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Dependence of Insulin Secretion from Permeabilized Pancreatic β-Cells on the Activation of Ca^{2+}/Calmodulin-Dependent Protein Kinase II

A RE-EVALUATION OF INHIBITOR STUDIES

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ABSTRACT. Previous studies utilizing inhibitors of the Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) to address the role of this enzyme in insulin secretion have produced contradictory results. In the current study, these inconsistencies have been addressed by evaluating the effect of various CaM kinase II inhibitors to decrease Ca^{2+}-induced insulin secretion from permeabilized β-cells. KN-93 (2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxy-benzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) markedly inhibited both CaM kinase II activation and insulin secretion in parallel in α-toxin-permeabilized β-cells. These effects were specific since they were not mimicked by the inactive analog, KN-92 (2-[*N*-(4-methoxybenzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine). In contrast, KN-62 (1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-l-tyrosyl]-4-phenylpiperazine), while reported to be similar to KN-93 with respect to mechanism of action, did not inhibit Ca^{2+}-induced activation of CaM kinase II or insulin secretion in these cell preparations. All three agents suppressed Ca^{2+} influx in intact β-cells induced by depolarization in the presence of elevated extracellular potassium although to different extents. The synthetic peptide inhibitors of CaM kinase II, [Ala^{286}]CaMK 281–302 and AIP (autocamtide-2-related inhibitory peptide), strongly inhibited Ca^{2+}-induced insulin secretion from electropermeabilized islets, an effect that also correlated with an equivalent inhibition of CaM kinase II activation. This re-evaluation (i) explains a lack of effect of KN-62 on insulin secretion from permeabilized cells based on its inability to inhibit CaM kinase II activation in these preparations; (ii) has revealed that CaM inhibitors, either chemical or peptide in nature, that are capable of preventing enzyme activation uniformly suppress Ca^{2+}-sensitive insulin secretion; and (iii) cautions the use of KN-62/93/92 as selective inhibitors of CaM kinase II in intact cell studies. These observations reinforce the suggestion that CaM kinase II plays an important role in insulin exocytosis in the β-cell. BIOCHEM PHARMACOL **60**;11: 1655–1663, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. calcium; protein kinase; insulin secretion; calmodulin; protein phosphorylation; exocytosis

Disparate effects of inhibitors of the multifunctional CaM kinase II§ on insulin secretion are reported in the literature and hinder an understanding of the function of this kinase in the β-cell. In a positive sense, the peptide inhibitor of CaM kinase II, CaMK 290–302, significantly suppresses insulin exocytosis triggered by cell depolarization and Ca^{2+} influx [1]. An important role for CaM kinase II in this process was also initially supported from observations that the pharmacological inhibitors KN-62 (1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) [2] and KN-93 (2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxy-benzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) [3] both suppressed glucose-induced insulin secretion from isolated islets [4, 5], and that KN-62 inhibited mixed nutrient-induced insulin secretion from insulinoma clonal β-cells, HIT [6]. Subsequent studies, however, revealed a potent effect of KN-62 to interfere with Ca^{2+} influx in clonal β-cells (HIT) [6]. Since the primary mechanism of glucose-induced insulin secretion is mediated via the activation of voltage-dependent Ca^{2+} channels [7], the effect of KN-62 to inhibit insulin secretion cannot be fully ascribed to the inhibition of CaM kinase II. Consequently, when KN-62 failed to inhibit Ca^{2+}-induced insulin secretion in permeabilized HIT cells, conditions that presumably bypass the requirement for Ca^{2+} channel activity, it was concluded that CaM kinase II is not required for Ca^{2+}-sensitive secretion [6]. Thus, a contradiction exists between the effects of KN-62 and CaMK 290–302 on insulin secretion that remains unresolved.

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§ *Abbreviations*: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; and AIP, autocamtide-2-related inhibitory peptide.

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A considerable body of evidence from enzyme activation studies has now accumulated in support of a role for CaM kinase II in physiologic insulin secretion (reviewed in Ref. [8]). Most compelling, perhaps, is the close correlation of CaM kinase II activation with the initial and sustained phases of glucose-induced insulin secretion from perifused rat islets [4, 9]. CaM kinase II activation is induced in islets by insulin secretagogues that induce calcium influx from the extracellular space (e.g. glucose, depolarizing concentrations of K^+ [4]) or mobilize Ca^{2+} from intracellular stores (e.g. carbachol [10]). Enzyme activation in the β-cell also results in the phosphorylation of proteins that are implicated in secretory events, namely microtubule-associated protein-2 [11] and synapsin I [12]. Furthermore, indirect evidence that CaM kinase II regulates exocytosis in the β-cell is gained from the demonstration that a delta isoform of this enzyme is localized to the insulin secretory granule [8, 13, 14].

The intensifying support for a role of CaM kinase II in insulin secretion demands that the inconsistencies of inhibitor studies be re-evaluated. Therefore, this study was initiated to determine the effects of select CaM kinase II inhibitors on insulin release in correlation with their effects on enzyme activation. The data obtained demonstrated that the agents that effectively inhibit CaM kinase II also uniformly suppress insulin secretion and thus support a role of CaM kinase II in exocytosis mechanisms of the β-cell.

MATERIALS AND METHODS

Materials

INS-1 and βTC3 cells were obtained from Dr. Mark Prentki (University of Montreal) and Dr. Shimon Efrat (Albert Einstein College of Medicine), respectively, and were cultured as previously described [15, 16]. ATP (disodium salt), calmodulin, leupeptin, and hemolysin-A (*Staphylococcus aureus* α-toxin) were purchased from the Sigma Chemical Co., and glucose (Dextrose) was purchased from the National Bureau of Standards. [γ-^{32}P]-ATP was purchased from NEN Research Chemicals. [Ala^{286}]CaMK 281–302 (MHRQEAVDCLKKFNARRKLKGA) and AIP (KKALRRQEAVDAL) were synthesized by Biosynthesis, Inc. KN-62 was purchased from LC Laboratories, the Sigma Chemical Co., or Calbiochem. KN-93 and KN-92 (2-[*N*-(4-methoxy-benzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)*N*-methylbenzylamine) were purchased from Calbiochem. Guinea pig anti-rat insulin antibody and rat insulin standard were obtained from Linco Research, Inc. All other chemicals were of the finest reagent grade available.

Permeabilization of βTC3 Cells

Permeabilization of βTC3 cells using α-toxin was achieved by methods reported previously [11, 17]. Cells were permeabilized using 100–125 U α-toxin/10^6 cells/0.1 mL in suspension or 100 U α-toxin/0.2 mL/well in a monolayer, respectively. Permeabilization was performed in a buffer with the following composition: 20 mM HEPES (pH 7.0), 140 mM potassium glutamate, 5 mM NaCl, 7 mM $MgSO_4$, 5 mM Na_2ATP, and either 1.0 mM EGTA (cell suspension) or 10.2 mM EGTA (monolayer). Then cells were incubated in permeabilization buffer supplemented with basal (0.05 μM) or stimulatory (10 μM) concentrations of Ca^{2+} at 37° for the indicated times before the determination of CaM kinase II activation or insulin secretion. The free Ca^{2+} concentrations of incubation and permeabilization buffers were estimated using an Orion Ca^{2+} electrode calibrated using Ca^{2+}/EGTA buffers, as previously described [11, 18].

Islet Isolation and Permeabilization

Pancreatic islets were isolated from male Wistar rats (250–350 g) by Collagenase P (Roche Molecular Biochemicals) digestion and subsequent enrichment by centrifugation on a Ficoll gradient as described previously [9]. Islets (200/group) were permeabilized in ice-cold permeabilization buffer [140 mM potassium glutamate, 15 mM HEPES (pH 6.7), 7 mM $MgSO_4$, 5 mM glucose, 1 mM EGTA, 5 mM Na_2ATP, and 0.5 mg/mL of BSA] supplemented with 0.05 μM free Ca^{2+} using a square-wave electrical pulse (1.7 kV/cm, 10 pulses, 30 μsec each, BTX 800 Transfector). Where indicated, islets were permeabilized in the presence of peptide inhibitors. Islets were placed immediately in incubation medium containing basal or stimulatory concentrations of Ca^{2+} (as for βTC3 cells) in the absence and presence of peptide inhibitors and used for the determination of insulin secretion (10–15 islets/tube) or CaM kinase II activation (100–150 islets/tube).

Assay of CaM Kinase II Activity and Activation

Cells were homogenized in an ice-cold buffer [20 mM Tris–HCl (pH 7.5), 0.5 mM EGTA, 1.0 mM EDTA, 10 mM sodium pyrophosphate, 0.4 mM ammonium molybdate, 100 μg/mL of leupeptin] by sonication (10 pulses, setting 3, 30% duty cycle). Ca^{2+}/calmodulin-dependent and independent (autonomous) CaM kinase II activity was assayed in the resultant homogenate by the determination of $^{32}P_i$ incorporation into exogenously added peptide, autocamtide-2 [19], in the presence and absence of Ca^{2+}, respectively [4]. Since autonomous kinase activity is the result of autophosphorylation on Thr$^{286/287}$ of CaM kinase II [20], the ratio of these activities (percent autonomy) is a measure of the extent of activation of CaM kinase II.

Measurement of Intracellular Ca^{2+} Concentrations ($[Ca^{2+}]_i$)

The determination of $[Ca^{2+}]_i$ was performed on a Nikon Diaphot microscope via dynamic video-imaging using a Metafluor Quantitative Fluoroescence System (Universal Imaging Co.). INS-1 or βTC3 cells were grown onto

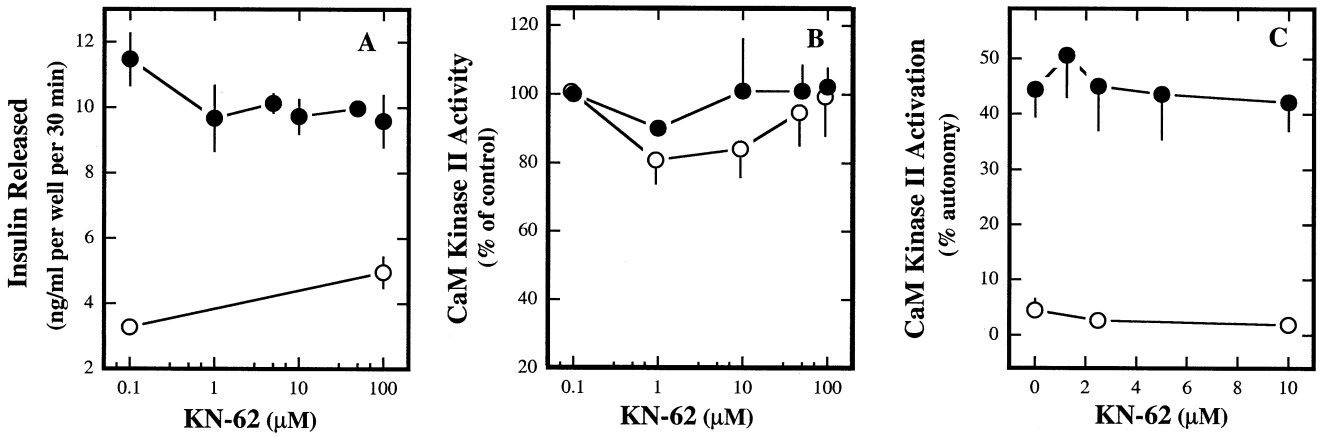

FIG. 1. Effect of KN-62 on Ca^{2+}-induced insulin secretion and CaM kinase II activation in permeabilized βTC3 cells. (A and C) Permeabilized βTC3 cells (approx. 0.1 × 10^6 cells/well) were incubated in 0.05 μM (○) or 10 μM (●) Ca^{2+} buffers containing various concentrations of KN-62 (0–100 μM) at 37°. For (A), the medium was recovered after 30 min, and the insulin content was determined. For (C), cells were incubated for 1 min, then homogenized, and CaM kinase II activation was estimated based on Ca^{2+}-dependent incorporation of ^{32}P into autocamtide-2. (B) βTC3 cell homogenates, generated from intact (●) or α-toxin-permeabilized cells (○), were assayed for Ca^{2+}-dependent CaM kinase II activity in the presence of the indicated concentrations of KN-62 (0–100 μM). For B and C, values are expressed as a percentage of control (8,980 and 16,450 cpm, for non-permeabilized and permeabilized cells, respectively) in the absence of KN-62. Values are means ± SEM from 6 independent determinations.

25-mm diameter glass coverslips and loaded with 3 μM Fura-2 AM (Molecular Probes) in RPMI for 30 min at 37°. Then cells were washed three times and maintained in an isotonic Krebs–Ringer bicarbonate (KRB) buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM HEPES, pH 7.4) supplemented with 3 mM glucose and 0.1% BSA. $[Ca^{2+}]_i$ measurements were made by monitoring the ratio of Fura-2 fluorescence (emission at 550 nm) at excitation wavelengths of 340 and 380 nm every 100 msec. Maximum and minimum ratios were determined in the presence of 1 μM ionophore (4-bromo-A23187) and 5 mM EGTA, respectively, and used in the calculation of $[Ca^{2+}]_i$ using the Grynkiewicz equation [21]. In high K^+ KRB buffers, isotonicity was maintained via equimolar substitution of KCl for NaCl.

Insulin Assay

Determination of the insulin content of the incubation medium was performed by Enzyme Immunoassay (EIA) [22] or two-antibody radioimmunoassay (RIA) [23] using rat insulin as standard.

Statistical Analysis

Results are expressed as means ± SEM determined from at least three independent experiments, unless otherwise stated. Statistical significance was assessed by Student's *t*-test.

RESULTS

The Experimental Model

The permeabilized β-cell was the primary model chosen for this study for a number of reasons. In previous studies, we established that Ca^{2+}-induced insulin secretion from α-toxin-permeabilized β-cells is correlated closely with the activation of CaM kinase II [17]. With respect to both parameters, the threshold concentration of Ca^{2+} beyond which activation occurred was estimated to be approximately 0.5 μM, and maximal induction (approximately 6-fold) was observed at 10 μM. This experimental model thus provided a means to study the relationship of these mechanisms in the absence of the potential complications derived from known effects of CaM kinase II inhibitors to suppress Ca^{2+} channel activity [6]. In addition, cell permeabilization provided a convenient means whereby peptide inhibitors of CaM kinase II could be introduced into the cell cytoplasm to study their effects on insulin secretion.

Effect of KN-62 on CaM Kinase II Activity and Insulin Secretion from Permeabilized β-Cells

Previously, KN-62 was reported to be incapable of inhibiting Ca^{2+}-induced insulin secretion from streptolysin-O-permeabilized HIT cells [6]. This observation was confirmed in the current study in α-toxin-permeabilized βTC3 cells where KN-62 (1–100 μM) had only a marginal inhibitory effect (a maximal inhibition of 17%) on insulin secretion induced by Ca^{2+} (Fig. 1A). In extension of these studies, KN-62 within the same concentration range (1–100 μM) was surprisingly ineffective as an inhibitor of CaM kinase II activity in βTC3 cells measured in homogenates obtained from intact or α-toxin-permeabilized cells (Fig. 1B). A modest inhibition approaching 20% was observed at a low inhibitor concentration (1 μM) in homogenates from permeabilized cells, but this was not maintained at higher concentrations and was less apparent in homogenates obtained from intact cells. This may reflect a compromised solubility of KN-62 that is accentuated at higher concen-

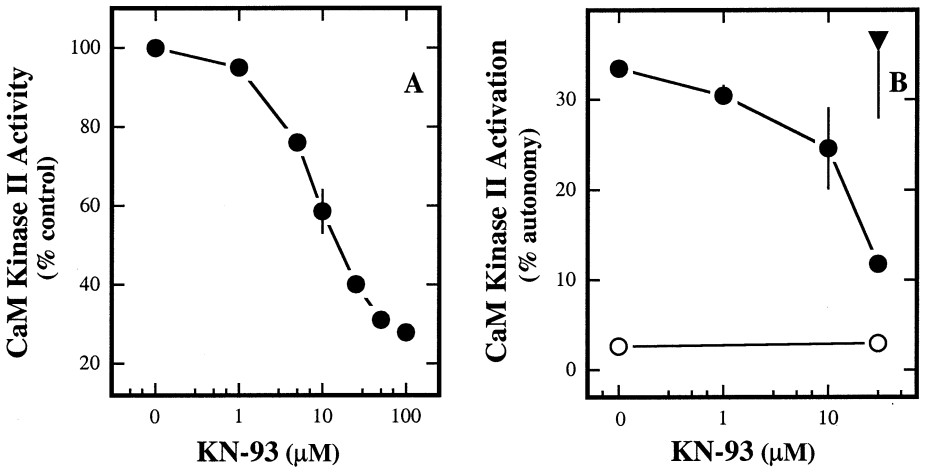

FIG. 2. Inhibition of β-cell CaM kinase II activity and activation by KN-93. (A) Ca^{2+}-dependent CaM kinase II activity was assayed in βTC3 cell homogenates in the absence or presence of KN-93 (0–100 μM). Data were recorded as counts per minute of ^{32}P incorporated into autocamtide-2, and ordinate values are expressed as a percentage of control (averaged 12,680 cpm, N = 6) in the absence of KN-93. (B) CaM kinase II activation was determined under the conditions described in the legend of Fig. 1. Cells were incubated at 37° in 0.05 μM Ca^{2+} (○) or 10 μM Ca^{2+} buffers (filled symbols) in the presence of the indicated concentrations of KN-93 (●) or KN-92 (▼). Values are means ± SEM from 4–6 independent determinations.

trations of inhibitor. Furthermore, KN-62 (1–10 μM) also failed to prevent Ca^{2+}-induced activation of CaM kinase II as determined from an estimation of its autophosphorylation-dependent conversion to an autonomous form (Fig. 1C). The inconsistent and modest effects of KN-62 indicate that this compound may not be an effective inhibitor of CaM kinase II in these β-cell preparations and may be unsuitable for studies of the role of this enzyme in insulin secretion.

Effect of KN-93 on CaM Kinase II Activation and Insulin Secretion from Permeabilized β-Cells

Another widely used pharmacological inhibitor of CaM kinase II is the methoxybenzene sulfonyl derivative KN-93 [3]. Like KN-62, KN-93 acts via competition of calmodulin binding to CaM kinase II ($K_i \sim 0.37$ μM *in vitro*) [3] but has superior water solubility relative to KN-62. KN-93 strongly inhibits (80% inhibition at 10 μM) glucose-induced insulin secretion from isolated rat islets [5] with the most prominent effect being on second phase secretion in perifused islets (Easom RA, unpublished observations). It was of interest to determine the effect of KN-93 on CaM kinase II activity and insulin secretion in permeabilized βTC3 cells.

As demonstrated in Fig. 2A, KN-93 was shown to be an effective inhibitor of CaM kinase II activity in βTC3 cell homogenates with half-maximal inhibition achieved at approximately 8 μM. At a maximally effective concentration (50–100 μM), KN-93 inhibited CaM kinase II activity by 72%. Since autocamtide-2 is selective for CaM kinase II, the remaining activity is possibly accounted for by the presence of autonomous kinase that is insensitive to inhibition by KN-93 [3]. The ability of KN-93 to prevent CaM kinase II activation (i.e. enzyme autophosphorylation) was also assessed in α-toxin-permeabilized β-cells. As shown in Fig. 2B, KN-93 markedly inhibited CaM kinase II activation at concentrations above 10 μM with approximately 70% inhibition being achieved at 30 μM. These effects of KN-93 were specific since they were not mimicked by the inactive analog KN-92 (Fig. 2B).

Importantly, the inhibition of CaM kinase II activation by KN-93 was accompanied by a profound inhibition of Ca^{2+}-induced insulin secretion from permeabilized βTC3 cells. These experiments were performed in cell monolayers to facilitate the direct correlation of kinase activation and insulin secretion under identical conditions. Determinations were made at 1 and 30 min to accommodate optimal measurements of kinase activation and insulin secretion measurements. As demonstrated in Fig. 3A, kinase activation was suppressed markedly (66 and 72% inhibition at 1 and 30 min, respectively) by 50 μM KN-93 but was not affected by KN-92 (also 50 μM). Thus, the inhibition of CaM kinase II by KN-93 was preserved throughout the period of secretion studied; this was in spite of a profound Ca^{2+}-induced and time-dependent reduction in kinase activity observed at 30 min relative to 1 min (data not shown), similar to a previous report [24]. Ca^{2+}-induced insulin secretion was similarly suppressed by 50 μM KN-93, amounting to 78% inhibition at 30 min (Fig. 3B). While KN-93 also tended to inhibit secretion after a 1-min incubation in 10 μM Ca^{2+}, this effect was not statistically significant. Again, these effects of KN-93 were not mimicked by KN-92 (Fig 3B and inset). Therefore, and in contrast to KN-62 (see Fig. 1), KN-93 was determined to be an effective inhibitor of CaM kinase II in permeabilized β-cells, and its specific effect on insulin secretion supports a role of this enzyme in insulin secretion.

Effect of KN-93 on Ca^{2+} influx in INS-1 Cells

It is not known whether KN-93 also suppresses Ca^{2+} channel activity in the β-cell. To test this, the effect of depolarizing concentrations of K^+ to increase cytosolic Ca^{2+} concentrations in INS-1 cells in the presence of KN-93 was studied. The protocol employed a repeated

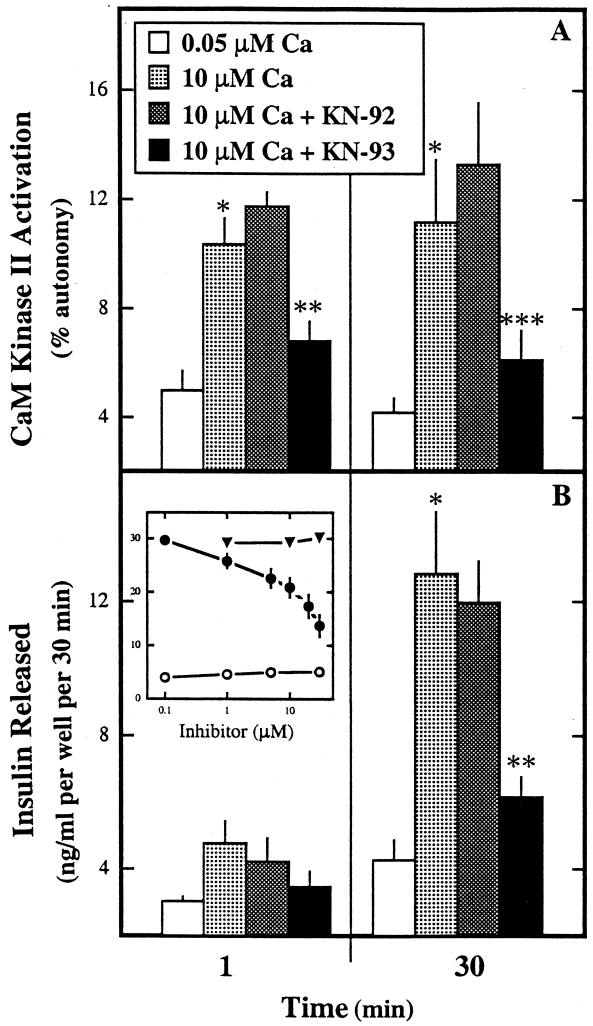

FIG. 3. Inhibition of Ca^{2+}-induced activation of CaM kinase II (A) and insulin secretion (B) by KN-93 in permeabilized βTC3 cells. βTC3 cells were cultured in 12-well plates and were permeabilized in monolayers using α-toxin as described in Materials and Methods. Cells were then incubated at 37° in 0.05 μM Ca^{2+} or 10 μM Ca^{2+} buffers in the absence or presence of 50 μM KN-92 or 50 μM KN-93. After 1 and 30 min, the medium was recovered for insulin assay (RIA) (panel B), and the cells were homogenized for the determination of CaM kinase II activation (panel A). Inset: Cells were incubated for 30 min in 0.05 μM Ca^{2+} (○) or 10 μM Ca^{2+} buffers (filled symbols) in the presence of the indicated concentrations of KN-93 (●) or KN-92 (▼). Values are means ± SEM from 6–9 independent determinations. Key: (*) $P < 0.002$ vs 0.05 μM Ca^{2+}; (**) $P < 0.01$ vs 10 μM Ca^{2+}; and (***) $P < 0.05$ vs 10 μM Ca^{2+}.

stimulation of selected cells loaded with the Ca^{2+}-sensitive fluoroprobe Fura-2, with 40 mM KCl in the presence of 3 mM glucose. In control cells, this manipulation produced quantitatively similar responses to sequential exposures of KCl that were characterized by a rapid, sharp response followed by a sustained second phase. In this latter phase, $[Ca^{2+}]_i$ remained elevated by 2- to 3-fold relative to basal concentrations (Fig. 4A, Table 1). KN-62 (10 μM) markedly inhibited the elevation of cytosolic Ca^{2+} induced by K^+ (Fig. 4B), which was consistent with observations made previously in HIT cells [6]. As indicated in Fig. 4C, KN-93 also profoundly inhibited K^+-induced Ca^{2+} influx, having profound effects on both peak and sustained phases (Table 1). In fact, in all cell comparison studies, KN-93 was a more effective inhibitor of Ca^{2+} influx than KN-62. The pharmacological inactive analog of KN-93, KN-92 [25], had an intermediate quenching effect on Ca^{2+} influx (compare Fig. 4D with Fig. 4A) and was less effective than KN-93 (Fig. 4C). Quantitatively similar effects were observed for all inhibitors in studies when βTC3 cells were used (data not shown). These data indicate that both KN-93 and its inactive analog, KN-92, suppress Ca^{2+} influx in the β-cell although to different extents.

Inhibition of Insulin Secretion by Peptide Inhibitors of CaM Kinase II

To further substantiate the involvement of CaM kinase II in Ca^{2+}-induced insulin secretion, the effect of peptide inhibitors of CaM kinase II to mimic inhibition by KN-93 was assessed. The two synthetic peptides chosen were [Ala^{286}]-CaMK 281–302, designed from the primary sequence of the autoinhibitory domain of CaM kinase II [26], and AIP, a non-phosphorylatable analog of the peptide substrate autocamtide-2 [27]. Both peptides virtually abolished Ca^{2+}-dependent CaM kinase II activity in βTC3 cell homogenates with ic_{50} values estimated at 1 and 100 μM for AIP and [Ala^{286}]-CaMK 281–302, respectively (Fig. 5A).

Initial attempts to inhibit CaM kinase II in α-toxin-permeabilized cells were not successful and likely due to an inability of the peptides to navigate the charged channel of the toxin. However, both strongly inhibited (by 60–70%) the activation of CaM kinase II by high Ca^{2+} (10 μM) buffer added to islets that had been permeabilized by electroporation (Fig. 5, B and C). As in the case with studies using KN-93, the inhibition of CaM kinase II activation correlated with a marked inhibition of Ca^{2+}-induced insulin secretion. Insulin secretion was inhibited by greater than 70% by the addition of either AIP or [Ala^{286}]-CaMK 281–302 (Fig. 5, B and C).

DISCUSSION

The elevation of $[Ca^{2+}]_i$ is a critical signal in the initiation of insulin secretion from the β-cell but exactly how this cation triggers granule exocytosis is not understood completely. The localization of L-type Ca^{2+} channels to exocytotically active regions of the β-cell promotes high Ca^{2+} concentrations sufficient for granule fusion [28], possibly through direct binding to synaptotagmin [29]. Earlier Ca^{2+}-sensitive steps are, however, dependent on ATP [30, 31], implying the involvement of Ca^{2+}-dependent kinases in insulin secretion (reviewed in Refs. 8 and 32). The current study demonstrates that inhibitors of CaM kinase II that are effective in the prevention of enzyme activation by Ca^{2+} in permeabilized β-cells also markedly inhibit insulin secre-

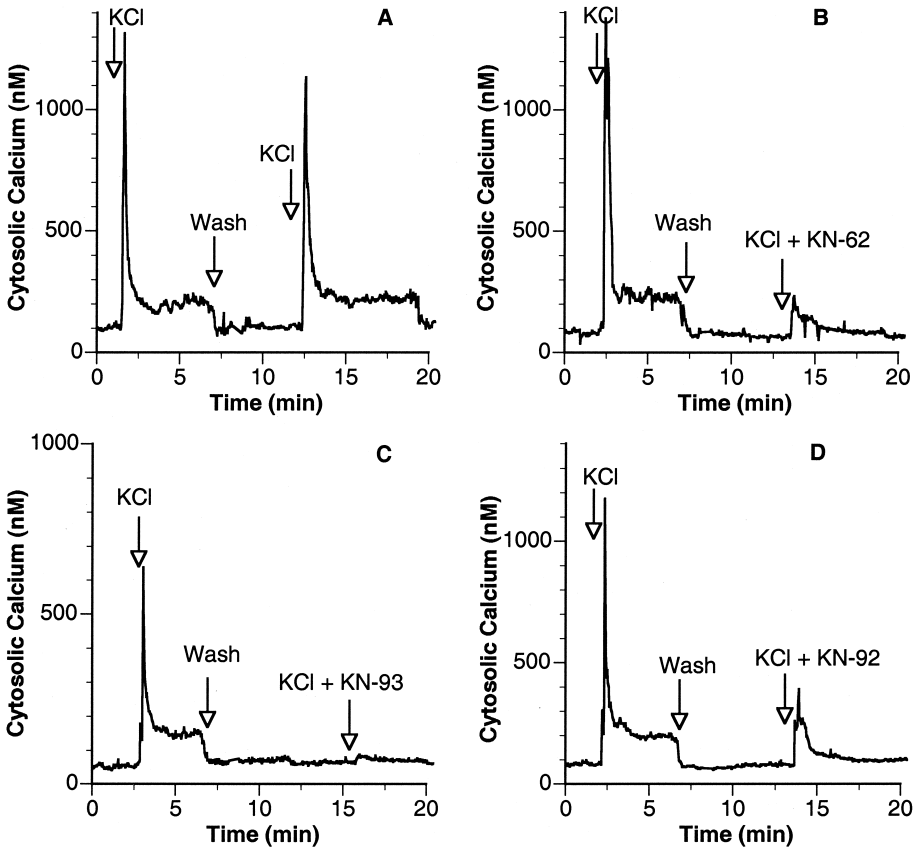

FIG. 4. Effect of CaM kinase II inhibitors on K^+-induced Ca^{2+} influx in INS-1 cells. INS-1 cells loaded with Fura-2 were subjected to consecutive stimulations with 40 mM KCl, the second of which contained: (A) control (DMSO); (B) 10 μM KN-62; (C) 10 μM KN-93; or (D) 10 μM KN-92. Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) were estimated by calculations described in Materials and Methods. Values are means from 9–12 individual cells.

tion. This was true irrespective of whether chemical inhibitors (KN-93) or peptide inhibitors (Ala or AIP) were used. Conversely, KN-62, which was ineffective as an inhibitor of insulin secretion in the same β-cell population, failed to affect Ca^{2+}-induced activation of CaM kinase II. These data are significant for two reasons. First, while still correlative, these data support an important role of CaM kinase II in the regulation of insulin secretion in the β-cell and complement previous studies that demonstrate the timely activation of CaM kinase II by insulin secretagogues [4, 9, 10]. Second, the discovery that KN-62 is incapable of inhibiting CaM kinase II nullifies a previous conclusion [6] that CaM kinase II is not involved in insulin secretion based on the insensitivity of this process to KN-62.

The reason for the inability of KN-62 to inhibit CaM kinase II in the permeabilized β-cell is not clear. With respect to mechanism, KN-62 is similar to KN-93 in exhibiting competitive inhibition with calmodulin, although it also shows non-competitive inhibition with ATP [2, 3]. Since the β-cell is suggested to have a high concentration of calmodulin relative to other cells [33], it is possible that the inhibitor is unable to overcome this

TABLE 1. Quantification of KN-62, KN-92, and KN-93 effects on KCl-induced Ca^{2+} influx in INS-1 cells

Treatment	Basal Ca^{2+} (nM)	Stimulated Ca^{2+} (nM)			
		Peak	+1	+2	+3
Control	102 ± 14	1134 ± 318 (11.1)	231 ± 49 (2.26)	229 ± 42 (2.25)	311 ± 37 (3.05)
KN-62	78 ± 10	243 ± 33 (3.1)	136 ± 25 (1.33)	96 ± 18 (1.23)	95 ± 17 (1.21)
KN-93	63 ± 9	87 ± 8* (1.38)	73 ± 8* (1.16)	65 ± 7 (1.03)	67 ± 8 (1.06)
KN-92	77 ± 9	389 ± 143 (5.05)	198 ± 25 (2.57)	121 ± 17 (1.57)	104 ± 13 (1.35)

Elevation in cytosolic Ca^{2+} concentrations achieved by cell depolarization in the absence and presence of KN-62, KN-93, and KN-92 (see Fig. 4) were quantitated by procedures described in Materials and Methods. Basal Ca^{2+} and Stimulated Ca^{2+}: average cytosolic Ca^{2+} level immediately prior to or during KCl stimulation, respectively. Stimulated Ca^{2+} levels are shown for peak response and for 1, 2, or 3 min following this peak response (+1, +2, +3). Numbers in parentheses represent fold stimulation with respect to basal Ca^{2+} concentrations for that set of cells. Statistical analyses (independent *t*-test): Peak: $P < 0.0023$ for all inhibitors; +1 to +3: $P < 0.012$ for all KN-93 vs KN-92; no significant difference between all KN-92 and KN-62 values.

* $P < 0.013$ vs KN-62.

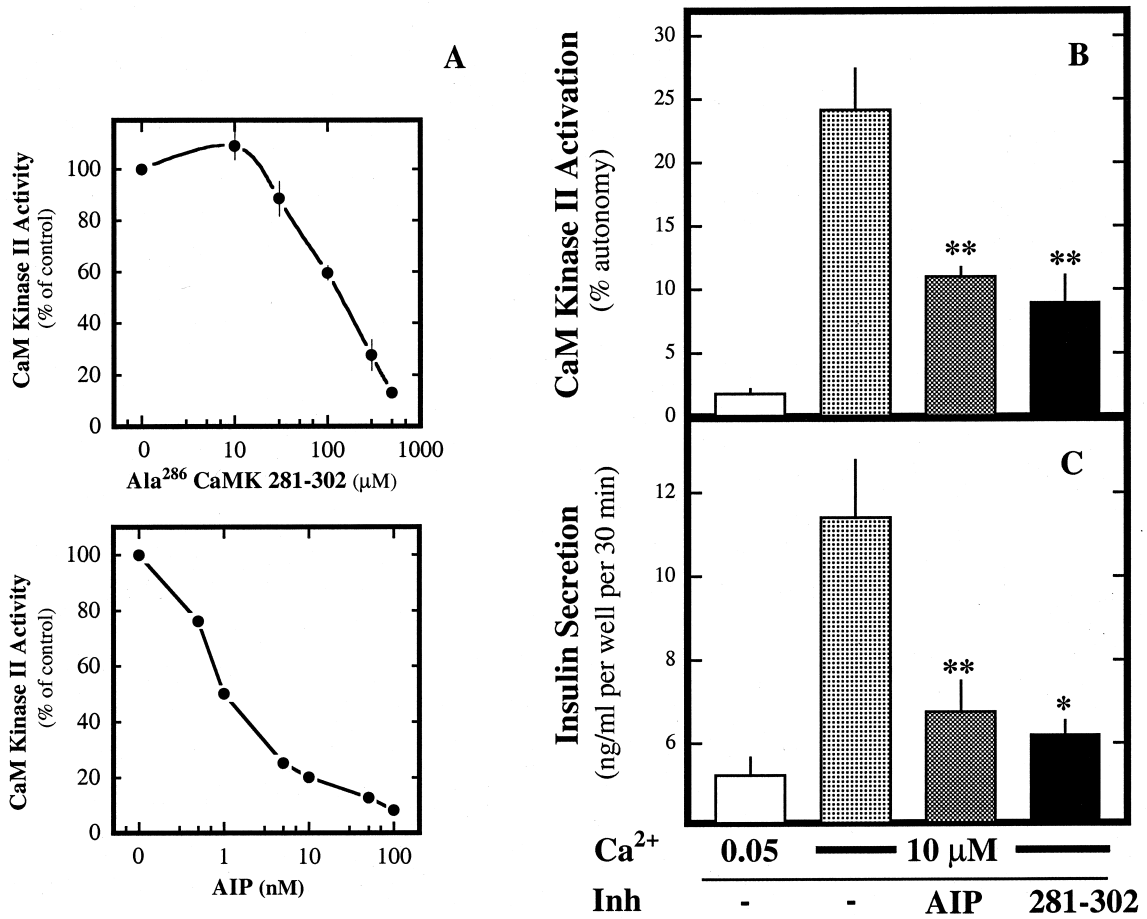

FIG. 5. Correlative inhibition of CaM kinase II activation and insulin secretion in permeabilized islets by peptide inhibitors. (A) CaM kinase II activity was assayed in βTC3 cell homogenates in the presence of increasing concentrations of [Ala^{286}]-CaMK 281–302 (0–500 μM) or AIP (0–100 μM). Data were recorded as counts per minute of ^{32}P incorporated into autocamtide-2, and ordinate values were expressed as a percentage of control in the absence of inhibitors. Average control values were 62,600 and 36,470 cpm, respectively (N = 4). (B and C) Isolated rat islets were permeabilized by electroporation and incubated in 0.05 μM Ca^{2+} buffer or 10 μM Ca^{2+} buffer in the absence or presence of AIP (100 μM) or [Ala^{286}]-CaMK 281–302 (300 μM). In panel B, islets (150/tube) were incubated for 1 min before homogenization and assay of enzyme activation. In panel C, islets (10/tube) were incubated for 30 min before the medium was recovered and used for the determination of insulin content. Values are means ± SEM from 15–20 determinations. Key: (*) $P < 0.002$ vs 10 μM Ca^{2+}; and (**) $P < 0.001$ vs 10 μM Ca^{2+}.

cofactor within the intracellular location of the enzyme. This seems unlikely because the effectiveness of KN-62 was not improved by the 10-fold elevation of its concentration and because KN-93 demonstrated competent inhibition within a similar concentration range. The more potent effect of KN-93 relative to KN-62 (K_i of 0.37 vs 0.9 μM [2, 3]) is insufficient to account for the dramatic difference in effectiveness of these drugs to inhibit CaM kinase II *in situ*. A more likely explanation may be the superior solubility of KN-93 in aqueous solutions and thus its projected ability to access CaM kinase II within the cytosol of the permeabilized cell. Nonetheless, the resident calmodulin concentrations of the β-cell may partially explain the higher concentrations of KN-93 required to inhibit CaM kinase II activity in βTC3 cells (IC_{50} ~10–20 μM) relative to purified enzyme *in vitro*.

An effect of KN-62 to inhibit Ca^{2+} influx has been demonstrated previously in a variety of cell types including the β-cell [6, 34–37]. Unfortunately, KN-93 also proved to be a powerful suppressor of Ca^{2+} influx in β-cells producing effects similar to, and in fact more potent than, KN-62. This observation was not surprising in light of similar effects of KN-93 reported on cardiac myocytes [38, 39], although the current data indicate that KN-92 also influences Ca^{2+} influx mechanisms. However, because glucose-induced insulin secretion is heavily dependent on Ca^{2+} influx [40], to conclude that CaM kinase II is involved in this process based merely on the inhibitory effects of these agents is insufficient. Furthermore, since KN-92 had a partial effect on Ca^{2+} influx relative to KN-93, it is not possible to use a differential effect of these compounds to assess a direct involvement of CaM kinase II in cellular events, a method recently employed within the context of the β-cell [41].

The possibility that the effect of KN-93 on Ca^{2+} influx is mediated through an interference of CaM kinase II regulation of Ca^{2+} channel activity, as proposed in the case of Ca^{2+}-induced enhancement of L-type Ca^{2+} channel

activity in cardiac myocytes [39, 42], cannot formally be eliminated. However, this suggestion is difficult to reconcile with observations herein, that KN-62, while effective against Ca^{2+} influx, had no effect on CaM kinase II activation. Furthermore, concentrations of KN-93 that inhibit Ca^{2+} influx by > 90% only inhibited CaM kinase II activation by approximately 30%. The differential effects of KN-93 versus KN-92 thus may be an inherent property of the compounds and independent of CaM kinase II. It is intriguing that two structurally distinct compounds, KN-93 and KN-62, common in their competitive antagonism of calmodulin, similarly inhibit Ca^{2+} influx. In spite of evidence that KN-62 does not bind calmodulin [2], these compounds may interfere with some calmodulin-dependent function of Ca^{2+} influx, by direct competition at a calmodulin-binding site on the Ca^{2+} channel. Calmodulin is constitutively associated with L-type Ca^{2+} channels in myocytes and mediates feed-back inhibition of these channels by Ca^{2+} [43, 44]. In the β-cell, calmidazolium inhibits Ca^{2+} influx, suggesting an opposite, positive mode of regulation of Ca^{2+} channel activity by calmodulin [45]. Clearly, more detailed studies are required before the precise actions of KN-62 and KN-93 (and KN-92) on ion fluxes are fully delineated. Until then, the employment of these compounds as CaM kinase II inhibitors in intact cells that are functionally dependent on Ca^{2+} should be conducted with extreme care.

In summary, the current study provides an explanation for contradictions that exist in the literature surrounding the effect of CaM kinase II inhibitors on insulin secretion. Importantly, this study shows that inhibitors of CaM kinase II effective in the prevention of CaM kinase II activation uniformly suppress Ca^{2+}-induced insulin secretion, reinforcing existing evidence in support of an important role of this kinase in insulin secretion.

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