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INHIBITION OF INSULIN SECRETION BY KN-62, A SPECIFIC INHIBITOR OF THE MULTIFUNCTIONAL Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II

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SUMMARY: The effects of KN-62, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CamPKII), on insulin secretion and protein phosphorylation were studied in rat pancreatic islets and RINm5F cells. KN-62 was found to dose-dependently inhibit autophosphorylation of CamPKII in subcellular preparations of RINm5F cells ($K_{0.5}$ = 3.1 ± 0.3 μM), but had no effect on protein kinase C or myosin light chain kinase activity. KN-62, but not the inactive analogue KN-04, dose-dependently inhibited glucose-induced insulin release ($K_{0.5}$ = 1.5 ± 0.5 μM) in a manner similar to the inhibition of CamPKII autophosphorylation. KN-62 (10 μM) inhibited carbachol (in the presence of 8 mM glucose) and potassium-stimulated insulin secretion from islets by 53% and 59%, respectively. These results support a role of CamPKII in glucose-sensitive insulin secretion.

It is well known that increased intracellular free Ca^{2+} concentrations play a central role as a second messenger in the stimulation of insulin secretion. Insulin secretion induced by glucose, considered the primary physiological stimulus, is dependent on the presence of extracellular Ca^{2+} (1,2). Current hypotheses postulate that metabolism of glucose within islets results in a transient increase in the ATP/ADP ratio leading to depolarization and opening of voltage-dependent Ca^{2+} channels, promoting the influx of Ca^{2+} into the β-cell (3,4). However, the mechanism by which this increase in intracellular Ca^{2+} is translated into insulin release remains largely unknown. Since insulin secretion is accompanied by protein phosphorylation (5) it is logical to suggest that the effects of Ca^{2+} in the β-cell, as in other cell types, are mediated through the activation of Ca^{2+}-dependent protein kinases. Indeed, both CamPKII (5-8) and the

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Ca^{2+}/calmodulin-dependent myosin light chain kinase (5,9) have been implicated in the regulation of the insulin secretion process.

The strongest evidence for a role of a Ca^{2+}/calmodulin-dependent protein kinase to date comes from observations that alloxan, a diabetogenic agent, inactivates both glucose-induced insulin secretion and CamPKII activity (10,21). Alloxan, however, also has a potent effect on β-cell glucokinase (11,12). Until recently, selective inhibitors of CamPKII have not been available to assess the involvement of this enzyme in insulin secretory processes. One such newly-developed inhibitor is KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) (13,14). KN-62 has been shown to compete for the calmodulin binding site on brain CamPKII and inhibits both substrate phosphorylation and autophosphorylation with no significant effect on purified preparations of cAMP-dependent protein kinase (PKA), myosin light chain kinase (MLCK), or protein kinase C (PKC) (13,14). In this study, we have used KN-62 to assess a possible involvement of CamPKII in insulin secretion induced by glucose, depolarizing concentrations of potassium (K^+), and the muscarinic agonist, carbachol.

MATERIALS AND METHODS

Materials: KN-04 and KN-62 were synthesized according to the method of Tokumitsu *et al.* (14). Male Wistar rats were purchased from Sasco and fed *ad libitum*. Tissue culture supplies (CMRL-1066, RMPI-1640, glutamine, streptomycin, and fetal bovine serum) were from Gibco BRL (Grand Island, NY), and Hank's Balanced Salt Solution was purchased from Whittaker Bioproducts (Walkersville, MD). Glucose (Dextrose) was from the National Bureau of Standards (Gaithersburg, MD), and carbachol (carbamylcholine chloride) and TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) were purchased from Sigma (St. Louis, MO). All other chemicals were of the finest reagent grade available.

Pancreatic Islets: Islets were isolated from the pancreati of 2 to 3 rats by collagenase digestion as described by Johnson *et al.* (15). Islets were hand-picked from contaminating acinar tissue under a stereo microscope and either used immediately or stored overnight in CMRL 1066 media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% fetal bovine serum at 25°C in an atmosphere of 95% air/5% CO_2.

Insulin Secretion: Intact islets (20/tube) were preincubated (30 min.) in a modified Krebs-Ringer-Bicarbonate buffer (KRB) (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM HEPES, 3 mM Glucose, 0.1% BSA, pH 7.4) containing vehicle or inhibitor (0 - 10 μM) under an atmosphere of 95% O_2/5% CO_2. Following the preincubation, fresh KRB buffer containing basal concentrations of glucose (3 mM) or secretagogue in the presence of inhibitor (0 - 10 μM) or vehicle was added. After 30 minutes incubation, medium was removed from the islets and the insulin content was determined by double antibody radioimmunoassay (16). All incubations contained 200 μl medium and were performed at 37°C.

CamPKII Autophosphorylation: CamPKII activity was measured by the method of Landt *et al.* (17). Briefly, incubations (100 μl) contained 45 μl of RINm5F microsomal preparation, 50 μM [^{32}P]ATP (8 Ci/mmol), 0.45 mM EDTA, 0.20 mM EGTA, 50 mM PIPES-NaOH, 10 mM $MgCl_2$ and, when added, 1 μg/ml calmodulin/1.0 mM $CaCl_2$. Reactions were conducted at 37°C and terminated after 5 sec. by addition of a SDS solution and placement in a boiling water bath for 2 min. Relative activity was quantitated by measurement of the incorporation of [^{32}P]phosphate into the subunits of calmodulin-dependent protein kinase (M_r = 54000-57000) (18) by excision and scintillation counting of the bands subsequent to separation of phosphoproteins by SDS-PAGE electrophoresis.

Assay of Protein Kinase C Activity: PKC activity was measured by the method of Landt *et al.* (19). Briefly, islet cytosol (10 μl) was incubated at 37°C in a total volume of 100 μl, containing

100 mM TES-NaOH pH 7.4, 400 μg/ml histone III-S, 5 mM $MgCl_2$, and 1.0 mM EGTA. Calcium was added to yield total Ca^{2+} concentrations of 0-1.30 mM. Phosphatidylserine, when added, was at a final concentration of 175 μM. Assays were started by addition of [γ^{32}P]ATP (20 μM final concentration, 5-10 μCi of radioactivity). At 0.5 min., the reactions were terminated by the addition of 50 μl of 9 mM SDS, 6 mM 2-mercaptoethanol, 186 mM Tris-HCl (pH 6.7), 15% (v/v) glycerol. The proteins were denatured (100°C, 2 min.) and then separated by SDS-PAGE (12% w/v gels). ^{32}P-incorporation into histones excised from the gel was determined by liquid scintillation spectrometry.

Assay of Myosin Light Chain Kinase Activity: MLCK activity in RIN cell homogenates was performed as described by Penn *et al.* (9). Chicken gizzard myosin light chain, purified by the method of Adelstein and Klee (20) was added as exogenous substrate.

Analysis of Data: All results are expressed as the mean ± the standard error of the mean (SEM) of three experiments (n=3) unless otherwise noted.

RESULTS

Effect of KN-62 on Enzyme Activity

In order to characterize KN-62 inhibition of CamPKII in β-cells, its effects on Ca^{2+}/calmodulin-stimulated autophosphorylation of the enzyme were investigated in a subcellular preparation of the insulin-secreting tumor cell line, RINm5F. KN-62 dose-dependently inhibited CamPKII autophosphorylation activity ($K_{0.5}$ = 3.1 ± 0.3 μM) (Fig.1). Maximal inhibition (37 ± 5%) was achieved at an inhibitor concentration of 10 μM. By contrast, the same concentration of KN-62 (10 μM) failed to significantly inhibit either RIN cell PKC or MLCK; activities in the presence of inhibitor were 93 ± 1% and 100 ± 5% of control activity respectively.

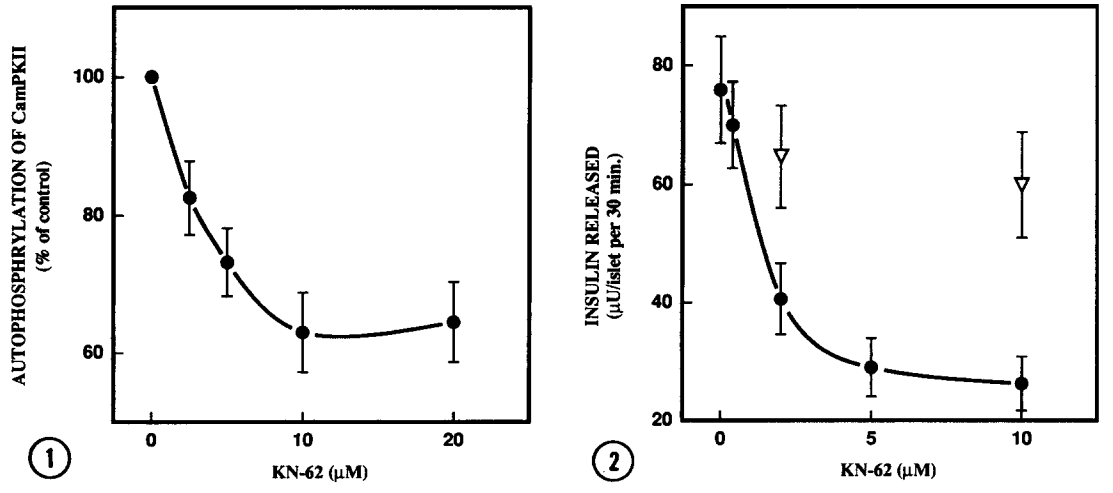

Figure 1. Inhibition of β-cell CamPKII Autophosphorylation by KN-62. Ca^{2+}/calmodulin-stimulated autophosphorylation of CamPKII was measured in microsomal preparations of RIN cells as described in Methods.

Figure 2. Effect of KN-62 on Glucose-Induced Insulin Secretion from Isolated Islets Insulin secretion was measured from intact islets incubated in the presence of increasing concentrations of KN-62 (•) or its pharmacologically inactive analogue, KN-04 (▽).

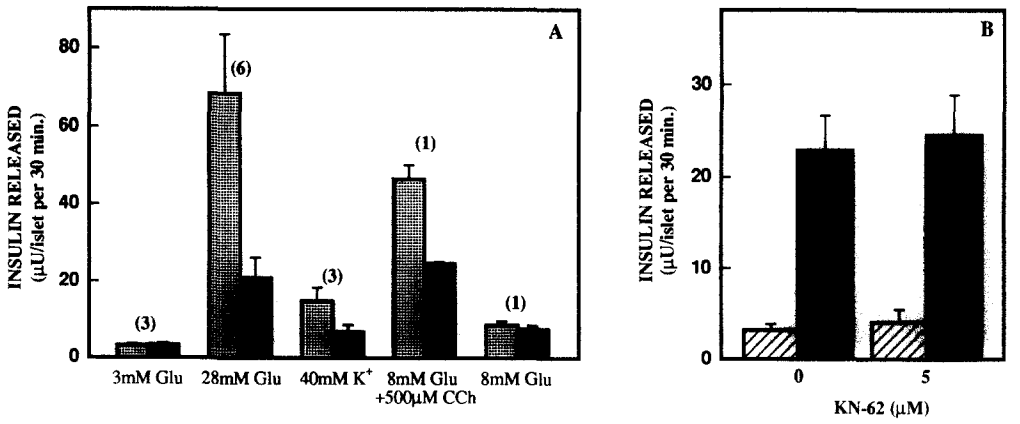

Figure 3. Effect of KN-62 on Secretagogue-Induced Insulin Secretion from Isolated Islets. **(A)** Insulin secretion from intact islets incubated with various secretagogues in the absence (light shading) or presence (heavy shading) of 10 μM KN-62 was determined. Number in parentheses indicates number of determinations made. **(B)** Insulin secretion induced by 100nM TPA (solid bars) relative to basal concentrations (3mM) of glucose (hatched bars) in the absence or presence of 5 μM KN-62 (n=3).

Effects of KN-62 on Insulin Secretion

The effects of KN-62 on glucose-induced insulin secretion were examined by incubating isolated islets in the presence of 28 mM glucose and increasing concentrations of KN-62 or its pharmacologically inactive analogue, KN-04. Insulin secretion was suppressed in a dose-dependent manner by KN-62, with half-maximal effect achieved at an inhibitor concentration of 1.5 ± 0.5 μM (Fig. 2). By contrast, KN-04 at the highest concentration tested (10 μM) only had a modest effect on glucose-induced insulin release (Fig. 2). Therefore the concentration of KN-62 required to half maximally inhibit insulin secretion was similar to that required to half maximally inhibit β-cell CamPKII activity ($K_{0.5}$ = 1.5 μM vs. 3.1 μM). Also similar to the effect of the inhibitor on CamPKII activity, secretion was only partially inhibited at high concentrations of KN-62, with maximal inhibition (67 ± 11%) achieved at 10 μM KN-62.

The effects of KN-62 on islet insulin secretion induced by other secretagogues, whose action is thought to involve elevated intracellular Ca^{2+} concentrations, were also studied. KN-62 inhibited K^+-induced insulin secretion by 59 ± 11% (Fig. 3A). Furthermore, the potentiation of glucose (8 mM)-induced insulin secretion by the muscarinic agonist, carbachol, a stable analogue of acetylcholine, was inhibited by 53% (n=1) relative to control in the presence of 10 μM KN-62 (Fig. 3A). By contrast, KN-62 at a similar concentration (5 μM) had no effect on insulin secretion when islets were incubated with basal (3 mM) glucose or basal medium containing 100 nM TPA (a potent activator of PKC) (Fig. 3B). A 7.1 ± 1.4 fold increase in insulin secretion relative to basal was induced by TPA and 109 ± 3% of stimulated insulin secretion was retained in the presence of inhibitor.

DISCUSSION

Several reports have provided preliminary evidence for a positive role of CamPKII in the modulation of glucose-induced insulin secretion (5-8,10). However, a definitive assessment of

the role of this enzyme in this process has been hampered by the lack of specific inhibitors and insufficient knowledge of its intracellular substrates in the β-cell. The recent development of KN-62 as a selective inhibitor of CamPKII has offered a more specific pharmacological means to explore the possible involvement of this enzyme in the regulation of the insulin secretory process. This reported selectivity of KN-62 was confirmed for the β-cell in initial experiments which demonstrated that KN-62 selectively inhibited β-cell CamPKII activity. Importantly, the activities of other Ca^{2+}-dependent protein kinases with potential roles in the modulation of insulin secretion, PKC and MLCK, were not inhibited by concentrations of KN-62 which elicited a maximal effect on CamPKII activity and insulin secretion. Thus, for the β-cell at least, KN-62 appears to be selective tool to probe for the role of CamPKII in Ca^{2+}-sensitive secretion processes.

Inhibition of β-cell CamPKII was achieved within the concentration range effective to inhibit brain CamPKII (14). Confirmation of inhibitory capacity in β-cells was important because of the previous demonstration of tissue-specific distribution of CamPKII isozymes, which raised the possibility that susceptibility to inhibition by KN-62 could be different for the non-neuronal isozymes of CamPKII (22). Inhibition of CamPKII by KN-62 in subcellular fractions of β-cells was not complete, with more than half of the activity apparently resistant to inhibition. The reason for this resistance is not known. A possible explanation is that a heterogeneous population of CamPKII molecules, a portion of which is resistant to inhibition by KN-62, is generated in β-cell homogenate as the result of phosphorylation or proteolytic modification.

Significantly, a close correlation was observed between the inhibitory effects of KN-62 on CamPKII autophosphorylation, an indicator of enzyme activity, and glucose-induced insulin secretion with respect to both sensitivity and the extent of inhibition. These observations suggest that the site of action of KN-62 on insulin secretion is CamPKII. Further support for this interpretation is provided from the demonstration that KN-62 also inhibited islet insulin secretion induced by depolarizing concentrations of K^+ and carbachol by a similar extent. In fact, in experiments not described here, the dose-dependence of the inhibition of K^+-induced insulin secretion from RIN-cells ($K_{0.5}$ = 1.5 ± 0.2 μM) was similar to that observed for the inhibitory effect of KN-62 on CamPKII autophosphorylation and glucose-induced insulin secretion. Furthermore, preliminary data from our laboratory (unpublished results) have indicated that the concentrations of alloxan required to inhibit glucose-induced secretion also profoundly inhibit K^+-stimulated secretion from isolated islets. These data collectively provide significant evidence implicating a role for CamPKII in Ca^{2+}-mediated insulin secretion.

In summary, KN-62 appears to be a selective probe for the involvement of CamPKII in the insulin secretory processes and its potent inhibitory effect on glucose- and potassium-induced insulin secretion suggest a positive role for CamPKII in these processes.

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