Biochemical Pharmacology, Vol. 53, pp. 1107–1114, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00089-0

Inhibition of M_3 Muscarinic Acetylcholine Receptor-Mediated Ca^{2+} Influx and Intracellular Ca^{2+} Mobilization in Neuroblastoma Cells by the Ca^{2+}/Calmodulin-Dependent Protein Kinase Inhibitor 1-[*N*,O-Bis(5-isoquinolinesulfonyl)-*N*-methyl-L-trosyl]-4-phenylpiperazine (KN-62)

Henry L. Puhl, Padma S. Raman, Carol L. Williams and Robert S. Aronstam*

LABORATORIES OF NEUROBIOLOGY AND MOLECULAR PHARMACOLOGY, GUTHRIE RESEARCH INSTITUTE, SAYRE, PA 18840, U.S.A.

ABSTRACT. The role of Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase; EC 2.7.1.123) in the generation of Ca^{2+} signals by muscarinic acetylcholine receptors (mAChR) was studied. Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) induced by mAChR activation were monitored in SK-N-SH human neuroblastoma cells using the dye Fura-2. SK-N-SH cells express M_3 mAChR, as well as CaM kinase types II and IV, which are specifically inhibited by the CaM kinase antagonist KN-62 (1-[*N*,O-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine). Carbamylcholine (100 μM) elicited an initial transient peak in $[Ca^{2+}]_i$ due to mobilization of Ca^{2+} from internal stores, followed by a sustained elevation in $[Ca^{2+}]_i$ that depended on the influx of extracellular Ca^{2+} and which was inhibited by EGTA and Ni^{2+}. These mAChR-induced Ca^{2+} signals were diminished to an equal extent by preincubating the cells with 0.01 to 100 μM KN-62. KN-62 inhibited mAChR-induced Ca^{2+} influx and mobilization from internal stores by about 25–30%, producing a half-maximal effect at ≈ 1μM. In contrast, KN-62 (25 μM) almost completely abolished carbamylcholine-stimulated entry of divalent cations through Mn^{2+}-permeant channels, as revealed by Mn^{2+} quenching of Fura-2 fluorescence. KN-62 also almost completely abolished Ca^{2+} influx induced by depolarization of the cells with 25 mM K^+ (IC_{50} ≈ 3 μM). These results suggest that CaM kinases regulate both the mobilization of intracellular Ca^{2+} and the stimulation of Ca^{2+} influx that are induced by mAChR activation, and indicate that the mAChR-induced influx of Ca^{2+} occurs through Ca^{2+} channels other than, or in addition to, the voltage-gated calcium channels or Mn^{2+}-permeant channels which are inhibited by KN-62. BIOCHEM PHARMACOL 53;8:1107–1114, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Ca^{2+}/calmodulin-dependent protein kinase; muscarinic acetylcholine receptor; Ca^{2+} channels; KN-62; SK-N-SH human neuroblastoma

Activation of the M_3 mAChR† mobilizes Ca^{2+} from internal stores and induces an influx of extracellular Ca^{2+} [1–4]. These Ca^{2+} signals play an important role in regulating proximal events in mAChR signal transduction [5, 6] as well as activating downstream effectors such as protein kinase C [7]. The mechanisms by which these Ca^{2+} signals are generated, including the identification of the enzymes and Ca^{2+} channels involved in this process, have not been characterized completely. Toward this aim, we are investigating the role of CaM kinases in mAChR-mediated Ca^{2+} signaling.

The CaM kinases are likely candidates to participate in mAChR-induced Ca^{2+} signaling. Stimulation of the M_3 mAChR in SK-N-SH cells induces both Ca^{2+} mobilization and calmodulin translocation, which may affect the activity of CaM kinase [8]. Additionally, recent studies indicate that CaM kinases regulate Ca^{2+} channel activity. The regulation of VGCC by CaM kinases is well documented: inactivation of CaM kinase with the CaM kinase antagonist KN-62 or other specific inhibitors reduces VGCC activity in several cell types [9–14], diminishing both L-type [11, 12] and T-type [13] VGCC activity. The phosphorylation of L-type [15] and N-type [16] VGCC by CaM kinase type

* Corresponding author: Dr. Henry L. Puhl, Guthrie Research Institute, One Guthrie Square, Sayre, PA 18840. Tel. (717) 882-4642; FAX (717) 882-5151.

† *Abbreviations:* CaM kinase, calcium/calmodulin-dependent protein kinase (EC 2.7.1.123); $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; DMEM, Dulbecco's modified Eagle's medium; KN-04, *N*-[1-[*N*-methyl-*p*-(5-isoquinolinesulfonyl)benzyl]-2-(4-phenylpiperazine)ethyl]-5-isoquinolinesulfonamide; KN-62, 1-[*N*,O-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; mAChR, muscarinic acetylcholine receptor(s); and VGCC, voltage-gated calcium channels.

Received 18 July 1996; accepted 31 October 1996.

II provides additional evidence for CaM kinase modulation of VGCC activity.

The effects of CaM kinases on second messenger-operated Ca^{2+} channels are less well understood. Inactivation of CaM kinase with KN-62 only modestly diminishes the increase in $[Ca^{2+}]_i$) induced by activating the bombesin receptor in HIT-T15 cells [9], and does not alter significantly the $[Ca^{2+}]_i$ increase induced by activating M_3 mAChR in small cell lung carcinoma cells [14]. The $[Ca^{2+}]_i$ increase induced by activating these receptors is due to both Ca^{2+} influx and mobilization from internal stores [1, 9]. The possibility that CaM kinases may have different effects on these components of the Ca^{2+} response has not been evaluated.

To resolve these questions, we examined the effects of a CaM kinase inhibitor, KN-62, on Ca^{2+} responses induced by mAChR expressed by cultured SK-N-SH cells. These human neuroblastoma cells express M_3 mAChR as well as CaM kinases type II and IV [8, 17]. Both of these CaM kinase types are inhibited by the CaM kinase antagonist KN-62 [18, 19]. We have demonstrated that KN-62 diminishes Ca^{2+} influx from the external medium as well as Ca^{2+} mobilization from intracellular stores induced by mAChR activation. However, KN-62 is a much more efficient inhibitor of Ca^{2+} influx mediated by K^+-induced depolarization. These findings suggest a role for CaM kinases in the regulation of Ca^{2+} signaling mediated by mAChR activation.

MATERIALS AND METHODS

SK-N-SH human neuroblastoma cells (American Type Culture Collection, Rockville, MD; ATCC HTB-11) were grown to approximately 70% confluency in 150 cm^2 culture flasks in complete DMEM medium consisting of DMEM (Fisher Scientific, Springfield, NJ) supplemented with 10% fetal bovine serum (Biofluids, Rockville, MD), penicillin, streptomycin, and non-essential amino acids (Fisher Scientific). The cells were then dislodged by Viokase treatment, washed, and resuspended in Hanks' salt solution. The cells were counted, pelleted, and resuspended in complete DMEM medium at a concentration of 1×10^5 cells/mL. Aliquots (2 mL) of the cell suspension were plated on sterile glass coverslips (0.11 × 31 mm) in 35 × 10 mm petri dishes. The cells were grown at 37° in the presence of 5% CO_2 for 4 days prior to determining intracellular Ca^{2+} concentrations.

Ca^{2+} measurements were performed using the fluorescent dye Fura-2 as follows. The medium was removed and the SK-N-SH cells were incubated for 30 min in complete DMEM containing 2 μM Fura-2-AM (Molecular Probes, Eugene, OR). The medium was replaced with fresh complete DMEM, and the cells were incubated for a further 30 min. Fura-2 fluorescence was measured using a SPEX Industries dual wavelength fluorometer. Excitation wavelengths were 340 and 380 nm, and the emission wavelength was 505 nm. The ratio of fluorescence intensities upon excitation at 340 and 380 nm was converted to $[Ca^{2+}]_i$ values by comparison to the corresponding ratios obtained when the cells were permeabilized with digitonin in the presence of different extracellular Ca^{2+} concentrations. These standard values were used to solve the equations of Grynkiewicz *et al.* [20]. Manganese permeability was determined by the quenching of Fura-2 fluorescence after excitation at a calcium-insensitive wavelength (360 nm) following the addition of $MnCl_2$ to the medium to a final concentration of 1 mM.

Depolarization measurements were made by replacing the complete DMEM medium with a Kreb's buffer consisting of 1.2 mM $MgCl_2$, 24.9 mM $NaHCO_3$, 10 mM glucose, 1.2 mM $CaCl_2$, 118 mM NaCl, and 4.7 mM KCl at pH 7.4. The K^+ concentration was raised to 25 mM by the addition of an appropriate quantity of a modified Kreb's buffer in which the concentrations of NaCl and KCl were 32.8 and 90 mM, respectively.

KN-62 and KN-04 (Seikagaku America, Rockville, MD) were prepared in DMSO as 0.01 M stock solutions, and were diluted in complete DMEM medium immediately prior to use. The drugs were added to the coverslips following Fura-2 loading, and incubated at 37° in 5% CO_2 for 20 min prior to fluorescence measurements. To measure cellular responses in the absence of extracellular calcium (and, thus, Ca^{2+} influx), 6 mM EGTA was added to the medium 2 min before the addition of carbamylcholine. Drug solutions were prepared in deionized, distilled water.

RESULTS

Activation of mAChR with 100 μM carbamylcholine increased the $[Ca^{2+}]_i$ in SK-N-SH cells (Fig. 1). This $[Ca^{2+}]_i$ increase could be resolved into two components: an initial transient peak in $[Ca^{2+}]_i$ that reflected Ca^{2+} release from internal stores, and a sustained elevation in $[Ca^{2+}]_i$ that was dependent on influx of extracellular Ca^{2+}. The sustained $[Ca^{2+}]_i$ elevation was decreased by inclusion of 6 mM EGTA or 100 mM Ni^{2+} in the extracellular medium (Fig. 1). EGTA alone did not affect the concentration of intracellular calcium in unstimulated cells over the time course of the present experiments (up to 10 min exposure). All of the cells responded to carbamylcholine, although there was considerable variation in the magnitudes of both the initial transient peak in $[Ca^{2+}]_i$ and the sustained elevation in $[Ca^{2+}]_i$ induced by carbamylcholine. Carbamylcholine-induced increases in $[Ca^{2+}]_i$ were inhibited by the selective M_3 mAChR antagonist 4-DAMP (Fig. 2).

Preincubation of SK-N-SH cells with the CaM kinase antagonist KN-62 diminished the increase in $[Ca^{2+}]_i$ induced by carbamylcholine (Fig. 3). A maximal effect of KN-62 required a preincubation of 20 min, and this preincubation time was utilized in all of the experiments presented here. Maximal inhibition caused by 100 μM KN-62 in eight experiments ranged from 20 to 72%; the reason for

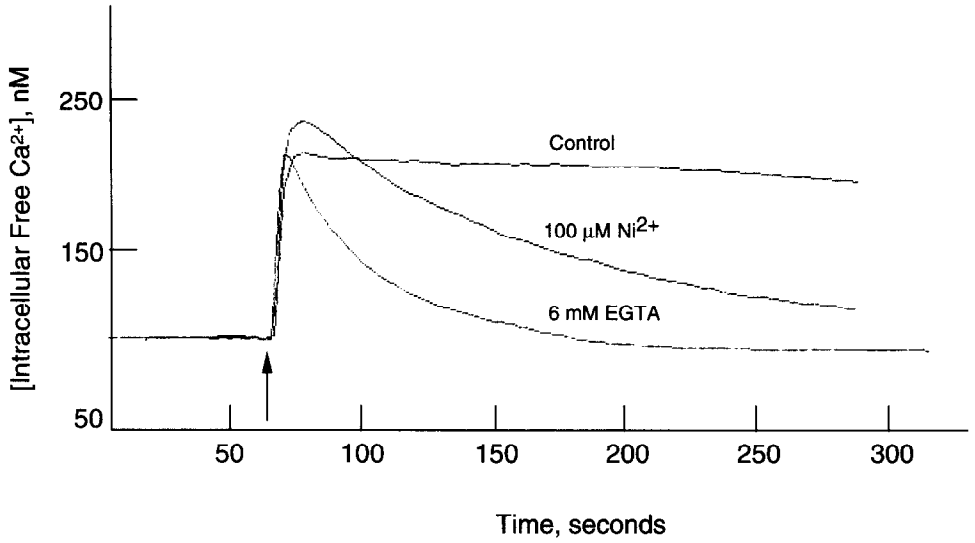

FIG. 1. Carbamylcholine-induced increase in intracellular free calcium concentration of SK-N-SH cells. The calcium concentration was calculated from the ratio of Fura-2 fluorescent emission intensities measured at 505 nm following excitation at 340 and 380 nm. Carbamylcholine (100 μM) was added at the point indicated by the arrow. The cells were incubated in complete DMEM (control) or in complete DMEM containing 100 μM Ni^{2+} or 6 mM EGTA, as indicated. Tracings are representative results from at least ten experiments.

this variation was unclear. Solubility limitations dictated that 100 μM was the highest concentration of KN-62 that could be used. Significant inhibition of the carbamylcholine-induced increase in $[Ca^{2+}]_i$ was always noted with 0.5 μM KN-62. Examples of the effects of KN-62 from one series of experiments are shown in Fig. 3.

The KN-62 concentrations that diminished the initial peak and sustained increases in $[Ca^{2+}]_i$ induced by mAChR activation determined in nine experiments are summarized in Fig. 4. While there was considerable variability in the absolute magnitude of the Ca^{2+} responses in separate experiments, KN-62 inhibited these responses in every case. The initial $[Ca^{2+}]_i$ peak measured 15 sec after the application of carbamylcholine (197 ± 17 nM; N = 9) was depressed by KN-62, and the sustained $[Ca^{2+}]_i$ elevation measured 60 sec after the application of carbamylcholine (165 ± 16; N = 9) was depressed to a commensurate degree. KN-62 diminished both of these Ca^{2+} responses by an average of 25–30%, and the half-maximal concentration of KN-62 that diminished these Ca^{2+} responses was ≈1 μM.

The initial peak in $[Ca^{2+}]_i$ induced by carbamylcholine was diminished by KN-62 even when 6 mM EGTA was included in the medium to eliminate Ca^{2+} influx (Fig. 5A). KN-04, a structural analogue of KN-62 which does not inhibit CaM kinase activity [21], had little effect on either the initial peak or sustained increases in $[Ca^{2+}]_i$ induced by mAChR activation (Fig. 5B). This suggests that the action of KN-62 is related to its inhibition of CaM kinase activity rather than a structural blockade of divalent cation channels.

Manganese quenching of Fura-2 fluorescence can be used as an indication of divalent cation entry in cells [22]. When excited at 360 nm, Fura-2 fluorescent emission at 505 nm is insensitive to the Ca^{2+} concentration. Carbamylcholine increased Mn^{2+} entry in SK-N-SH cells as evidenced by the quenching of Fura-2 fluorescence upon excitation at 360 nm (Fig. 6). Preincubation of SK-N-SH cells with 25 μM KN-62 decreased carbamylcholine-induced Mn^{2+} entry in the cells by 80–90% (N = 6) (Fig. 6). Preincubation of SK-N-SH cells with KN-04 was much less effective than

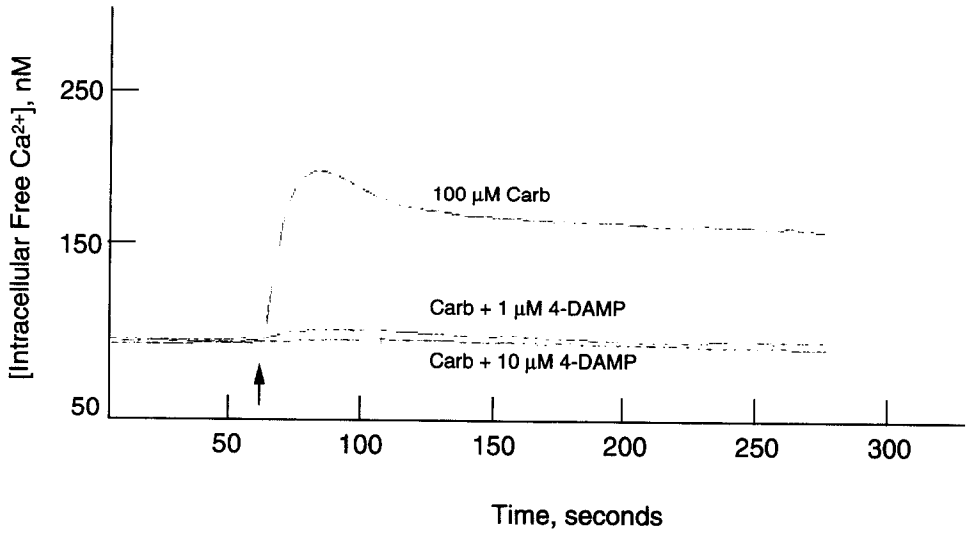

FIG. 2. Influence of 4-DAMP, a specific M_3 receptor antagonist, on carbamylcholine-induced increases in the intracellular calcium concentration of SK-N-SH cells. Carbamylcholine (100 μM) was added at the point indicated by the arrow. The cells were preincubated with 4-DAMP for 10 min as indicated. Tracings are from representative experiments repeated at least five times.

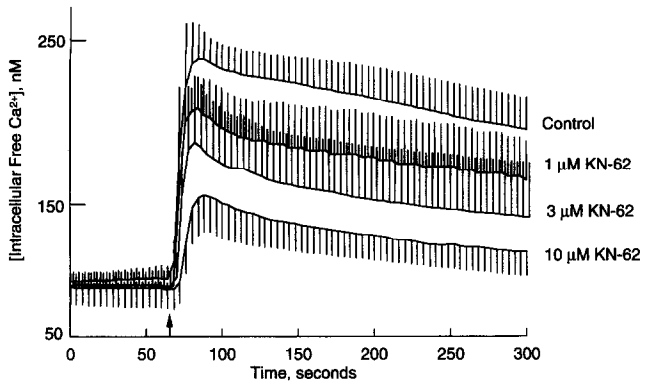

FIG. 3. Influence of KN-62 on carbamylcholine-induced increases in the intracellular free calcium concentration of SK-N-SH cells. Carbamylcholine (100 μM) was added at the point indicated by the arrow. The cells were incubated with the indicated concentrations of KN-62 for 20 min before the calcium responses were measured. Averages and standard deviations from responses obtained from six different cells in two separate experiments are depicted. The time course associated with treatment with 3 μM KN-62 is shifted 2 sec to the right to allow visualization of the standard deviations.

KN-62 at inhibiting carbamylcholine-induced Mn^{2+} entry (Fig. 6).

SK-N-SH cells possess VGCC which can be activated by raising the extracellular K^+ concentration to depolarize the cells [23]. The effects of 25 mM K^+ on $[Ca^{2+}]_i$ are shown in Fig. 7. The increase in $[Ca^{2+}]_i$ induced by 25 mM K^+ did not exhibit the initial transient peak in $[Ca^{2+}]_i$ that is induced typically by mAChR activation. The response to K^+ was totally eliminated by inclusion of 6 mM EGTA in the extracellular medium, reflecting the extracellular source of Ca^{2+}. KN-62 potentially inhibited K^+-stimulated entry of Ca^{2+}, inhibiting 90% of the response at 50 μM (Fig. 7). The concentration of KN-62 that half-maximally inhibited the K^+-stimulated Ca^{2+} entry was ≈3 μM (data not shown).

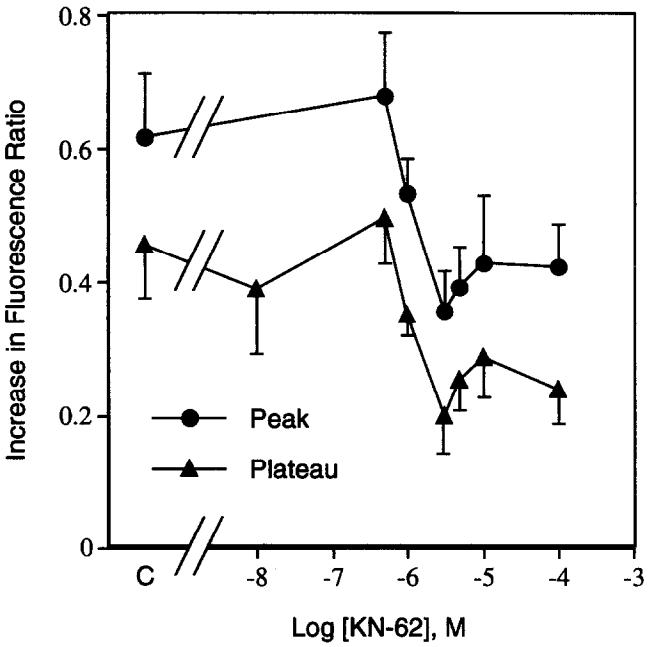

FIG. 4. Concentration dependence of KN-62 inhibition of carbamylcholine-induced increases in the intracellular calcium concentration of SK-N-SH cells. The cells were incubated with the indicated concentrations of KN-62 for 20 min before the calcium responses were measured. $[Ca^{2+}]_i$ was measured 15 (●) and 60 (▲) sec after the application of 100 μM carbamylcholine. The means ± SEM from nine experiments performed on separate days are indicated.

DISCUSSION

The present studies demonstrated that KN-62, CaM kinase inhibitor, disrupts Ca^{2+} signaling mediated by the M_3 mAChR. Specifically, pretreatment with KN-62 depresses the initial transient peak in $[Ca^{2+}]_i$, which is due to release of Ca^{2+} from intracellular stores, and the sustained elevation in $[Ca^{2+}]_i$, which reflects Ca^{2+} influx from the extracellular medium. This suggests that CaM kinases participate in mAChR signaling mechanisms that involve both Ca^{2+} influx and mobilization from internal stores. The precise site of this action is not certain; possible sites include Ca^{2+} channels, enzymes involved in generating inositol polyphosphates, and proximal signal transduction by the mAChR.

Several CaM kinases have been cloned and characterized, including CaM kinase type II [24] and CaM kinase type IV [17, 25–27]. Although KN-62 was characterized originally as a specific inhibitor of CaM kinase type II [18, 24], it is now clear that KN-62 inhibits both CaM kinases type II and type IV [18, 19, 24]. Both of these CaM kinases are expressed by SK-N-SH cells [8, 17]. A previous report suggested that KN-62 may physically block VGCC [9] in addition to inactivating CaM kinase. However, the effects of KN-62 in the present study did not appear to involve such an effect insofar as the mAChR-mediated responses were not affected by KN-04, which is structurally similar to KN-62 but unable to inactivate CaM kinases [21]. The structural specificity of membrane ion channel blockers is typically less strict than that required for enzyme inhibition.

We found that mAChR activation induced a sustained elevation in $[Ca^{2+}]_i$ that was diminished but not abolished by KN-62 at concentrations up to 100 μM. This sustained $[Ca^{2+}]_i$ elevation was due to Ca^{2+} influx since it was abolished by chelation of extracellular Ca^{2+} with EGTA and by Ca^{2+} channel blockade with Ni^{2+}. Although activation of mAChR induces a sustained $[Ca^{2+}]_i$ elevation in many different cell types [2–6, 14, 28–31], the effectors and Ca^{2+} channels involved in this response have not been characterized completely. We found that KN-62 partially inhibits mAChR-mediated Ca^{2+} influx but almost completely abolishes depolarization-dependent VGCC activity in SK-N-SH cells, consistent with the inhibition of VGCC by KN-62 in other cell types [9–14]. There are several possible interpretations of these data. The most plausible interpretation is that mAChR activation induces Ca^{2+} influx through channels other than (or in addition to) VGCC and

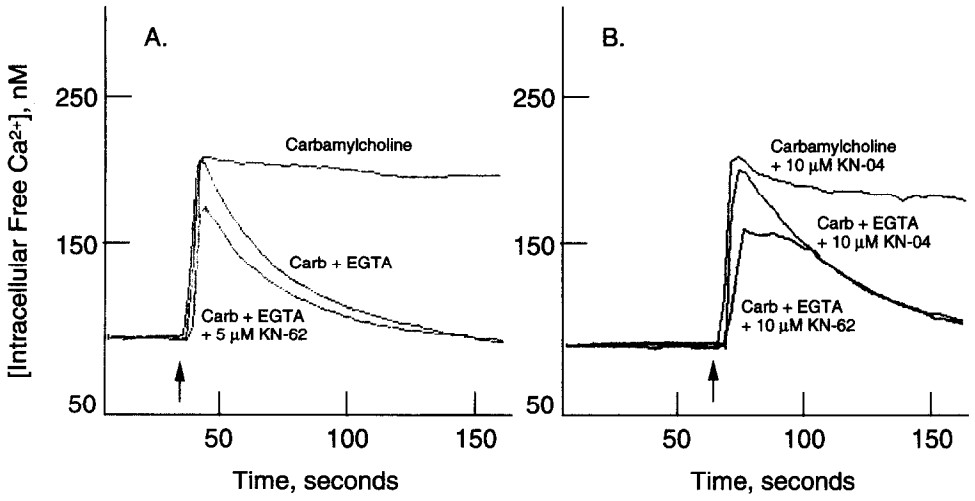

FIG. 5. Influence on KN-62 and KN-04 on carbamylcholine-induced increases in the intracellular calcium concentration of SK-N-SH cells. $[Ca^{2+}]_i$ was measured at the indicated times after the addition of 100 μM carbamylcholine at the times indicated by the arrows. (A) Responses to 100 μM carbamylcholine are depicted. Responses were also measured in the presence of 6 mM EGTA to eliminate calcium influx in untreated cells and in cells treated with 5 μM KN-62, as indicated. (B) Effect of pretreatment of cells with 10 μM KN-04 in the absence or presence of 6 mM EGTA. Pretreatment of the cells with 10 μM KN-62 is included for comparison. Tracings are representative results from experiments repeated at least four times with qualitatively similar results.

that these channels are less sensitive to VGCC to inhibition by KN-62. This is consistent with reports that mAChR-mediated Ca^{2+} influx is insensitive to pharmacological inhibitors of VGCC [2, 28, 30]. An alternative interpretation is that mAChR activation induces Ca^{2+} influx through VGCC, but the specific pathway by which mAChR activation induces this influx is relatively insensitive to the inhibitory effects of KN-62.

We found that mAChR stimulation also activates Mn^{2+}-permeant channels in SK-N-SH cells. Previous studies demonstrated that stimulation of mAChR activates Mn^{2+}-permeant channels [3, 31], which are insensitive to the VGCC blockers nifedipine [31] or diltiazem [3]. Interestingly, KN-62 almost completely abolished the mAChR-mediated activation of Mn^{2+}-permeant channels in SK-N-SH cells, but was less effective in inhibiting

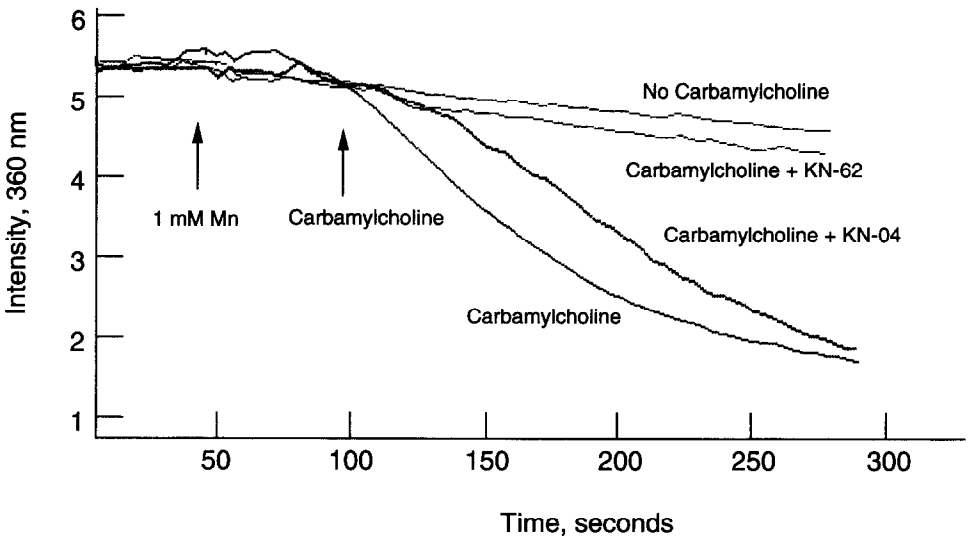

FIG. 6. Influence of KN-62 and KN-04 on carbamylcholine-induced increases in Mn^{2+} entry into SK-N-SH cells. Mn^{2+} permeability was determined by its quenching of Fura-2 fluorescence after excitation at a calcium-insensitive wavelength, 360 nm. Mn^{2+} (1 mM) was added to the medium at the time indicated by the first arrow; carbamylcholine (100 μM) was added at the time indicated by the second arrow. Thus, Mn^{2+} quenching of Fura-2 fluorescence serves as an indication of cation entry through Mn^{2+}-permeant pathways, including VGCC. The cells were treated with 25 μM KN-62 or KN-04, as indicated, for 20 min before measuring the fluorescence intensity at 505 nm. Tracings are from representative experiments repeated at least six times.

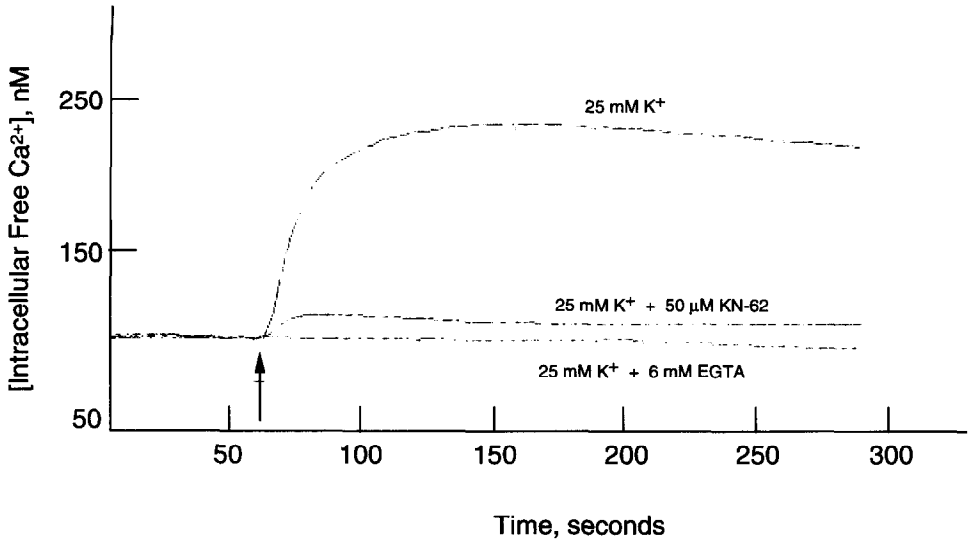

FIG. 7. Influence of KN-62 on depolarization-induced increases in the intracellular calcium concentration of SK-N-SH cells. $[Ca^{2+}]_i$ was measured at the indicated times after addition of 25 mM K^+. In some experiments as indicated, the medium included 6 mM EGTA to eliminate calcium influx. In other experiments, as indicated, the cells were pretreated with 50 μM KN-62 for 20 min to inhibit CaM kinase activity. Tracings are from representative experiments repeated three times.

mAChR-mediated Ca^{2+} influx. This suggests that mAChR activation induces Ca^{2+} influx through channels other than (or in addition to) Mn^{2+}-permeant channels, and these mAChR-activated Ca^{2+} channels are less sensitive than the Mn^{2+}-permeant channels to inhibition by KN-62.

Activation of mAChR also induced an initial transient peak in $[Ca^{2+}]_i$, which was diminished by KN-62. This initial $[Ca^{2+}]_i$ peak was due to mobilization of Ca^{2+} from internal stores because it was unaffected by chelation of extracellular Ca^{2+} with EGTA or by Ca^{2+} channel blockade with Ni^{2+}. KN-62 diminished the initial $[Ca^{2+}]_i$ peak even when Ca^{2+} influx was abolished by EGTA, indicating that this effect of KN-62 occurs independently of the inhibitory effects of KN-62 on Ca^{2+} influx. However, other studies found that inactivation of CaM kinase has minimal effects on Ca^{2+} mobilization induced by activating mAChR in small cell lung carcinoma cells [14] or bombesin receptors in HIT-T15 cells [9]. The basis for the differences between the SK-N-SH cells and the other cell lines is not apparent. The earlier studies did not examine the effects of CaM kinase inactivation on the individual components of the Ca^{2+} response (i.e. Ca^{2+} influx and intracellular Ca^{2+} mobilization) induced by these G protein-coupled receptors. Alternately, certain of these actions of KN-62 could reflect effects on molecules other than the CaM kinases.

It has been suggested that mAChR activation depletes internal stores of Ca^{2+}, and this is responsible for the subsequent influx of Ca^{2+} from the extracellular medium [2, 3, 28]. According to this model of capacitive refilling [2], a greater release of Ca^{2+} from internal stores will induce a greater influx of Ca^{2+} to refill these internal stores. If this model is correct, inhibition of Ca^{2+} release from internal stores would diminish Ca^{2+} influx. Thus, it is plausible that inactivation of CaM kinase with KN-62 initially inhibits mAChR-mediated Ca^{2+} release from internal stores, and this in turn results in the apparent inhibition of Ca^{2+} influx. Consistent wtih this notion, we found that KN-62 dose–response curves for inhibition of the initial $[Ca^{2+}]_i$ peak (due to Ca^{2+} release from internal stores) and inhibition of the sustained $[Ca^{2+}]_i$ elevation (due to Ca^{2+} influx) were identical, suggesting that a common site of action underlies both effects of KN-62.

There are several ways that inactivation of CaM kinase could alter calcium signaling by mAChR. CaM kinase may directly regulate the activity of Ca^{2+} channels involved in this response. This possibility is supported by the demonstration that inactivation of CaM kinase type II causes L-type VGCC to have lower amplitude and inactivate more quickly in cardiac myocytes [11, 12], suggesting that phosphorylation of VGCC by CaM kinase affects the activity of these channels [15, 16]. Similarly, CaM kinase type II can phosphorylate and regulate the activity of the ryanodine-sensitive Ca^{2+} channel [32, 33]. Many other proteins have been identified as substrates for CaM kinase [24], and phosphorylation of these proteins by CaM kinase may also affect mAChR signaling. It is intriguing to speculate that CaM kinase inactivation may directly or indirectly alter the phosphorylation state of the mAChR itself. It is clear that mAChR are phosphorylated by multiple G protein-linked kinases as well as by protein kinase C [34–37]. The phosphorylation by G protein-linked kinases is agonist dependent and is enhanced by G protein βγ subunits [38]. Phosphorylation of mAChR is associated with receptor desensitization, G protein uncoupling, and internalization [39, 40].

Our studies demonstrate that the CaM kinase inhibitor KN-62 interferes with mAChR-mediated mobilization of intracellular Ca^{2+} and activation of Ca^{2+} influx. The present results raise the possibility that there is a CaM kinase substrate other than VGCC whose state of phosphorylation affects muscarinic calcium signaling.

This work was supported by PHS Grants GM46408, NS31518, and CA52471, and Department of the Army Grant DAMD17-94-J-4011.

References

1. Williams CL and Lennon VA, Activation of M_3 muscarinic acetylcholine receptors inhibits voltage-dependent calcium influx in small cell lung carcinoma. *J Biol Chem* **265:** 1443–1447, 1990.
2. Lambert DG and Nahorski SR, Carbachol-stimulated calcium entry in SH-SY5Y human neuroblastoma cells: Which route? *J Physiol (Paris)* **86:** 77–82, 1992.
3. Roche S, Bali J-P and Magous R, Receptor-operated Ca^{2+} channels in gastric parietal cells: Gastrin and carbachol induce Ca^{2+} influx in depleting intracellular Ca^{2+} stores. *Biochem J* **289:** 117–124, 1993.
4. Yang CM, Yo YL and Wang YY, Intracellular calcium in canine cultured tracheal smooth muscle cells is regulated by M_3 muscarinic receptors. *Br J Pharmacol* **110:** 983–988, 1993.
5. del Rio E, Nicholls DG and Downes CP, Involvement of calcium influx in muscarinic cholinergic regulation of phospholipase C in cerebellar granule cells. *J Neurochem* **63:** 535–543, 1994.
6. Wojcikiewicz RJ, Tobin AB and Nahorski SR, Muscarinic receptor-mediated inositol 1,4,5-trisphosphate formation in SH-SY5Y neuroblastoma cells is regulated acutely by cytosolic Ca^{2+} and by rapid desensitization. *J Neurochem* **63:** 177–185, 1994.
7. Trejo J and Brown JH, *c-fos* and *c-jun* Are induced by muscarinic receptor activation of protein kinase C but are differentially regulated by intracellular calcium. *J Biol Chem* **266:** 7876–7882, 1991.
8. Mangels LA and Gnegy ME, Carbachol stimulates binding of a photoreactive calmodulin derivative to calmodulin-binding proteins in intact SK-N-SH human neuroblastoma cells. *J Biol Chem* **267:** 5847–5854, 1992.
9. Li G, Hidaka H and Wollheim CB, Inhibition of voltage-gated Ca^{2+} channels and insulin secretion in HIT cells by the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-62: Comparison with antagonists of calmodulin and L-type Ca^{2+} channels. *Mol Pharmacol* **42:** 489–498, 1992.
10. Ammala C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM and Rorsman P, Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *J Physiol (Lond)* **472:** 665–688, 1993.
11. Anderson ME, Braun AP, Schulman H and Premack BA, Multifunctional Ca^{2+}/calmodulin-dependent protein kinase mediates Ca^{2+}-induced enhancement of L-type Ca^{2+} current in rabbit ventricular myocytes. *Circ Res* **75:** 854–861, 1994.
12. Xiao RP, Cheng H, Lederer WJ, Suzuki T and Lakatta EG, Dual regulation of Ca^{2+}/calmodulin-dependent kinase II activity by membrane voltage and by calcium influx. *Proc Natl Acad Sci USA* **91:** 9659–9663, 1994.
13. Lu HK, Fern RJ, Nee JJ and Barrett PQ, Ca^{2+}-dependent activation of T-type Ca^{2+} channels by calmodulin-dependent protein kinase II. *Am J Physiol* **267:** F183–F189, 1994.
14. Williams CL, Porter RA and Phelps SH, 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). *Biochem Pharmacol* **50:** 1979–1985, 1995.
15. Chang CF, Gutierrez LM, Mundina-Weilenmann C and Hosey MM, Dihydropyridine-sensitive calcium channels from skeletal muscle. II. Functional effects of differential phosphorylation of channel subunits. *J Biol Chem* **266:** 16395–16400, 1991.
16. Hell JW, Appleyard SM, Yokoyama CT, Warner C and Catterall WA, Differential phosphorylation of two size forms of the N-type calcium channel α1 subunit which have different COOH termini. *J Biol Chem* **269:** 7390–7396, 1994.
17. Bland MM, Monroe RS and Ohmstede C-A, The cDNA sequence and characterization of the Ca^{2+}-calmodulin-dependent protein kinase-Gr from human brain and thymus. *Gene* **142:** 191–197, 1994.
18. Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M and Hidaka H, KN-62, 1-[*N*,O-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. *J Biol Chem* **265:** 4315–4320, 1990.
19. Enslen H, Sun P, Brickey D, Soderling SH, Klamo E and Soderling TR, Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. *J Biol Chem* **269:** 15520–15527, 1994.
20. Grynkiewicz G, Poenie M and Tsien R, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
21. Ishikawa N, Hashiba Y and Hidaka H, Effect of a new Ca^{2+}-calmodulin-dependent protein kinase II inhibitor on GABA release in cerebrospinal fluid of the rat. *J Pharmacol Exp Ther* **254:** 598–602, 1990.
22. Merritt JE, Jacob R and Hallman TJ, Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J Biol Chem* **264:** 1522–1527, 1989.
23. Lesser SS and Lo DC, Regulation of voltage-gated ion channels by NGF and ciliary neurotropic factor in SK-N-SH neuroblastoma cells. *J Neurosci* **15:** 253–261, 1995.
24. Schulman H, The multifunctional Ca^{2+}/calmodulin-dependent protein kinases. *Curr Opin Cell Biol* **5:** 247–253, 1993.
25. Jones DA, Glod J, Wilson-Shaw D, Hahn WE and Sikela J, cDNA sequence and differential expression of the mouse Ca^{2+}/calmodulin-dependent protein kinase IV gene. *FEBS Lett* **289:** 105–109, 1991.
26. Means AR, Cruzalegul F, LeMagueresse B, Needleman DS, Slaughter GR and Ono T, A novel Ca^{2+}-calmodulin-dependent protein kinase and a male germ cell-specific calmodulin-binding protein are derived from the same gene. *Mol Biol Cell* **11:** 3960–3971, 1991.
27. Ohmstede CA, Bland MM, Merrill BM and Sahyoun N, Relationship of genes encoding Ca^{2+}/calmodulin-dependent protein kinase Gr and calspermin, a gene within a gene. *Proc Natl Acad Sci USA* **88:** 5784–5788, 1991.
28. Pacaud P and Bolton TB, Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. *J Physiol (lond)* **441:** 477–499, 1991.
29. Fatatis A, Bassi A, Monsurro MR, Sorrentino G, Mita GD, Di Renzo GF and Annunziato L, LAN-1, a human neuroblastoma cell line with M1 and M3 muscarinic receptor subtypes coupled to intracellular Ca^{2+} elevation and lacking Ca^{2+} channels activated by membrane depolarization. *J Neurochem* **59:** 1–9, 1992.
30. Oettling G, Gotz U and Drews U, Characterization of the Ca^{2+} influx into embryonic cells after stimulation of the embryonic muscarinic receptor. *J Dev Physiol* **17:** 147–155, 1992.
31. Forsberg EJ, Li Q and Xu Y, Cation channel activated by muscarinic agonists on porcine adrenal chromaffin cells. *Am J Physiol* **269:** E43–E52, 1995.
32. Wang J and Best PM, Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. *Nature* **359:** 739–741, 1992.

33. Hohenegger M and Suko J, Phosphorylation of the purified cardiac ryanodine receptor by exogenous and endogenous protein kinases. *Biochem J* **296:** 303–308, 1993.
34. Richardson RM, Kim C, Benovic JL and Hosey MM, Phosphorylation and desensitization of human m2 muscarinic cholinergic receptors by two isoforms of the β-adrenergic receptor kinase. *J Biol Chem* **268:** 13650–13656, 1993.
35. Kameyama K, Haga K, Haga T, Moro O and Sadee W, Activation of a GTP-binding protein and a GTP-binding-protein-coupled receptor kinase (β-adrenergic-receptor kinase 1) by a mouse m2 mutant lacking phosphorylation sites. *Eur J Biochem* **226:** 267–276, 1994.
36. Tobin AB, Willars GB, Burford NT and Nahorski SR, Relationship between agonist binding, phosphorylation and immunoprecipitation of the m3-muscarinic receptor, and second messenger responses. *Br J Pharmacol* **116:** 1723–1728, 1995.
37. Haga K, Kameyama K, Kikkawa U, Shiozaki K and Uchiyama H, Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C. *J Biol Chem* **271:** 2776–2782, 1996.
38. Debburman SK, Kunapuli P, Benovic JL and Hosey MM, Agonist-dependent phosphorylation of human muscarinic receptors in *Spodoptera frugiperda* insect cell membranes by G protein-coupled receptor kinases. *Mol Pharmacol* **47:** 224–233, 1995.
39. Tsuga H, Kameyama K, Haga T, Kurose H and Nagao T, Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G-protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. *J Biol Chem* **269:** 32522–32527, 1994.
40. Pals-Ryaarsdam R, Xu Y, Witt-Enderby P, Benovic JL and Hosey MM, Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. *J Biol Chem* **270:** 29004–29011, 1995.