA. SCC-9 cells loaded with Fura-2 were exposed to 90 mM K^+ (at 200 sec) in the absence of EGTA (A) or after addition of 2.5 mM EGTA (at 100 sec) (B). Three other independent experiments produced similar results.

of 0.4 μM KN-62. Incubation of SCC-9 cells with a submaximal concentration of carbachol markedly inhibited VGCC activity in SCC-9 cells (Fig. 7), in agreement with our previous findings [9]. The VGCC activity was inhibited to an even greater extent if the cells were preincubated with KN-62 before treatment with carbachol (Fig. 7). This indicates that inactivating CaM kinase with KN-62 does not suppress the ability of mAChR agonists to inhibit VGCC activity.

DISCUSSION

Because CaM kinase interacts with Ca^{2+} channels in other cell types [27, 28, 30], we investigated the role of CaM kinase in regulating Ca^{2+} channel activity in SCLC cells. We recently determined that SCLC cells express functional CaM kinase, which is inactivated by the CaM kinase-specific antagonist KN-62.* In the present study, we found that incubation with KN-62 inhibited VGCC activity in all of the SCLC cell lines tested. This is consistent with previous reports that incubation with KN-62 inhibits VGCC activity in the insulin-secreting cell line HIT-T15 [21] and in mouse pancreatic β cells [20]. Similar concentrations of KN-62 half-maximally inhibit VGCC activity in SCLC cells and in HIT-T15 cells [21], suggesting that KN-62 inactivates VGCC by a similar mechanism in these cell types.

The KN-62-mediated inhibition of VGCC in SCLC cells may be due to inactivation of CaMKII. Consistent with this is our finding that the KN-62 concentrations that inhibit VGCC activity in SCLC cells (Fig. 1) are

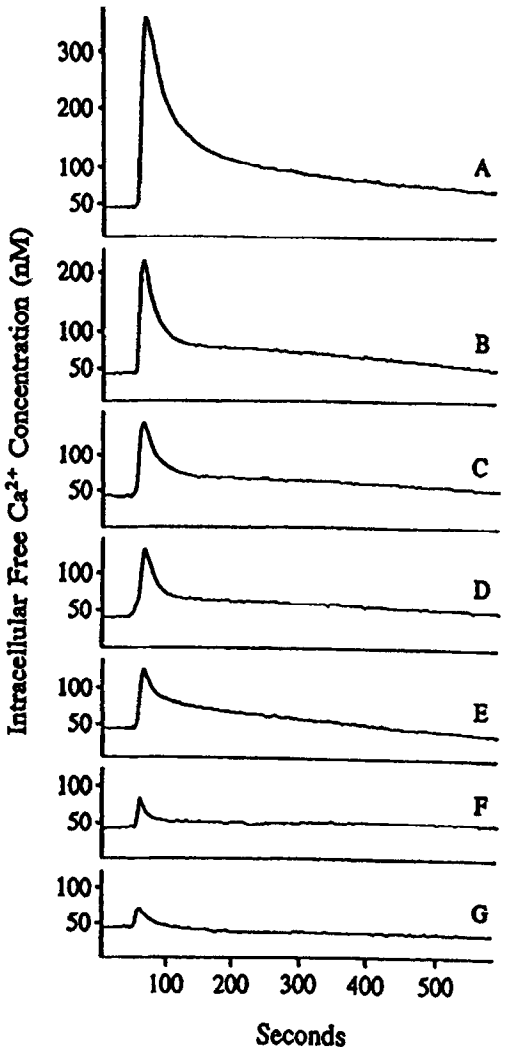

Fig. 4. Inhibition of depolarization-dependent Ca^{2+} influx by KN-62 in cells loaded with Fura-2. SCC-9 cells loaded with Fura-2 were exposed to 90 mM K^+ (at 50 sec) after incubation with no drug (A) or incubation with 10 μM KN-62 for 5 min (B), 7 min (C), 9 min (D), 11 min (E), 15 min (F), or 20 min (G). Two other independent experiments produced similar results.

similar to those that inactivate CaMKII in SCLC cells* and in other cells [22]. It was found recently that CaMKII mediates the Ca^{2+}-dependent enhancement of L-type VGCC activity in cardiac myocytes [24, 25]. If CaMKII is inactivated in these cells, L-type VGCC currents have lower amplitude and inactivate more quickly [24, 25]. Thus, it is plausible that inactivation of CaMKII with KN-62 produces the same effect in SCLC cells. Consistent with this, we found that treatment of SCC-9 cells with KN-62 caused the depolarization-dependent increase in $[Ca^{2+}]_i$ to have lower amplitude and return more quickly to baseline levels (Fig. 4). The ability of CaMKII to phosphorylate VGCC [27, 28] is one way CaMKII could alter VGCC activity.

Another possibility is that the KN-62-mediated inhibition of VGCC is due to direct blockage of VGCC with KN-62. This hypothesis is favored by Li *et al.* [21], who studied the effects of KN-62 on cells that have fully

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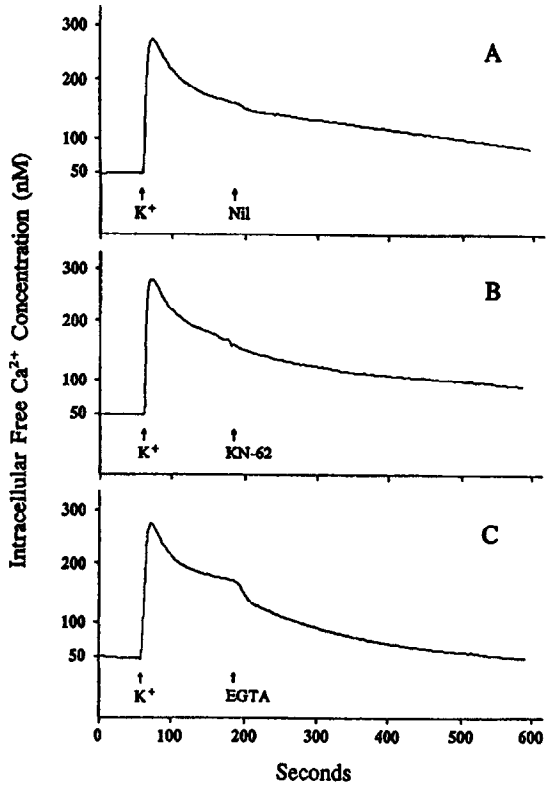

Fig. 5. Effects of KN-62 on Ca^{2+} influx in previously depolarized cells. SCC-9 cells loaded with Fura-2 were exposed to 90 mM K^+ (at 60 sec) and 2 min later were exposed to buffer containing no drug (A), 10 μM KN-62 (B), or 2.5 mM EGTA (C). Three other independent experiments produced similar results.

activated CaMKII due to prior depolarization. It was shown previously that KN-62 cannot inhibit CaMKII once the kinase has been fully activated by exposure to Ca^{2+}/calmodulin [22]. Because depolarization increases intracellular Ca^{2+}/calmodulin, and thus activates CaMKII, depolarized cells should be insensitive to the inhibitory effects of KN-62 on CaMKII. Li *et al.* [21] found that KN-62 inhibits VGCC in previously depolarized cells that should have fully activated CaMKII and that should be insensitive to KN-62. This led these authors to suggest that the KN-62-mediated inhibition of VGCC does not involve CaM kinase, but instead results from blockade of the channels by KN-62.

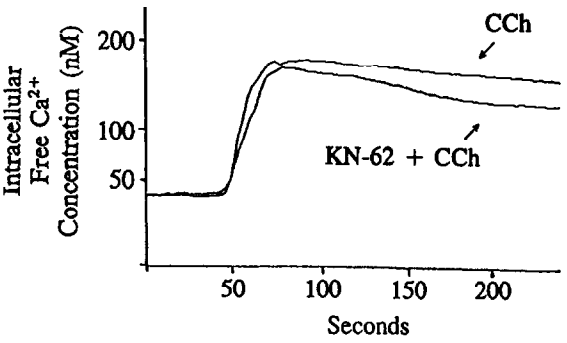

Fig. 6. Effects of KN-62 on Ca^{2+} mobilization induced by mAChR activation. SCC-9 cells loaded with Fura 2 were exposed to 1 μM carbachol (at 50 sec) without pretreatment (CCh) or after incubation with 10 μM KN-62 for 20 min (KN-62 + CCh). Four other independent experiments produced similar results.

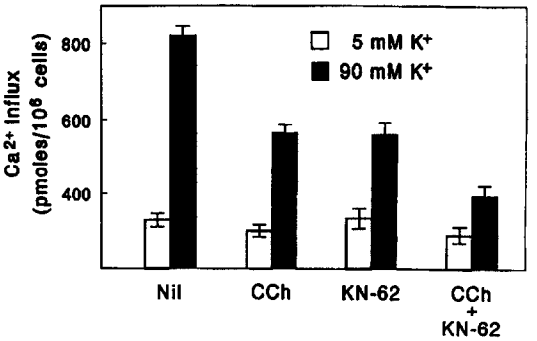

Fig. 7. Effects of carbachol and KN-62 on depolarization-dependent Ca^{2+} influx in SCC-9 cells. Influx of $^{45}Ca^{2+}$ in the presence of 5 mM K^+ or 90 mM K^+ was measured using SCC-9 cells that were untreated (Nil) or incubated with carbachol (CCh), KN-62 (KN-62), or KN-62 plus carbachol (CCh + KN-62). Submaximal concentrations of carbachol (1 μM) and KN-62 (0.4 μM) were used. Cells were exposed to carbachol for 20 min or to KN-62 for 40 min before depolarization. When both drugs were used, cells were incubated with KN-62 for 20 min, followed by incubation with both KN-62 and carbachol for an additional 20 min before depolarization. Results shown are the means (±SEM) of 12 determinations from 4 independent experiments.

We used an approach similar to that described by Li *et al.* [21] to address the possibility of direct blockade of VGCC by KN-62 in SCC-9 cells. To do this, we measured Ca^{2+} influx in SCC-9 cells that were depolarized before application of KN-62 (Fig. 5). We have shown that depolarization activates CaMKII in SCC-9 cells.* We found that KN-62 did not significantly inhibit VGCC activity in previously depolarized SCC-9 cells (Fig. 5), which have fully activated CaMKII. These findings are consistent wtih the hypothesis that the inhibition of VGCC activity by KN-62 in SCC-9 cells is due to inactivation of CaMKII, rather than direct blockade of VGCC.

We found that the maximum level of depolarization-dependent Ca^{2+} influx, and the response to KN-62, varied among different SCLC cell lines. The NCI-H146 cell line exhibited the greatest depolarization-dependent Ca^{2+} influx, whereas the NCI-H69 cell line exhibited the smallest influx. This agrees with previous studies demonstrating heterogeneity of VGCC activity among SCLC cell lines [2, 5, 10, 11]. Using patch clamp techniques, Pancrazio *et al.* [10] found that the inward Ca^{2+} current in NCI-H146 cells has greater amplitude and inactivates more slowly than the inward Ca^{2+} current in NCI-H69 cells. Similarly, Sher *et al.* [5] tested seven SCLC cell lines for ω-conotoxin GVIA binding sites, which are indicative of N-type VGCC, and found that the NCI-H146 cell line has the greatest number of binding sites, and the NCI-H69 cell line has the least. Interestingly, we found that the NCI-H146 cell line was the least suscep-

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tible to the inhibitory effects of KN-62 on VGCC activity. If KN-62 inactivates only a certain type of VGCC, then cells expressing VGCC which are insensitive to KN-62 would be less susceptible to the inhibitory effects of KN-62. Although previous studies indicate that KN-62 inhibits depolarization-dependent Ca^{2+} influx through L-type VGCC [21], the effects of KN-62 on N-type VGCC have not been characterized.

The SCC-9 cell line used in this study has been found previously to express transcripts for P-type, L-type, and N-type VGCC [6, 7] and to express functional channels with the pharmacological characteristics of L-type and N-type VGCC [2]. Thus, depolarization-dependent Ca^{2+} influx in SCC-9 cells most likely reflects activation of both L-type and N-type VGCC. We found that maximal concentrations of KN-62 abolished almost all depolarization-dependent Ca^{2+} influx in SCC-9 (Figs. 1, 2, and 4), suggesting that KN-62 inactivates both L-type and N-type VGCC expressed by these cells.

In contrast to the effects of KN-62 on VGCC activity, Ca^{2+} mobilization induced by mAChR activation was not inhibited significantly by KN-62. We previously presented evidence that the initial transient peak in $[Ca^{2+}]_i$ induced by mAChR activation is due to mobilization of Ca^{2+} from internal stores, whereas the subsequent sustained elevation in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+} [9]. We found that KN-62 did not alter significantly the transient peak or sustained elevation of $[Ca^{2+}]_i$ following mAChR activation. This insensitivity to KN-62 does not reflect a general insensitivity of the mAChR-mediated Ca^{2+} mobilization to pharmacological manipulation, because drugs such as the Ca^{2+} chelator EGTA and the mAChR antagonist 4-diphenylacetoxy-*N*- methylpiperidine methiodide significantly alter Ca^{2+} mobilization induced by mAChR activation in SCC-9 cells [9]. Previous studies have shown that KN-62 only slightly diminishes Ca^{2+} mobilization induced by bombesin, which activates a G protein-coupled receptor similar to mAChR [21]. These results indicate that Ca^{2+} channels activated by second messengers (such as inositol triphosphate) are less susceptible than VGCC to the inhibitory effects of KN-62.

Although KN-62 did not alter significantly mAChR-mediated Ca^{2+} mobilization, it is possible that inactivation of CaM kinase by KN-62 interferes with the mAChR-mediated inhibition of VGCC activity. To test this, we measured depolarization-dependent Ca^{2+} influx in cells incubated with submaximal concentrations of carbachol and KN-62. Treatment of cells with both drugs resulted in almost twice as much inhibition of VGCC activity as in cells treated with only one of the drugs. This may happen because carbachol and KN-62 inhibit VGCC activity by two independent mechanisms, resulting in twice as much VGCC inhibition when both mechanisms occur. Because KN-62 inactivates CaM kinase in SCLC cells,* our findings indicate that inactivation of CaM kinase does not alter the ability of mAChR agonists to inhibit VGCC activity. This suggests that the mAChR-mediated inhibition of VGCC in SCLC cells does not require active CaM kinase.

SCLC cells offer an excellent model system for studying the factors that regulate VGCC and SMOCC activity. Additionally, the characterization of Ca^{2+} channel activity in SCLC cells may provide important clinical information. Autocrine growth factors stimulate Ca^{2+} mobilization through SMOCC in SCLC cells [34, 35]. The secretion of autocrine growth factors by SCLC cells is also stimulated by Ca^{2+} influx through VGCC [8]. It is possible that factors that inhibit Ca^{2+} channel activity may suppress SCLC proliferation. Our findings identify KN-62 as a potent inhibitor of VGCC activity in SCLC cells. Our recent demonstration that KN-62 inhibits SCLC proliferation* provides further impetus to characterize the effects of inactivating CaM kinase with KN-62 in SCLC cells. We are currently investigating this.

Acknowledgements–This study was supported by NIH Grant CA52471 from the National Cancer Institute to C.L.W.

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