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EVIDENCE FOR A ROLE OF THE MULTIFUNCTIONAL CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II IN INSULIN SECRETION

THESIS

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Calcium/calmodulin-dependent protein kinase II (CaM kinase II) is demonstrated to exist in the β-cell and immunoprecipitation. Glucose and potassium significantly stimulate the rapid autophosphorylation of CaM kinase II and proportionally induce autonomous activity of the kinase in a dose-dependent manner that parallels insulin secretion. The activation of CaM kinase II is inhibited by the presence of mannoheptulose indicating that metabolism of the hexose, as in insulin secretion, is necessary. Inhibitors of CaM kinase II, alloxan, KN-62 and KN-93, suggest that the enzyme is an integral component of insulin secretion and/or related processes in the β-cell.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
AC-2	autocamtide-2
BSA	bovine serum albumin
CCh	carbachol (carbamylcholine chloride)
CaM kinase II	Ca^{2+}/calmodulin-dependent protein kinase II
Ca^{2+}-PDE	Ca^{2+}/calmodulin-dependent phosphodiesterase
DAG	diacylglycerol
DTT	dithiothreitol
HBSS	Hank's Balanced Salts Solution
HEPES	N-(2-hydroxyetheyl)piperazine-N'-2-ethane-sulfonic acid
HIT	hamster insulin tumor
IP_3	inositol-1,4,5-trisphosphate
KN-62	1-[N,O-bis(5-isoquinolinesulfonyl) -N-methyl-L-tyrosyl]-4-phenyl-piperazine
KN-93	2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine
MAP-2	microtubule associated protein-2
MES	2-(N-morpholino)ethanesulfonic acid
MLCK	myosin light chain kinase
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
PLC	phospholipase C

PKA	cAMP-dependent protein kinase (protein kinase A)
PKC	protein kinase C
TPA	12-O-tetradecanoyl-phorbol-13-acetate
Tris	Tris[hydroxymethyl]aminomethane)
RINm5F	rat insulinoma mouse 5F

INTRODUCTION

Diabetes Mellitus and its complications is a leading cause of death in Europe and the United States (1). The disease effects up to 5% of North Americans and is the most common human endocrine disorder except for obesity (2). It is characterized by abnormally high levels of blood glucose due to the inefficacy of insulin to mediate glucose uptake and utilization by the body. This results in very high fasting blood glucose levels (≥ 7.8 mM or 140 mg/dl) (3) causing glucose to be lost through the kidneys. The result is copious, sweet urine, from which diabetes derives its name.

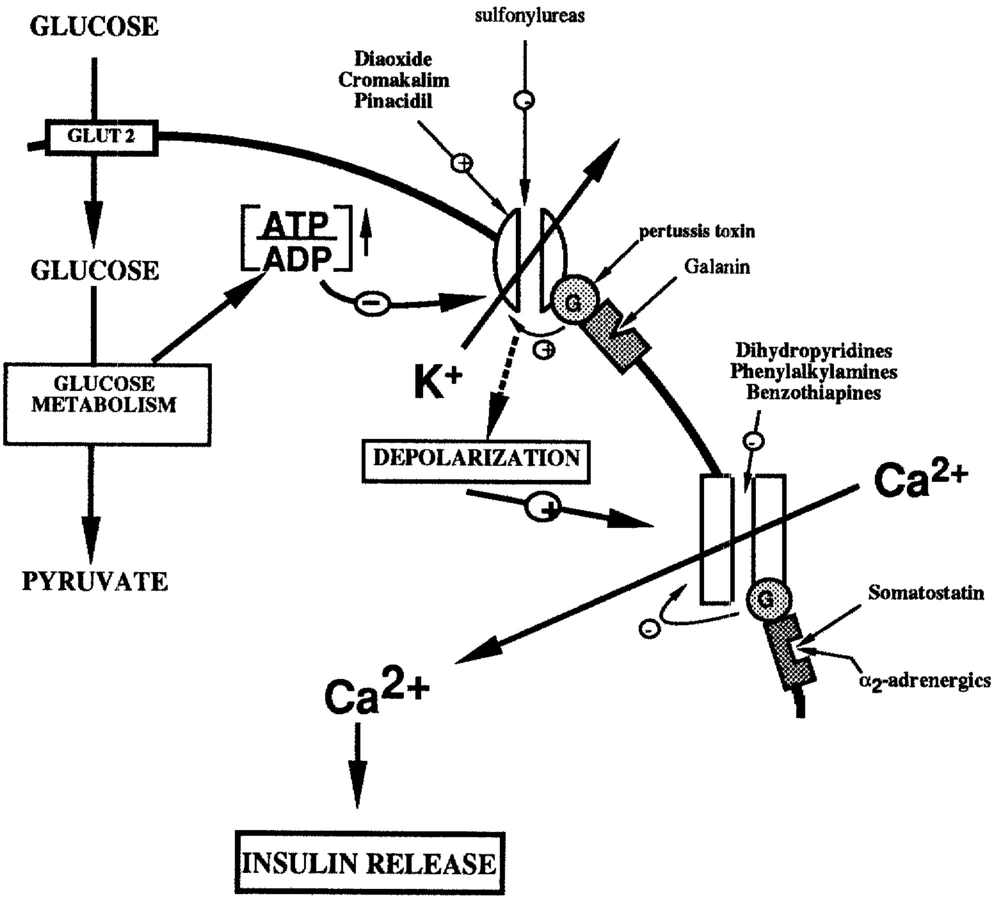
Two distinct forms of this disease exist. Type I, early-onset or insulin-dependent diabetes mellitus (IDDM), comprises about 10-20% of the diabetic population and appears to be the result of an autoimmune response which selectively destroys the insulin-secreting β-cells in the islets of Langerhans of the endocrine pancreas (4). Type I diabetics can therefore neither synthesize nor secrete insulin, and are dependent on administration of exogenous insulin for the preservation of life. Type II, late-onset or non-insulin dependent diabetes mellitus (NIDDM), accounts for the majority of the diabetic population and is a complex disease incorporating a number of different components including insulin resistance (5). In contrast to Type I, Type II diabetics retain the ability to synthesize and secrete insulin and may achieve endogenous insulin levels comparable to those in normal subjects in response to some secretagogues, such as L-arginine, but require elevated glucose concentrations to do so (6). A hallmark of NIDDM is the selective impairment of glucose-induced insulin secretion, particularly in that the early phase of secretion is absent or diminished.

β-cells comprise about 85% of the islet core and are responsible for the secretion of insulin (7). The remaining islet mantle is composed of α-, δ-, and pp-cells that secrete glucagon, somatostatin and pancreatic polypeptide respectively. The function of the latter protein is not clear, whereas the other two hormones, like insulin, are involved in maintaining metabolic homeostasis (8). The development of a procedure by Hellerström for the isolation of islets of Langerhans in 1969 greatly facilitated research directed toward the understanding of insulin secretion (9). Isolated islets retain the ability to secrete insulin and do so in a biphasic temporal pattern (10) that mimics physiological secretion in the intact pancreas *in situ* (11). The first phase is rapid and transient (peaks at 5 minutes) and is followed by a second, sustained phase that persists for at least 60 minutes; however, the cellular mechanisms creating this pattern are not understood(12). It is anticipated that the elucidation of the mechanisms involved in first and second phase secretion may provide important clues to the possible defects in Type II diabetes.

Efforts to understand the pathophysiology of type II diabetes are hindered by the incomplete knowledge of normal insulin release in response to glucose. D-glucose is the primary physiological stimulator of insulin release (12) but also potentiates insulin secretion induced by other hormones (*e.g.* acetylcholine (Ach), cholecystokinin (CCK), glucagon-like peptide (GLP)) (13). Our current understanding of glucose-induced insulin release is illustrated in Fig. 1. Glucose enters the β-cell by facilitated diffusion on Glut-2 transporters but this process is not rate limiting to secretion (14). However, glucose metabolism is required for secretion and the cellular receptor for glucose is considered to be the enzyme catalyzing the first step of this pathway, glucokinase (15).

It is widely accepted that an increased intracellular Ca^{2+} concentration is critical to glucose-induced insulin secretion (12,16). This is based on observations that this process is dependent on extracellular Ca^{2+} (11,16,17) and blocked by Ca^{2+}-channel blockers (18,19). Further studies employing Ca^{2+}-selective fluoroprobes (*e.g.* fura-2) have characterized glucose-induced increases in intracellular Ca^{2+} and suggest that it is the result primarily of Ca^{2+} influx into the β-cell rather than Ca^{2+} mobilization from

FIG. 1. **Current model of glucose-induced insulin secretion.**


GLUCOSE
GLUT 2
GLUCOSE
GLUCOSE METABOLISM
PYRUVATE
ATP
ADP
sulfonylureas
Diaoxide
Cromakalim
Pinacidil
K+
DEPOLARIZATION
pertussis toxin
Galanin
G
Dihydropyridines
Phenylalkylamines
Benzothiapines
Ca2+
Somatostatin
α2-adrenergics
Ca2+
INSULIN RELEASE

intracellular stores (20,21). Furthermore, insulin secretion is inhibited by calmodulin antagonists (22). The currently accepted model to explain glucose-induced increase in intracellular Ca^{2+} concentrations is that the metabolism of glucose in the β-cell by glycolysis increases the ATP:ADP ratio leading to cell depolarization through the closing of ATP-sensitive K^+-channels (23). This depolarization results in Ca^{2+} entry through the opening of L-type voltage-dependent Ca^{2+} channels (24) (see Fig. 1).

In contrast to our current understanding of these early steps in insulin secretion, the biochemical mechanisms by which increased cytosolic Ca^{2+} is translated into hormone release are not known. The final steps of insulin secretion can be dissected into 2 distinct phases: (1) the intracellular transport of insulin-containing secretory granules from the Golgi apparatus to the plasma membrane; and (2) the fusion of the granule with the plasma membrane and the release of insulin into the extracellular space (exocytosis) (12). Exocytosis is a complex process that, in the β-cell, remains mostly undefined but likely involves both Ca^{2+}-dependent and Ca^{2+}-independent events (25). However, studies demonstrating that drugs which interfere with microtubule and microfilament assembly and disassembly modulate insulin secretion suggest that the cell cytoskeleton is involved in the secretion process, possibly to regulate intracellular movement of insulin-containing secretory granules (26). Since insulin secretion is accompanied by protein phosphorylation and dependent upon Ca^{2+} and calmodulin, it has been proposed that the distal steps of insulin secretion are mediated by cytoskeleton-associated Ca^{2+}/calmodulin-dependent protein kinases (27). A logical candidate is the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), which has been shown to regulate cytoskeleton function in other cells (28). A second candidate is myosin light chain kinase (MLCK), which through the phosphorylation of its substrate myosin light chain (MLC) could trigger a contraction event sufficient to provide the motile force required for the physical movement of secretory granules to the exocytotic site on the plasma membrane. It is likely that both enzymes play important roles in β-cell function; however, the current thesis focuses on CaM kinase II.

Along with the cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), CaM kinase II is rapidly being considered one of the three major protein kinases. Widespread in nature, CaM kinase II has a broad substrate specificity and is proposed to play a multifunctional role in orchestrating a variety of cellular responses to Ca^{2+} (28,29). These include the regulation of intermediary metabolism, Ca^{2+} homeostasis, cytoskeletal function and gene expression (28). The enzyme is most abundant in neuronal cells where it is thought to regulate neurotransmitter synthesis and release by phosphorylation of synapsin I on synaptic vesicles (30). Since the pancreatic β-cell shares many properties common with neuronal cells, it is possible that CaM kinase II is equally important in the coordination of β-cell processes including insulin production and release.

Perhaps the most remarkable aspect of CaM kinase II is that activation of the enzyme by Ca^{2+}/calmodulin results in a rapid autophosphorylation of Thr 286 or Thr 287 (on the α- and β-subunits respectively) (29). The consequence of this autophosphorylation both *in vitro* and *in vivo* is the generation of an "autonomous", Ca^{2+}-independent activity which continues to phosphorylate itself and cellular substrates in the absence of Ca^{2+} and calmodulin (31,32). This property has lead to the proposed role of CaM kinase II in long term potentiation and memory, but may further function in the β-cell as a mechanism of prolonging insulin secretion initiated by a transient increase in intracellular Ca^{2+} (29). Although these events were initially characterized *in vitro*, recent evidence has suggested that similar enzyme autophosphorylation and generation of autonomous activity occurs in agonist-stimulated cells (33,34).

CaM kinase II has long been proposed to play a role in insulin secretion, although it has not been possible to definitively evaluate this. Most attempts to implicate this enzyme in insulin secretion were based on circumstantial observations. Several groups initially reported a Ca^{2+}/calmodulin-dependent protein kinase activity in insulin secreting cells (35-38). The principal endogenous substrate of the kinase was variously determined to have a molecular weight in the range of 53000 - 57000. It was found to be enriched in the microsomal fraction (35,37) and due to studies which indicated that the kinase was a component of the β-cell cytoskeleton, a role in granule-cytoskeleton interaction was suggested (39). This kinase was shown to be activated by Ca^{2+} concentrations thought to be obtained in the stimulated β-cell (35) and was blocked by calmodulin antagonists (*i.e.* trifluoroperazine and W7) (22). Furthermore, since the kinase activity could not be separated from phosphorylated peptide, it appeared likely that the observed phosphorylation represented autophosphorylation, a distinctive hallmark of CaM kinase II. Several properties of this kinase were similar to CaM kinase II. Purification from RINm5F cells of the kinase activity to near homogeneity demonstrated a similar low affinity for calmodulin (relative to other calmodulin-dependent enzymes, *e.g.* MLCK and Ca^{2+}-PDE) typical of brain CaM kinase II[1]. It was further observed that this purified kinase activity phosphorylated a number of known substrates of CaM kinase II including glycogen synthase, tubulin, synapsin-1 and MAP-2 (40); the latter of which has been proposed to function in the reversible polymerization of microtubules. The strongest evidence supporting a role of CaM kinase II in insulin secretion to date was provided by observations that the diabetogenic agent, alloxan, caused a parallel inhibition of CaM kinase II activity and glucose-induced insulin secretion (41). Therefore, while CaM kinase II has potential as a major regulator of glucose-induced insulin secretion, there was little evidence in support of this hypothesis, primarily due to the lack of the specific means necessary for a more thorough investigation.

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[1] M. Landt, personal communication.

It is the focus of this thesis to examine the possibility that CaM kinase II may have a role in insulin secretion. If this hypothesis is true, then it is expected that at least two minimum criteria be met. First, the enzyme should be activated by glucose in a manner that correlates both temporally and quantitatively with insulin secretion. Second, selective inhibitors should suppress glucose-induced insulin secretion within concentrations required to inhibit enzymatic activity and autophosphorylation in the β-cell. Despite the suitability of CaM kinase II as a mediator of insulin release, evaluation of this hypothesis to date has not been possible. This has been classically due to the lack of specific inhibitors and lack of knowledge concerning the intracellular substrates of the enzyme. The recent surge of activity that has lead to increased understanding of the regulation of this enzyme (*i.e.* autophosphorylation) together with the development of antibodies and putative inhibitors has provided a means to test the hypothesis that CaM kinase II mediates insulin release. This area of β-cell function is not well understood and it is anticipated that knowledge concerning such a role of CaM kinase II will contribute greatly to the understanding of the mechanisms mediating Ca^{2+}-sensitive and glucose-induced insulin release. Ultimately, these studies will have significant impact in understanding the pathophysiology of Type II diabetes and facilitate future efforts to develop treatments or a cure for this disease.

EXPERIMENTAL PROCEDURES

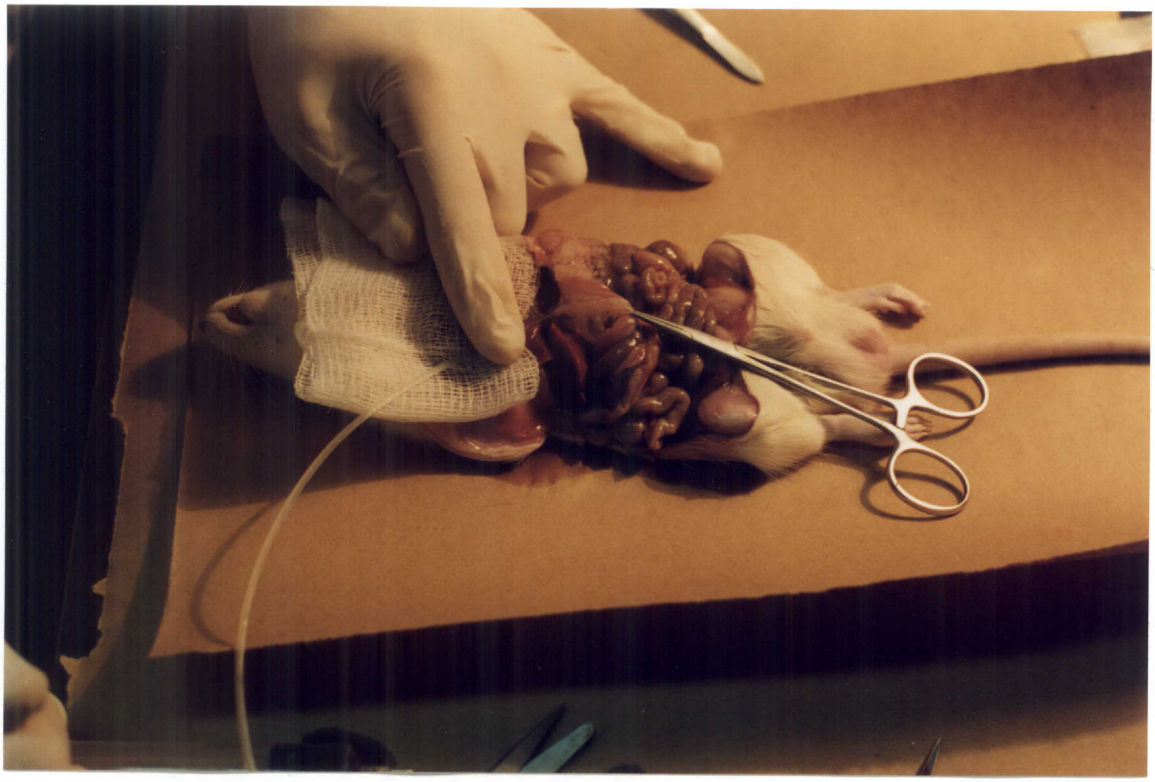
Materials—For the studies examining the inhibition of insulin release from intact islets, KN-04, KN-62 and KN-93 were obtained from Hiroyoshi Hidaka (Nagoya School of Medicine, Nagoya, Japan). KN-62 used in all other studies was purchased from Calbiochem (San Diego, CA). Male Wistar rats were purchased from either Sasco (O'Fallon, MO) or Harlan Sprague-Dawley (Indianapolis, IN) and maintained on Tekland Rodent Diet (Indianapolis, IN) *ad libitum* for 7-10 days prior to use. CMRL-1066, RMPI-1640, glutamine, streptomycin, penicillin, fetal calf serum and fetal bovine serum were from Gibco BRL (Grand Island, NY), and Hank's Balanced Salts Solution was purchased from Whittaker Bioproducts (Walkersville, MD). Ficoll, carbachol (carbamylcholine chloride), TPA (12-O-tetradecanoyl-phorbol-13-acetate), glyceraldehyde, Na^+ATP (adenosine-5'-triphosphate, sodium salt) and Protein A-Sepharose were purchased from Sigma (St. Louis, MO). Collagenase-P was from Boehringer Mannheim (Indianapolis, IN) and glucose (Dextrose) was from the National Bureau of Standards (Gaithersburg, MD). All radiochemicals (*i.e.* ^{32}P-orthophosphate and [γ-^{32}P]ATP) were from either ICN (Irvine, CA) or DuPont (Costa Mesa, CA). The peptide autocamtide-2 (sequence KKALRRQETVDAL) (42) was synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The polyclonal rabbit anti-rat CaM kinase IIα antibody was a gift from Paul Kelly (University of Houston, Houston, TX). All other chemicals were of the finest reagent grade available.

Pancreatic Islets—Islets were isolated from the pancreati of 2 to 10 rats by a modification of a collagenase digestion method described by Johnson *et al.* (43). Male Wistar rats (200-250 g, *i.e.* approx. 6 weeks old) were anaesthetized by an intraperitoneal injection of pentobarbital (65 mg per kg body weight) and allowed to lose consciousness.

When corneal reflexes were virtually absent, the body cavity was exposed and the tip of the duodenum located and clamped (Fig. 2). The bile duct was cannulated and approximately 15-20 ml of ice-cold Hank's Balanced Salts Solution (HBSS) (0.25 mg/ml collagenase-P, 1 mg/ml glucose, 0.2 mg/ml bovine serum albumin, pH 7.4) was instilled into the bile duct to distend the pancreas. Excess fat was trimmed from the pancreas which was then removed by blunt dissection and immersed in approximately 20 ml of the collagenase-containing HBSS. Collagenase digestion was initiated by placement of tubes containing pancreas in a water bath at 38.5 °C and a gas mixture of 95% O_2/5% CO_2 was bubbled through the HBSS (2 min) to maintain pH. During the remainder of the digestion, the tube was immersed in the water bath and the pancreas disrupted using a mechanical wrist-action shaker for approximately 8 min (modification of original method). The resulting solution was then transferred into 15 ml plastic tubes and washed 3 times with 10 ml volumes of ice cold HBSS (without collagenase) via rapid centrifugation and aspiration of supernatant. The final sediment was combined and vortexed in a tube containing 4 ml of 27% (w/v) Ficoll solution (44). Undigested material and remaining lymph nodes were removed and a discontinuous Ficoll gradient was formed by the sequential addition of 2 mls each of 23%, 20%, and 11% Ficoll solutions. Islets were separated from exocrine tissue by centrifugation at 650 x *g* for 15 minutes. Islets were harvested from the 11% to 20% interface using a siliconized pipette, resuspended in 40 mls HBSS and centrifuged at 300 x *g* for 2 minutes. Islets were removed from contaminating acinar tissue under a stereo microscope and either used immediately or stored overnight in CMRL 1066 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% fetal calf serum at 25 °C in an atmosphere of 95% air/5% CO_2.

Static Insulin Secretion Model—Intact islets (20/tube) were preincubated (30 min) in a modified Krebs-Ringer-Bicarbonate buffer (KRB) (115 mM NaCl, 5 mM KCl,

Fig. 2. **Surgical technique for the isolation of pancreatic islets.** Male Wistar rats (200-250 g) were anaesthetized by an intraperitoneal injection of pentobarbital and allowed to lose consciousness. When corneal reflexes were virtually absent, the body cavity was exposed and the tip of the duodenum located and clamped. The bile duct was cannulated and approximately 15-20 ml of ice-cold Hank's Balanced Salts Solution (HBSS) was instilled into the bile duct to distend the pancreas. Excess fat was trimmed from the pancreas which was then removed by blunt dissection.



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 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 or vehicle was added. After 30 minutes incubation, medium was removed from the islets and the insulin content was determined by double antibody radioimmunoassay (RIA) (45). When alloxan was used as inhibitor, groups of intact islets were treated (10 min at 37 ^{o}C) prior to preincubation with 1 ml KRB (no glucose) containing either alloxan (562.5 μM) or alloxan that had been inactivated (15 min, 37 ^{o}C, pH 7.4). Preincubation and incubation media for these studies did not contain the inhibitor, alloxan. All incubations contained 200 μl media and were performed at 37 ^{o}C in a shaking water bath.

Preparation of Anti-CaM kinase II αAntibody/Protein A Complex—For each precipitation, 4 mg of Protein A (immobilized on Sepharose CL-4B) was hydrated in 200 μl of PBSEBST buffer (20 mM $NaHPO_4$, pH 7.2, 2.0 mM EDTA, 1.0 mg/ml BSA, 0.01% SDS, 0.05% Tween-20). The suspension was washed once with the same volume of buffer. Liquid above the agarose was aspirated and 10.5 μl of anti-sera (anti CaM kinase II α) was added per precipitation and rocked overnight at 4 ^{o}C. The protein A-antibody conjugate was washed 5 times with the same volume of PBSEBST buffer and resuspended in a sixth volume.

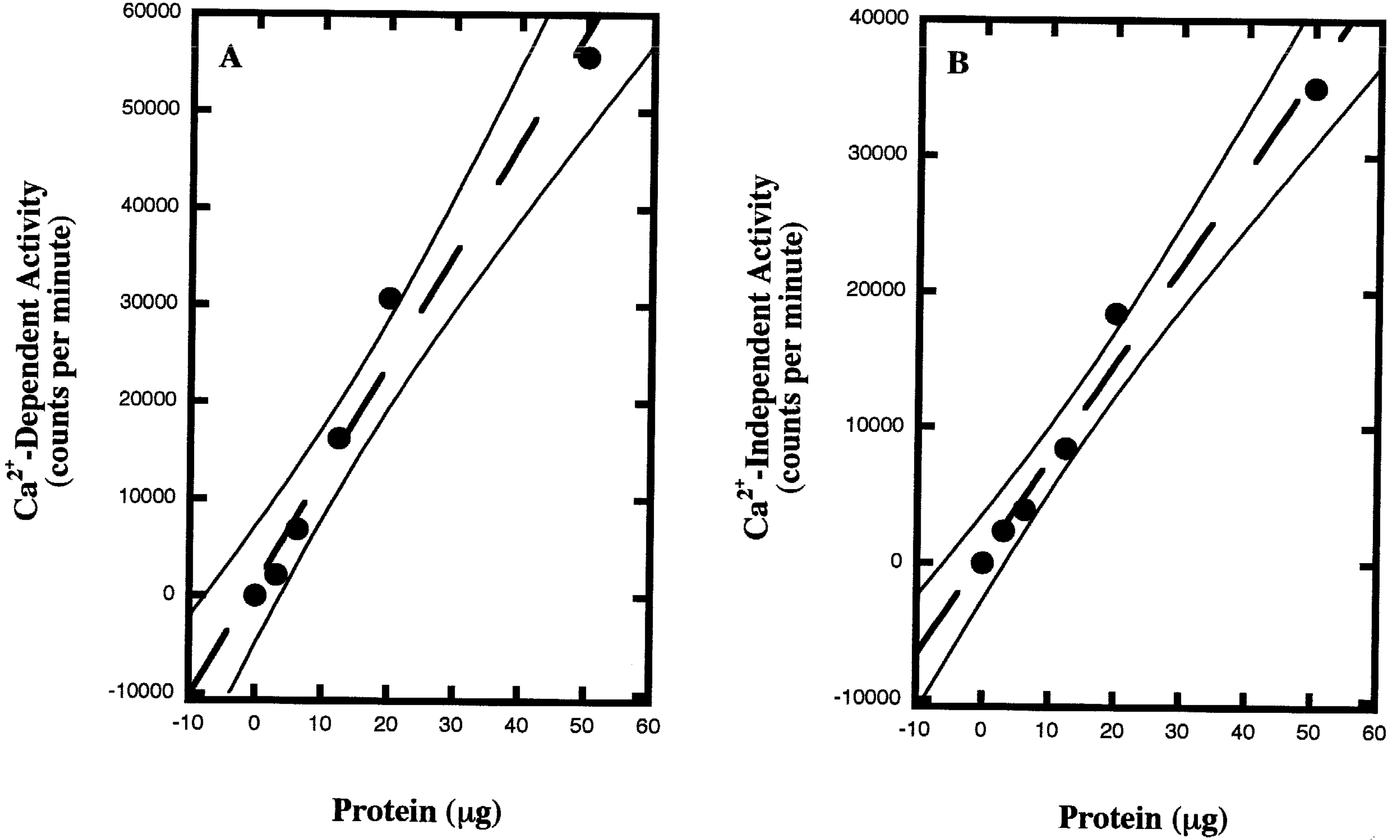
CaM kinase II Autophosphorylation—[A] RINm5F Cells: CaM kinase II activity was measured in RINm5F homogenates by the method of Landt *et al.* (46). RINm5F cell microsomal fraction was prepared by a centrifugal fractionation procedure of Colca *et al.* (47). Incubations (100 μl) contained 45 μl of RINm5F microsomal preparation, 50 μM [γ-^{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 s by addition of SDS sample buffer (186 mM Tris-HCl, pH 6.7, 9 mM SDS, 6 mM 2-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue) and placement in a boiling water bath for 2 min. Relative activity was quantitated by measurement of the incorporation of $^{32}P_i$ into the subunits of calmodulin-dependent protein kinase (M_r = 54000-57000) (48) by excision and scintillation counting of the bands subsequent to separation of phosphoproteins by SDS-PAGE electrophoresis. [B] Intact islets : Islets (400/tube) were labeled in 300 µl KRB containing basal glucose and 400 µCi $^{32}P_i$ for 1.5 hours at 37 °C under an atmosphere of 95% air/5% CO_2. Gentle agitation was applied to resuspend the islets at 30 min intervals. Reactions were initiated with 131.25 µl additions that made final reaction concentrations of either basal (3 mM) glucose, 28 mM glucose or 40 mM KCl (with appropriate adjustments made for maintaining iso-osmotic balance). Reactions were terminated by rapid centrifugation, aspiration and freezing in solid CO_2. Islets were then lysed by sonication (Branson sonicator; 10 pulses, setting 3, 30% duty cycle) in 100 µl ice cold homogenization buffer (50 mM MES, pH 7.2, 1 mM EDTA, 250 mM Sucrose, 50 mM $Na_4P_2O_7$, 50 mM NaF) . One hundred (100) µl of buffer containing 200 mM Tris-HCl, pH 7.2, 300 mM NaCl, 0.2% SDS, 2.0% Triton X-100, 2.0% deoxycholate, 50 mM $Na_4P_2O_7$, and 50 mM NaF was added, the tubes were vortexed and allowed to stand on ice for 2 min. Tubes were centrifuged for 2 min at 8000 x *g*. To remove unincorporated nucleotides, supernatants were passed over Centricon-30 columns (Amicon) and washed once with 200 µl ice-cold homogenization buffer. The retentate (~150-200 µl) was placed into new tubes containing 200 µl of the CaM kinase II α antibody/protein A complex (see Preparation of Anti-CaM kinase II α Antibody/Protein A Complex above) and agitated on a spin wheel for 2 hours at 4 °C. Tubes were centrifuged briefly (5 s at room temp) to pellet the complex, and the supernatant aspirated and discarded. This pellet was washed 4 times with 1.0 ml aliquots of PBSEBST buffer and 1 time with 20 mM $NaHPO_4$, pH 7.2, 2.0 mM EDTA, 0.01% SDS, 0.05% Tween-20 (to remove BSA). Liquid above the pellet was aspirated and discarded. Fifty (50) µl of SDS sample buffer (186 mM Tris-HCl, pH 6.7, 9 mM SDS, 6 mM 2-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue) was added and the mixture vortexed and boiled for 2 min. Fifteen (15) to 25 µl of each sample was separated by SDS-PAGE on a 10% (w/v) discontinuous gel. The gel was dried (Savant vacuum gel drier) and placed at -70 ^{o}C for autoradiography. Densitometric analysis was performed on an Optimus gel imager using BIOMED software.

Assay of CaM kinase II Activity—The CaM kinase II activity assay was performed using modification of previously described methods (33,49-51) [A] Islets: Islets (400/tube) were preincubated in basal KRB medium containing 3 mM glucose, 0.1% BSA 10 min at 37 ^{o}C, and then incubated in KRB containing 3 - 28 mM glucose or 40 mM KCl for 0 - 20 minutes. Media was then removed for insulin assay and islets quickly washed (200 µl) and sonicated (75 µl) in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 1.0 mM EDTA, 2.0 mM DTT, 10 mM sodium pyrophosphate, 0.4 mM ammonium molybdate, 0.1 mg/ml leupeptin). Ten (10) µl volumes of homogenate (approximately 20 µg protein) were immediately assayed at 30 ^{o}C for 30 s in tubes containing 40 µl of a reaction mixture which gave a final concentration of 50 mM PIPES, 10 mM $MgCl_2$, 0.1 mg/ml BSA (fraction V), 10 µM autocamtide-2, 20 µM [γ-^{32}P]ATP (40 Ci/mmol), and either 0.5 mM $CaCl_2$/5 µg/ml calmodulin for Ca^{2+}-stimulated activity or 0.9 mM EGTA for Ca^{2+}-independent activity. Termination of the reaction was achieved by the rapid addition of 25 µl ice cold 15% TCA and immediate, subsequent vortexing. Tubes were allowed to stand 20 min at 4 ^{o}C to precipitate large proteins followed by 1 min centrifugation at 5600 x *g*. Thirty (35) µl of supernatant was spotted onto 5 cm x 2 cm strips of phosphocelluose paper (Whatman

P81). Strips were washed 5 times for 20 min with constant stirring in distilled-deionized H_2O (10 ml/strip). The strips were subsequently dried at 115 ^{o}C for 20 min, and $^{32}P_i$ incorporation into autocamtide-2 determined by measurement of Cerenkov radiation. Autonomous kinase activity was expressed as percent of Ca^{2+}-stimulated activity. Such conditions were initially shown to be linear for the islet in respect to both protein concentration (Fig. 3) and time (data not shown). [B] RINm5F Cells : RINm5F cells were homogenized as described above and diluted to a final concentration of 20 μg protein/ml as determined by Bradford protein assay using BSA as a standard. Assays were also performed as above with the following exceptions. Reaction volumes were double but final concentrations were equivalent to those used for islets. Reactions were stopped by the addition of 25 μl of 25% TCA. When added to the reaction mixtures, TPA and cAMP were 1 μM and all assays had 0.2% DMSO (TPA vehicle). Radioactive Pi incorporation into peptide was performed by liquid scintillation spectroscopy.

Assay of Protein Kinase C Activity—PKC activity was measured by the method of Landt *et al.* (52). RIN cell cytosol (10 μl) was incubated at 37 ^{o}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 per assay). 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, 0.01% bromophenol blue. The proteins were denatured (100 ^{o}C, 2 min) and then separated by SDS-PAGE (12% w/v gels). $^{32}P_i$-incorporation into histones excised from the gel was determined by liquid scintillation spectrometry. PKC activity was calculated as the difference between $^{32}P_i$ incorporated into histone in the presence and absence of Ca^{2+} and phosphatidyl serine.

FIG. 3. **Linear protein concentration range for CaM kinase II autonomous activity assay.** Intact islets were homogenized by sonication and protein concentration determined by Bradford assay as described in Experimental Procedures. Assays (50 µl) for both (A) Ca^{2+}/calmodulin-dependent and (B) independent activity were immediately conducted in tubes containing between 0 and 50 µg homogenate protein. Dashed line is the first order linear regression with 95% confidence intervals shown (solid lines).


A
Ca^{2+}-Dependent Activity (counts per minute)
60000
50000
40000
30000
20000
10000
0
-10000
-10
0
10
20
30
40
50
60
Protein (µg)
B
Ca^{2+}-Independent Activity (counts per minute)
40000
30000
20000
10000
0
-10000
-10
0
10
20
30
40
50
60
Protein (µg)

Assay of Myosin Light Chain Kinase Activity—MLCK activity in RIN-cell homogenates was determined electrophoretically as described by Penn *et al.* (53). Chicken gizzard myosin light chain, purified by the method of Adelstein and Klee (54) was added as exogenous substrate.

Islet Permeabilization—Islets were permeabilized by a modification of the method of Jones *et al.* (55). Groups of 100 islets were washed 3 times in an ice-cold "permeabilization" buffer (140 mM potassium glutamate, 15 mM HEPES, pH 6.7, 7 mM $MgSO_4$, 5 mM ATP, 5 mM glucose, 1 mM EGTA and 0.5 mg/ml BSA) made 50 nM Ca^{2+} by the addition of $CaCl_2$. Free Ca^{2+} concentrations were estimated using a calcium electrode (Orion) calibrated using Ca^{2+}/EGTA buffers as described by Bers (56). Each group was then permeabilized by square-wave electrical pulse (3.4 KV/cm, 10 pulses, 30 µs each) using a BTX 800 transfector. Islets are washed 3 times in permeabilization buffer and used immediately.

Perifused Model of Insulin Secretion—Perifusions were performed by the method of Easom *et al.* (57). Groups of 100 islets were washed 3 times in either basal (3 mM glucose) KRB medium (intact islets) or permeabilization buffer (permeabilized islets) and loaded onto 8.0 µm (pore size) filters (Millipore) composed of an inert mixture of cellulose acetate and cellulose nitrate. The filters were placed in Swinex Disk Filter Holders (Millipore), sealed and immersed in a water bath at 37 °C, and perifused with basal and stimulatory media at a flow rate of 1 ml/min. Fractions were collected at indicated times (1 to 5 min intervals) and insulin content determined by RIA.

Measurement of Intracellular Ca^{2+}—RINm5F cells were cultured on glass coverslips in petri dishes in 3 ml of RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 10% fetal bovine serum at 37 °C with 5% CO_2. Prior to Ca^{2+} measurement, cells on the coverslip were incubated for 45 min in a HEPES buffered medium (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, 0.1% BSA, 10 mM glucose, pH 7.2) containing 5 μM fura-2/AM. The coverslip was mounted in a chamber and maintained at 37 ^oC. The fluorescence images obtained at 340 nm and 380 nm excitation wavelengths were recorded by a video camera with an image intensifier. The mean intracellular Ca^{2+} concentration of each cell was calculated from the ratio of fluorescence at these two excitation wavelengths using the equation according to Grynkiewicz *et al.* (58).

Protein Determinations—Estimation of protein concentration was performed by a method described by Bradford *et al.* (59).

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. Statistical analysis was performed using the unpaired Student's *t* test. Values were considered statistically significant at a level of $p < 0.05$.

RESULTS

Effect of KN-62 on β-cell Enzyme Activity—KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl) -N-methyl-L-tyrosyl]-4-phenyl-piperazine) had recently been synthesized as a selective inhibitor for CaM kinase II (60). Kinetic analyses of KN-62 on brain CaM kinase II demonstrated competitive inhibition with respect to Ca^{2+} and it inhibited 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) (60). In order to characterize KN-62 inhibition of CaM kinase II 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 CaM kinase II autophosphorylation activity ($K_{0.5} = 3.1 \pm 0.3$ μM) (Fig. 4). 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 (Table 1).

Inhibition of Glucose-Induced Insulin Release by KN-62 —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. 5). By contrast, KN-04 at the highest concentration tested (10 μM) only had a modest effect on glucose-induced insulin release (Fig. 5). Therefore the concentration of KN-62 required to half maximally inhibit insulin secretion

FIG. 4. **Effect of KN-62 on β-cell CaM kinase II activity.** CaM kinase II autophosphorylation was used to indicate CaM kinase II activity in RINm5F microsomal preparations as described in Experimental Procedures. Incubations (100 μl) contained 45 μl microsomal preparation and either inhibitor (10 μM) or vehicle in the absence or presence of Ca^{2+}/calmodulin. Reactions were conducted at 37 °C and terminated after 5 s 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.

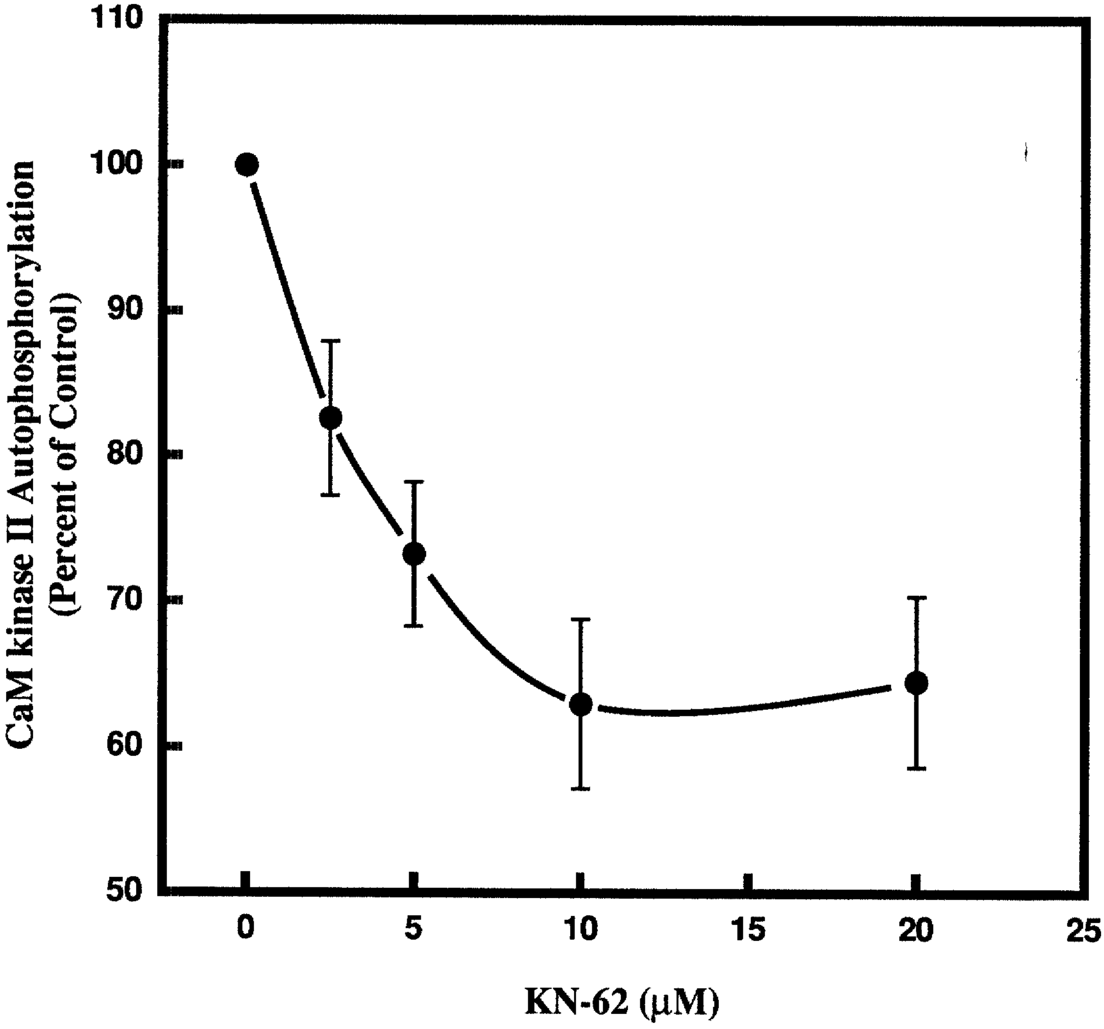

110
100
90
80
70
60
50
0
5
10
15
20
25
CaM kinase II Autophosphorylation
(Percent of Control)
KN-62 (μM)

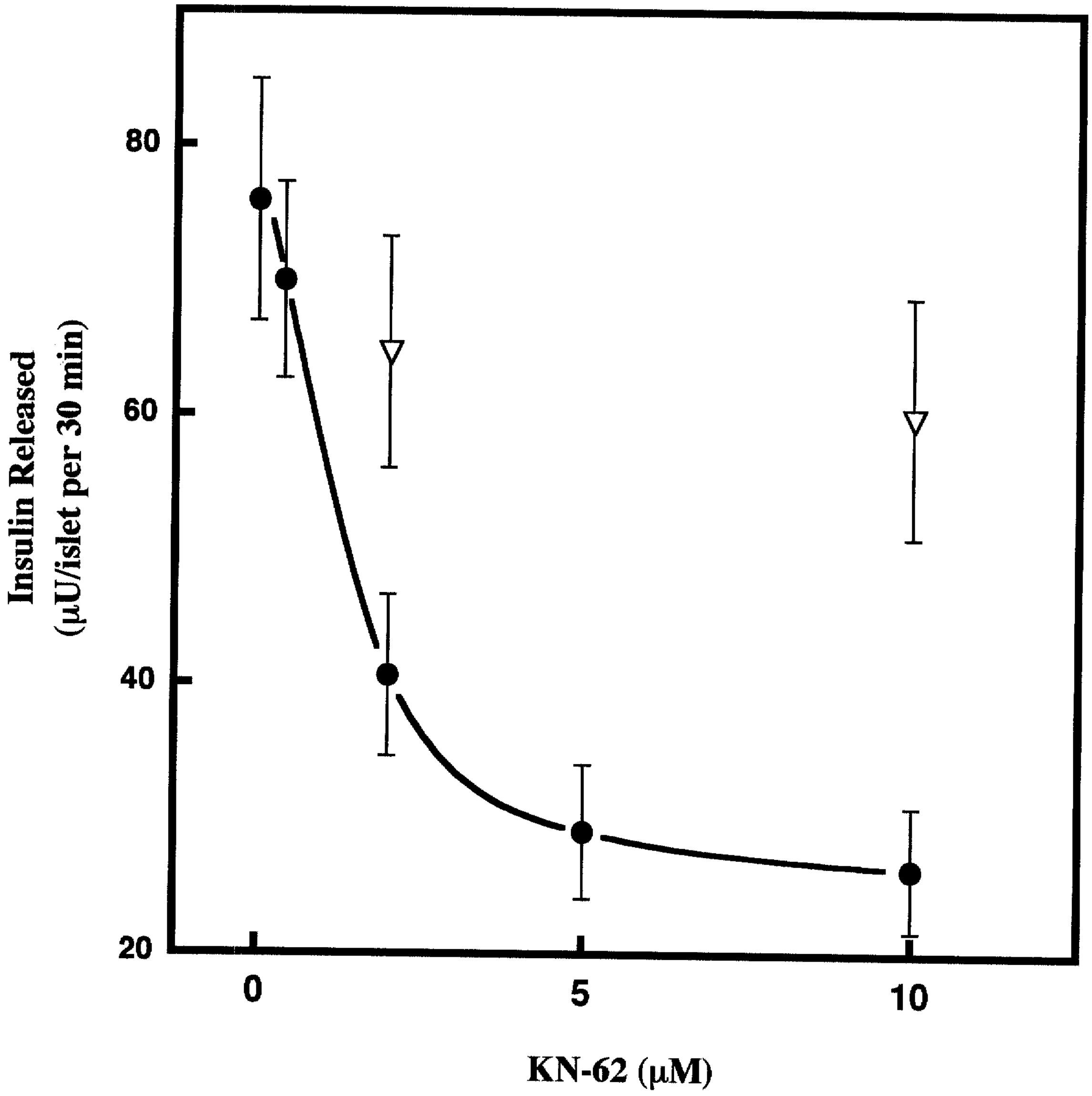
Table I

Effects of KN-62 on CaM kinase II, PKC and MLCK activity in RINm5Fcells

The activities of CaM kinase II, PKC, and MLCK were measured in the absence and presence of 10 μM KN-62 in assays described in Experimental Procedures.

Enzyme	Control	KN-62 (10μM)
	pmol/min	*pmol/min; % of control*
CaM kinase II	16.1 ± 2.9	10.1 ± 1.8 ; 63 ± 5%
PKC	9.6 ± 0.9	8.9 ± 0.8 ; 93 ± 1%
MLCK	0.35 ± 0.07	0.33 ± 0.05; 100 ± 5%

FIG. 5. **Inhibition of glucose-induced insulin release by KN-62 in a static model.** Insulin secretion induced by 28 mM glucose was measured from intact islets incubated in the presence of increasing concentrations of KN-62 (●) or its pharmacologically inactive analogue, KN-04 (▿).


Insulin Released
(µU/islet per 30 min)
80
60
40
20
0
5
10
KN-62 (µM)

was similar to that required to half maximally inhibit β-cell CaM kinase II activity ($K_{0.5}$ = 1.5 μM vs. 3.1 μM). Also similar to the effect of the inhibitor on CaM kinase II 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 glucose-induced secretion were also investigated using a perifusion model as described in Experimental Procedures. The primary advantage of the perifusion technique is that it allows a temporal examination of the changes in insulin secretion (*i.e.* first and second phases of secretion). It also appears to provide a more physiological situation in that the islets are not static in a milieu of their secretions, which may interfere with the secretory response. Islets were loaded upon membranes, placed in chambers (thermally controlled by immersion in a water bath), and perifused with basal (3 mM Glucose) KRB medium for 25 minutes. Basal medium containing either 1 μM KN-62 or vehicle was then introduced followed by perifusion with 28 mM glucose in the absence or presence of KN-62. As demonstrated in Fig. 6, insulin release was markedly inhibited (approximately 50%, n=2) in both the first and second phases by KN-62, seeming to suggest that CaM kinase II participated in both early and late insulin secretion.

Effect of KN-62 on Insulin Release by Other Secretagogues—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. A dose-dependent effect, much like that seen for glucose in islets, was observed for K^+-induced insulin secretion in RINm5F cells. Secretion was maximally inhibited (59 ± 2% of control) at 10 μM KN-62 with a half maximal inhibitory concentration of 1.5 ± 0.2 μM (Fig. 7). A quantitatively similar effect was observed in intact islets where the same concentration of KN-62 inhibited K^+-induced insulin secretion by 59 ± 11% (Fig. 8A). Furthermore, the potentiation of glucose (8 mM)-induced insulin secretion by the muscarinic agonist,

FIG. 6. **Inhibition of glucose-induced insulin release from by KN-62 in a perifusion model.** Intact islets (100 per chamber) were perifused with a stimulatory concentration of glucose (28 mM) in the absence (▽) or presence (●) of 1 μM KN-62 as described in Experimental Procedures (n=2).

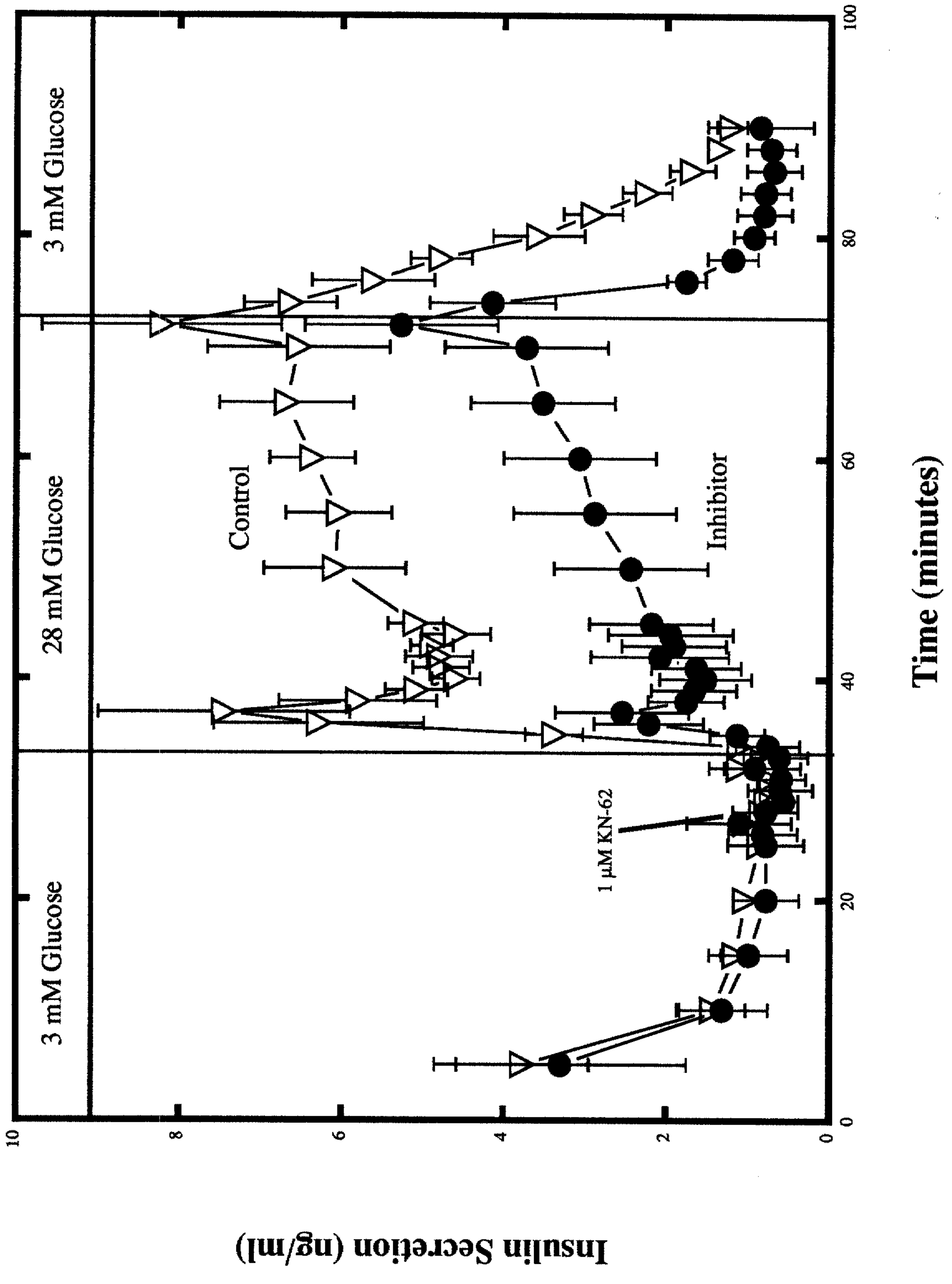

3 mM Glucose
28 mM Glucose
3 mM Glucose
Control
Inhibitor
1 µM KN-62
Insulin Secretion (ng/ml)
0
2
4
6
8
10
Time (minutes)
0
20
40
60
80
100

FIG. 7. **Dose-dependent inhibition of K^+-induced insulin secretion from RINm5F cells by KN-62.** Insulin secretion induced by 40 mM K^+ (●) or basal (5 mM K^+) (▿) medium was measured from RINm5F cells incubated in the presence of increasing concentrations of KN-62.

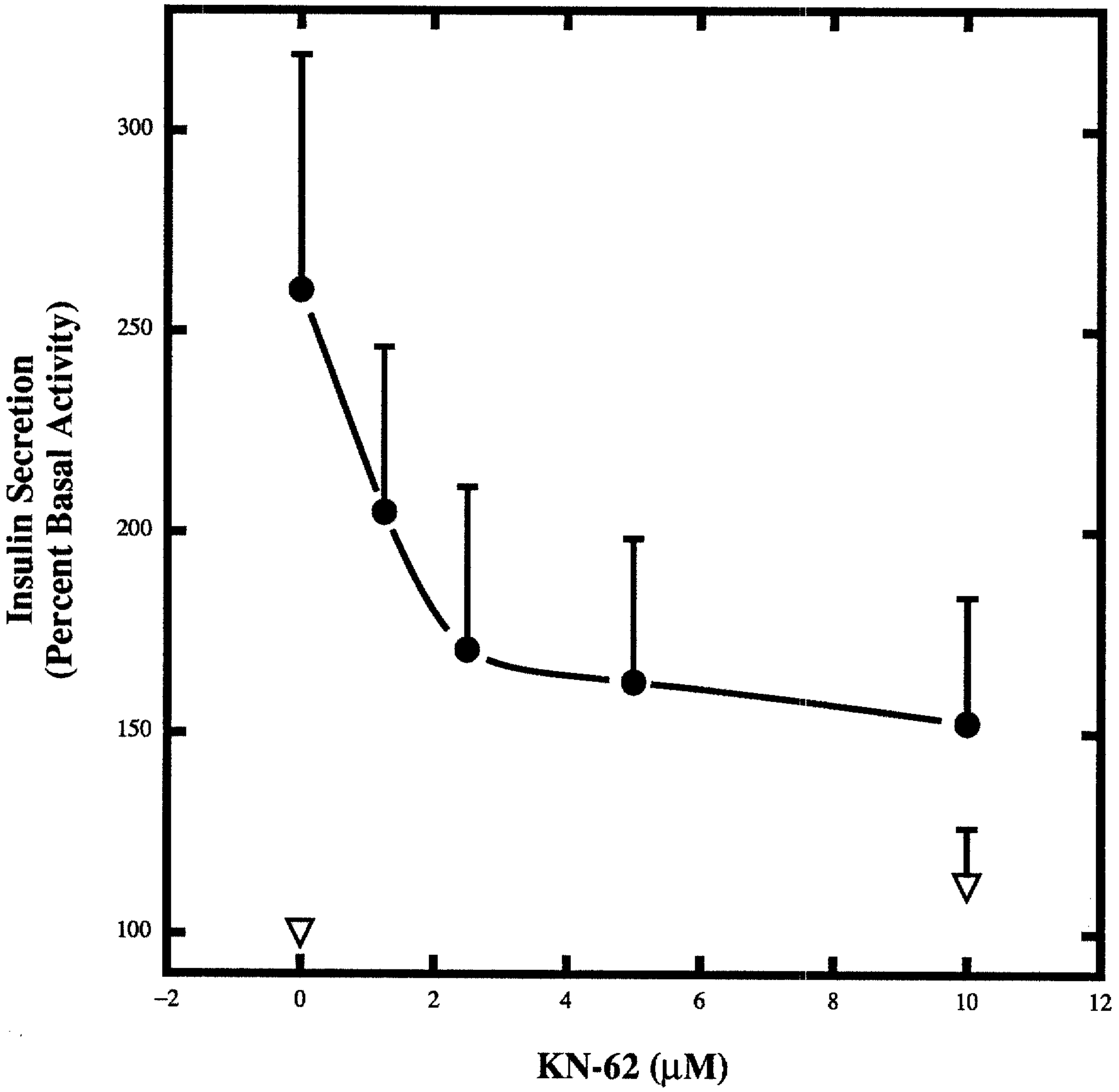
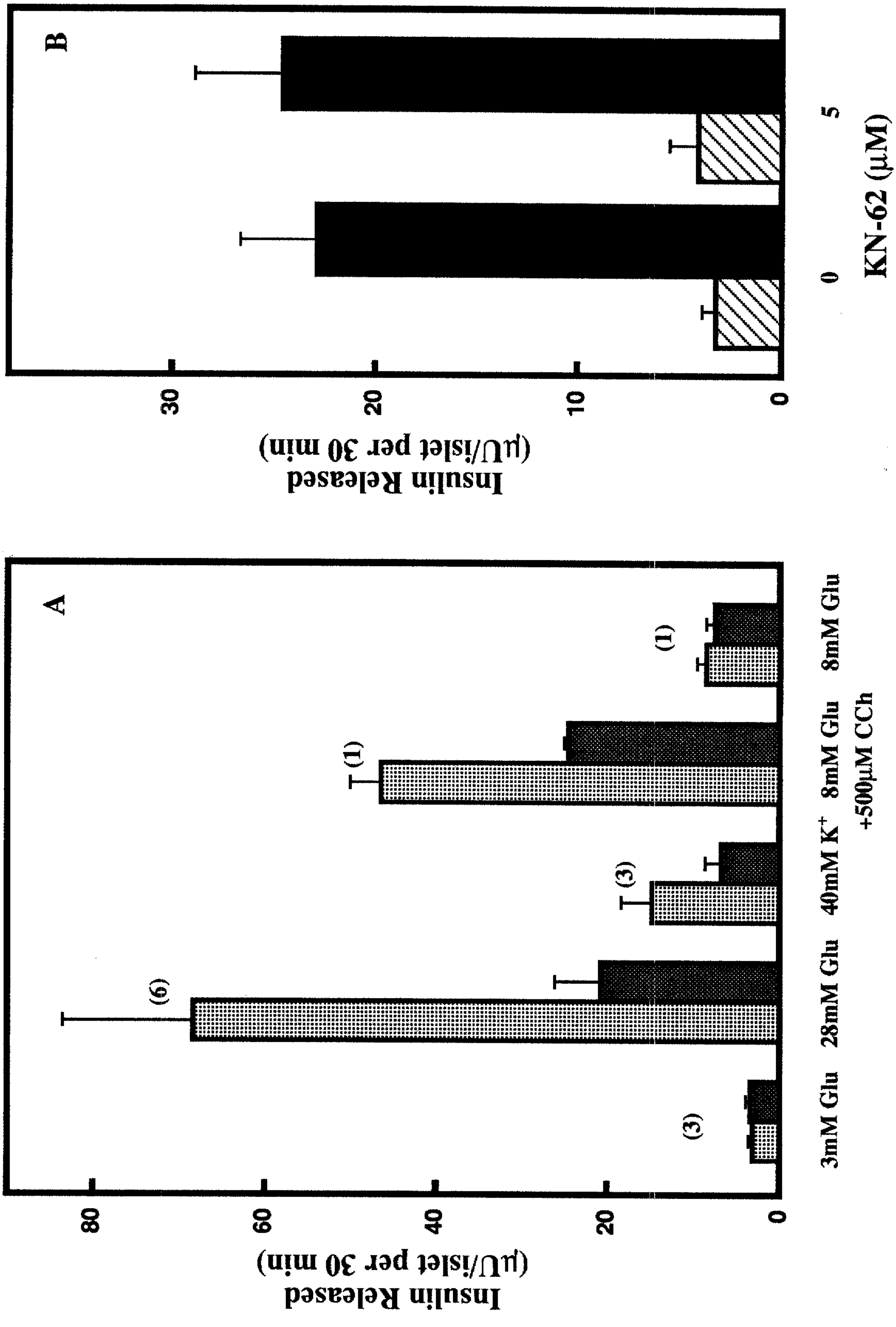

Insulin Secretion
(Percent Basal Activity)
300
250
200
150
100
−2
0
2
4
6
8
10
12
KN-62 (μM)

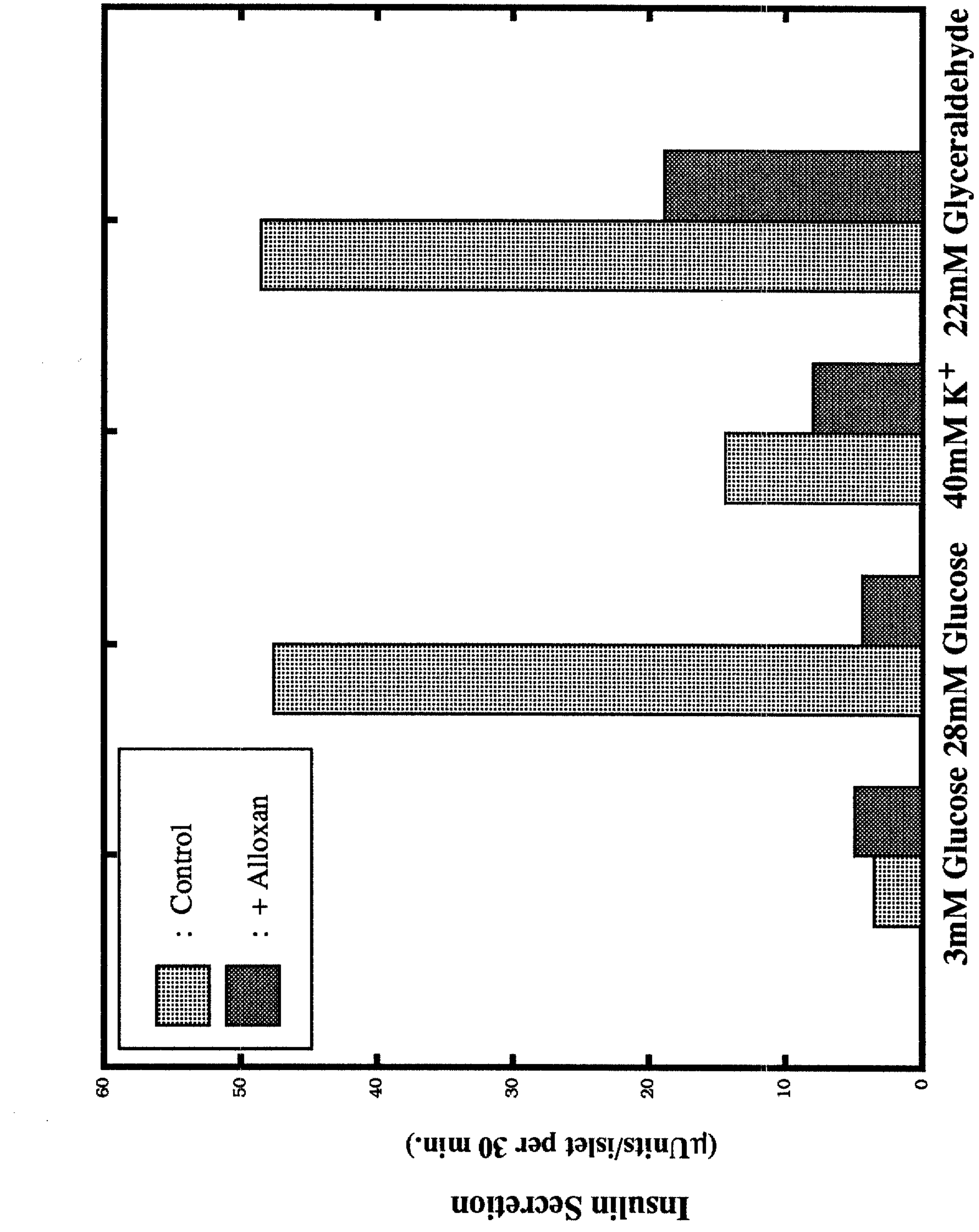
FIG. 8. **Effect of KN-62 on insulin release induced by various secretagogues.** (A) Insulin secretion from intact islets incubated with various secretagogues in the absence (light shading) or presence (heavy shading) of 10 μM KN-62 was determined. Number in parentheses indicates number of determinations made. (B) Insulin secretion induced by 100 nM TPA (solid bars) relative to basal concentrations (3 mM) of glucose (hatched bars) in the absence or presence of 5 μM KN-62 (n=3).


A
Insulin Released
(µU/islet per 30 min)
0
20
40
60
80
(3)
(6)
(3)
(1)
(1)
3mM Glu
28mM Glu
40mM K+
8mM Glu
+500µM CCh
8mM Glu
B
Insulin Released
(µU/islet per 30 min)
0
10
20
30
0
5
KN-62 (µM)

carbachol, a stable analogue of acetylcholine, was inhibited by 53% (n=1) relative to control in the presence of 10 μM KN-62 (Fig. 8A). Carbachol is known to promote inositol-trisphosphate (IP_3)-mediated mobilization of Ca^{2+} from intracellular stores through the activation of PLC coupled to muscarinic receptors. 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. 8B). 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.

Inhibition of K^+- and Glyceraldehyde-Induced Insulin Release by Alloxan—Alloxan, a potent diabetogenic agent, completely suppresses glucose-induced insulin secretion and concomitantly inhibits CaM kinase II activity (41). Alloxan, however, also inhibits glucokinase (61,62), an enzyme proposed to play a prominent role in glucose recognition by the β-cell (15). In order to probe for a role of CaM kinase II in insulin secretion, an assessment was made of the ability of alloxan to suppress insulin secretion induced by glyceraldehyde, whose metabolism by the β-cell is not dependent on glucokinase, and K^+, which increases intracellular Ca^{2+} and bypasses glucose metabolism. In initial experiments performed in islets, alloxan was found to dose-dependently inhibit K^+-induced insulin secretion with a half-maximal inhibitor concentration of 367.5 μM (n=1). Subsequently, at a near maximal inhibitory concentration, alloxan (562.5 μM) suppressed insulin secretion induced by glyceraldehyde and K^+ by 61% and 47%, respectively (n=1) (Fig. 9). Due to the ability of this approach to circumvent glucokinase, these data suggest that alloxan-induced suppression of glucose-induced insulin secretion is mediated at least in part by an inhibition of CaM kinase II.

FIG. 9. **Inhibition of K$^+$- and glyceraldehyde-induced insulin release by alloxan.** Intact islets (20/tube) were treated with alloxan (heavy shade) or inactivated alloxan (control) for 10 minutes prior to stimulation by either basal (3 mM) glucose, 28 mM glucose, 40 mM potassium, or 22 mM glyceraldehyde (n=1).

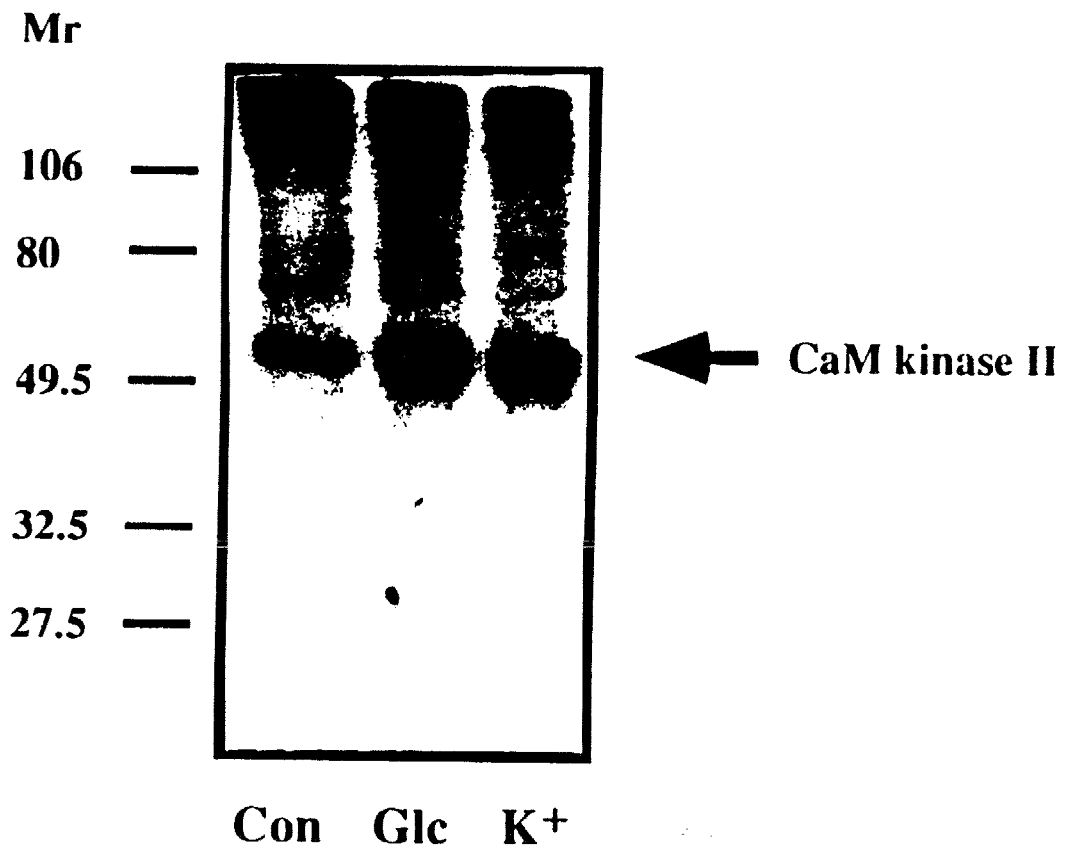

Insulin Secretion
(μUnits/islet per 30 min.)
60
50
40
30
20
10
0
: Control
: + Alloxan
3mM Glucose
28mM Glucose
40mM K+
22mM Glyceraldehyde

Induction of CaM kinase II Autophosphorylation by Glucose and K^+—An early consequence of the activation of CaM kinase II both *in vitro* and *in vivo* is the autophosphorylation of CaM kinase II. *In vitro* studies have demonstrated this to be the result of the phosphorylation of Thr 286 or Thr 287 on the α- and β-subunits, respectively (32,63-65). The phosphorylation of CaM kinase II has also been detected *in situ* in several cell systems (33,50,66,67) indicating that such a phosphorylation plays some physiological role in the regulation of the enzyme. Thus, the estimation of the phosphorylation of CaM kinase II can be used as a determinant of the activation of CaM kinase II in cells *in situ*.

Such a parameter of the activation of CaM kinase II was utilized in islets. In these studies an immunoprecipitation procedure using polyclonal anti-CaM kinase II α antibodies was employed to determine whether stimulatory concentrations of K^+ (40 mM) or glucose (28 mM) promote the increased phosphorylation of CaM kinase II. Such concentrations of K^+ have been shown to promote the depolarization of the β-cell and subsequent influx of Ca^{2+}, and as such are thought to mimic the cellular effects of glucose (23,59). It was reasoned that such an increase in intracellular concentration of Ca^{2+} should be sufficient to promote the activation of CaM kinase II and was therefore used as a positive control to which the effects of glucose could be compared. K^+ at these concentrations also promotes a profound but transient insulin secretion response (68).

Islets were prelabeled with ^{32}P-orthophosphoric acid as described in Experimental Procedures and subsequently stimulated by K^+ (40 mM, with adjustments made to maintain iso-osmotic balance) or glucose (28 mM). Control incubations using basal KRB medium (3 mM glucose, 5 mM K^+) were conducted in parallel. After stimulation for 2.5 min, the α-subunit of CaM kinase II was immunoprecipitated from islet homogenates and subjected to electrophoresis and autoradiography. As demonstrated in Fig. 10, a single

FIG. 10. **Induction of CaM kinase II autophosphorylation by glucose and K^+.** Islets (400 per tube) were prelabeled with ^{32}P-orthophosphoric acid for 90 min and then stimulated with 28 mM glucose (Glc) or 40 mM K^+ (K^+) at 37 °C. Control islets (Con) were incubated in basal KRB medium (3 mM glucose, 5 mM K+). CaM kinase II was immunoprecipitated, subjected to SDS-PAGE and autoradiography as described in Experimental Procedures. Markers to the left of the gel indicate positions of molecular weight markers. Autoradiogram is representative of 3 separate determinations.


Mr
106
80
49.5
32.5
27.5
CaM kinase II
Con
Glc
K+

phosphoprotein of Mr ~ 54000, consistent with that expected of the α-subunit of CaM kinase II, was immunoprecipitated by these procedures. Furthermore, $^{32}P_i$ incorporation into this protein was increased by stimulation of the islets with either 40 mM K^+ or 28 mM glucose as demonstrated by the increased intensity of this band by autoradiography (Fig. 10). Densitometric analyses of a series of immunoprecipitations using an Optimus gel imager are described in Table 2. Stimulatory concentrations of K^+ (40 mM) and glucose (28 mM) increased phosphate incorporation into CaM kinase II α by 175 ± 15% and 189 ± 19% respectively, suggesting that these secretagogues induce the phosphorylation of CaM kinase II.

In order to determine whether CaM kinase II phosphorylation occurred in the β-cell, similar experiments were performed using the clonal β-cell line derived from insulinoma, RINm5F. An unfortunate feature of these cells is that they do not respond to glucose within a physiological range. They do, however, secrete insulin in response to K^+ (69) and glyceraldehyde (63,70). A similar increased phosphate incorporation into CaM kinase II by stimulatory concentrations of K^+ was observed in RINm5F cells and this effect was further mimicked by glyceraldehyde (Table 2).

The autophosphorylation of CaM kinase II results in the generation of an autonomous, Ca^{2+}-independent protein kinase activity which continues to phosphorylate itself and available substrates in the absence of its cofactors. This activity can be assayed using a synthetic peptide, autocamtide-2, the sequence of which is that of the autophosphorylation site of the α-subunit of CaM kinase II (42). Therefore, a second method for the activation of CaM kinase II in islets, namely the quantification of the generation of autonomous CaM kinase II activity, was established.

Autonomous CaM kinase II activity in the β cell—Autocamtide-2 phosphorylation has been reported as being specific for CaM kinase II (28). That this is true for β-cell homogenates is demonstrated by the observation that the addition of

Table II

Effect of glucose and K^+ on autophosphorylation of CaM kinase II in islets and RINm5F cells.

Islets or RINm5F cells were prelabeled with $^{32}P_i$ and then stimulated for 2 min with either basal medium (3 mM glucose, 5 mM K^+) or medium containing 40 mM K^+, 28 mM glucose (islets) or 22 mM glyceraldehyde.(RIN cells) as indicated in the table. Relative increases in autophosphorylation were determined against basal controls as described in Experimental Procedures.

Secretagogue	Islets	RINm5F Cells
	percent stimulation over basal	
K^+	175 ± 15	178 ± 19
Glucose	189 ± 19	ND
Glyceraldehyde	ND	128 ± 9

ND not determined

activators of protein kinase A or protein kinase C (*i.e.* cAMP and TPA respectively) failed to increase phosphate incorporation into autocamtide-2 in RINm5F cell homogenates (Table 3). To establish this method as an indicator of CaM kinase II activation in islets, the ability of stimulatory concentrations of K^+ (40 mM) to generate autonomous CaM kinase II activity were studied. The effects of K^+ were studied over a time period in which K^+-induced insulin secretion occurs (0-20 min). In these experiments the autonomous activity is expressed as a proportion of Ca^{2+}-dependent activity to normalize activity to total amount of enzyme present in the homogenate. Under basal conditions (3 mM glucose, 5 mM K^+), Ca^{2+}-independent activity was assessed as 4.2 ± 1.6% of Ca^{2+}-dependent activity (see Fig. 12A). In these experiments, however, this control value varied considerably for unknown reasons but the fold stimulation was always consistent. Hence, the data in these experiments is expressed as a ratio of the percent stimulated and percent unstimulated activities (*i.e.* fold stimulation).

As illustrated in Fig. 11, K^+ (40 mM) induced a rapid and profound increase in autonomous CaM kinase II activity indicating a rapid activation of CaM kinase II. Autonomous CaM kinase II activity in K^+-stimulated islets increased rapidly relative to control, peaking at 2.5 min before declining to basal levels at 20 min. Maximal stimulation at 2.5 min was 3.0 ± 0.7 fold over control (3 mM glucose, 5 mM K^+). In contrast to its rapid appearance, increased autonomous CaM kinase II activity was prolonged and declined only slowly. This data clearly indicates that K^+ activates CaM kinase II activity and is likely due to increased Ca^{2+}-influx as the result of cell depolarization.

Induction of Autonomous CaM kinase II Activity by Glucose —The principal objective of this line of investigation was to determine whether glucose-activates CaM kinase II in isolated islets. Using conditions established in the previous experiments, the ability of glucose to promote the generation of autonomous CaM kinase II activity was

Table III

The effects of Ca^{2+}/CaM, TPA and cAMP on the phosphorylation of autocamtide-2 (AC-2) in RINm5f cell homogenates

Autocamtide-2 phosphorylation was measured in the presence of either Ca^{2+}/Cam, 1 μM TPA or 1 μM cAMP as described in Experimental Procedures.

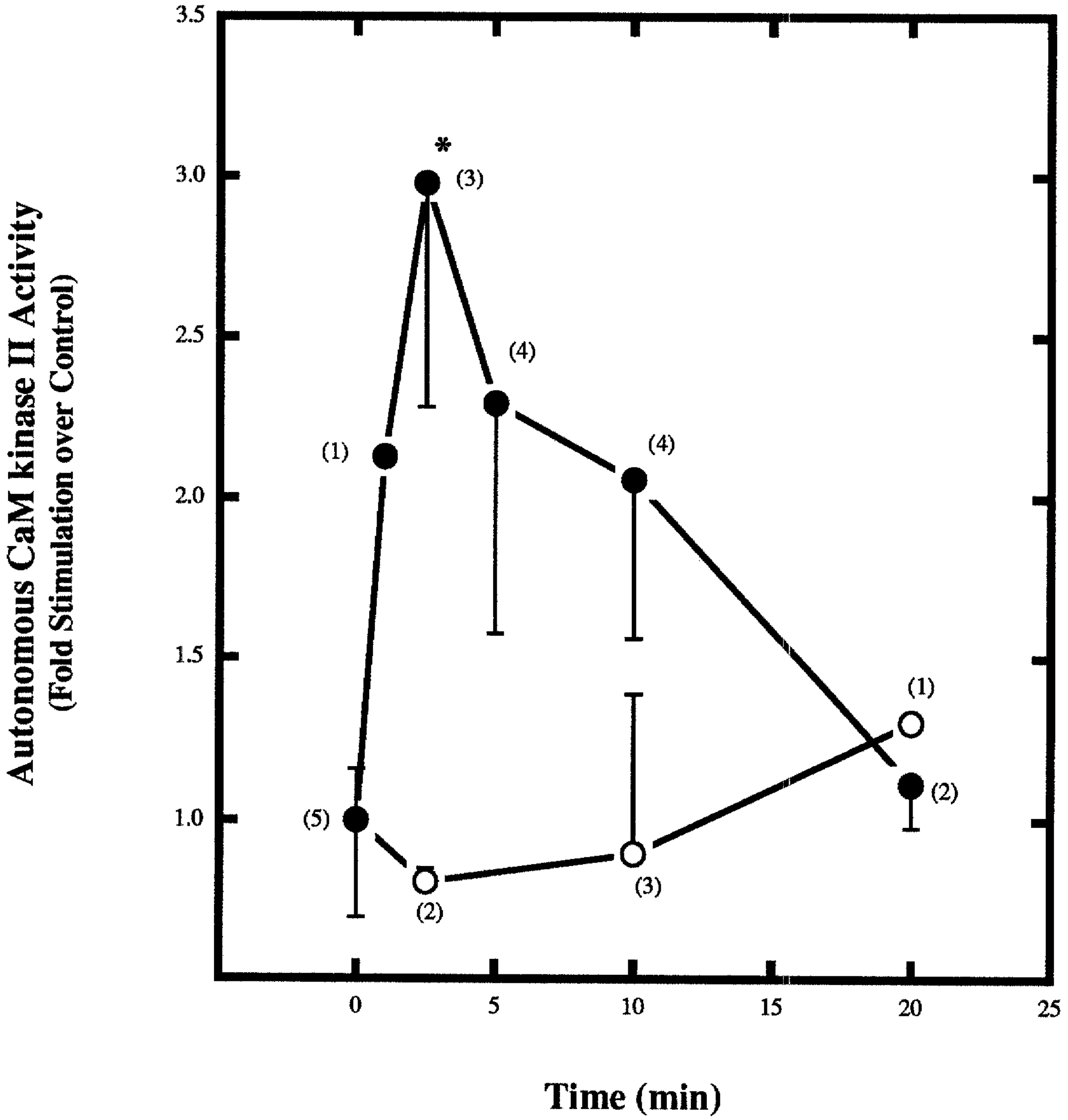
Condition	AC-2 Phosphorylation[b]	Fold Increase[c]
	pmol/min	
Control	1.2 ± 0.3	1.0 ± 0.2
Ca^{2+} /CaM	49.8 ± 1.6 [a]	70.0 ± 4.9
TPA	0.9 ± 0.3	1.5 ± 0.3
cAMP	1.2 ± 0.0	2.4 ± 0.1

[a] $p < 0.005$ vs. control

[b] Values represent the average of 3 separate determinations

[c] Values are expressed as the average fold stimulation calculated relative to each experiment's own control.

FIG. 11. **Induction of autonomous CaM kinase II activity in K^+-stimulated islets.** Islets (400 per tube) were incubated with K^+ (40 mM) for the times indicated, homogenized and CaM kinase II activity measured in the absence (autonomous activity) and presence (Ca^{2+}-dependent activity) of Ca^{2+} and calmodulin as described in Experimental Procedures. Autonomous CaM kinase II activity is expressed as a fold stimulation over control (3 mM glucose, 5 mM K^+) as described in text. ● = K^+; ○ = control. Values are means ± SEM and number in parentheses indicates number of determinations made. * $p < 0.03$.


Autonomous CaM kinase II Activity
(Fold Stimulation over Control)
3.5
3.0
2.5
2.0
1.5
1.0
*
(3)
(4)
(1)
(4)
(1)
(5)
(2)
(3)
(2)
0
5
10
15
20
25
Time (min)

assessed. Glucose at a concentration that maximally stimulates insulin secretion from isolated islets (28 mM) induced a marked activation of CaM kinase II as indicated by an increased autonomous CaM kinase II activity (Fig. 12A). In basal conditions autonomous CaM kinase II activity represented 4.2 ± 1.6% of total enzyme activity assayed in the presence of Ca^{2+} and calmodulin and this value did not vary over the time course studies (0-20 min). In a manner similar to K^+, glucose induced a rapid increase in autonomous CaM kinase II activity which peaked at 2.5 min (2.9 ± 0.2 fold induction over 3 mM glucose control) and declined slowly over the remaining times studied. In contrast to K^+, however, autonomous CaM kinase II activity was elevated over control at 20 min, although this increase was not statistically significant. Insulin secretion monitored from the same islets during this experiment is demonstrated in Fig. 12B. Cumulative insulin secretion increased steadily at early time points (1- 10 min).

To further characterize the effect of glucose to activate CaM kinase II, islets were incubated at the maximal time point (2.5 min) in medium containing increasing concentrations of the nutrient secretagogue (0 - 28 mM). The results of these experiments are demonstrated in Fig. 13A. Glucose dose-dependently stimulated the production of autonomous CaM kinase II activity. Using basal glucose as control, a threshold value of activation was observed between 8 and 11 mM glucose. By 28 mM, activation was 2.7 ± 0.2 fold of control and started to level off. This sigmoidal relationship correlated very closely with glucose-induced insulin secretion from isolated islets (Fig. 13B). Using values at 28 mM glucose as an approximation of maximal activation, the concentration of glucose required to produce a half-maximal effect was ~14-17 mM.

To further determine whether the activation of CaM kinase II by glucose was dependent upon the metabolism of the sugar, the ability of the mannoheptulose, which is known to suppress glucokinase activity, to prevent this effect was studied. In these

FIG. 12. **Time course for glucose-induced (A) autonomous CaM kinase II activity and (B) correlation with insulin secretion.** Islets (400 per tube) were incubated with glucose (28 mM, ●) or control (3 mM glucose, ○) for the times indicated. (A) Autonomous CaM kinase II activity was calculated as described in Experimental Procedures. (B) Insulin content of incubation medium from the same islets was determined by RIA. Values are means ± SEM for with numbers in parentheses indicating number of determinations. * $p < 0.004$; ** $p < 0.007$; *** $p < 0.01$.

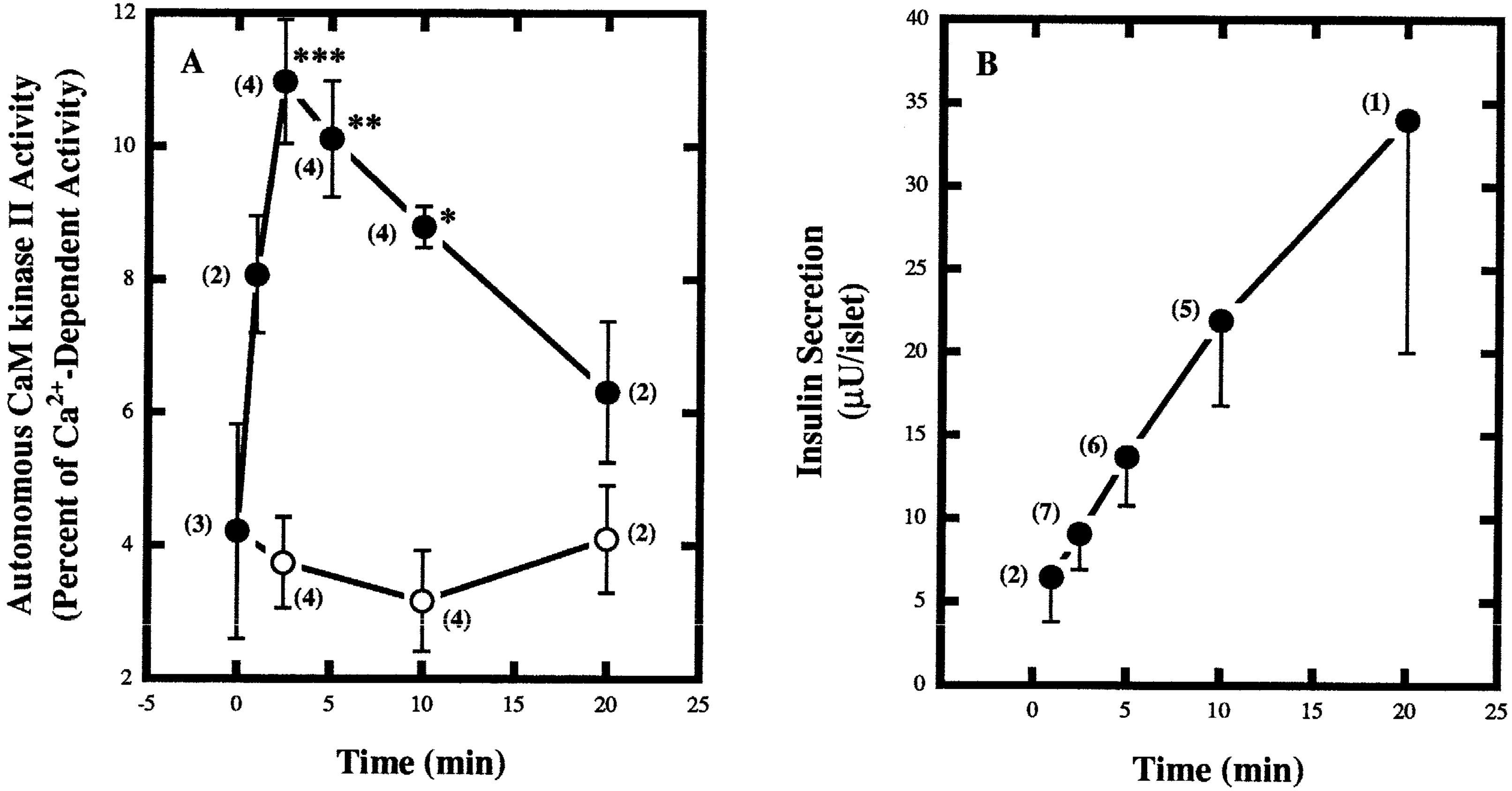
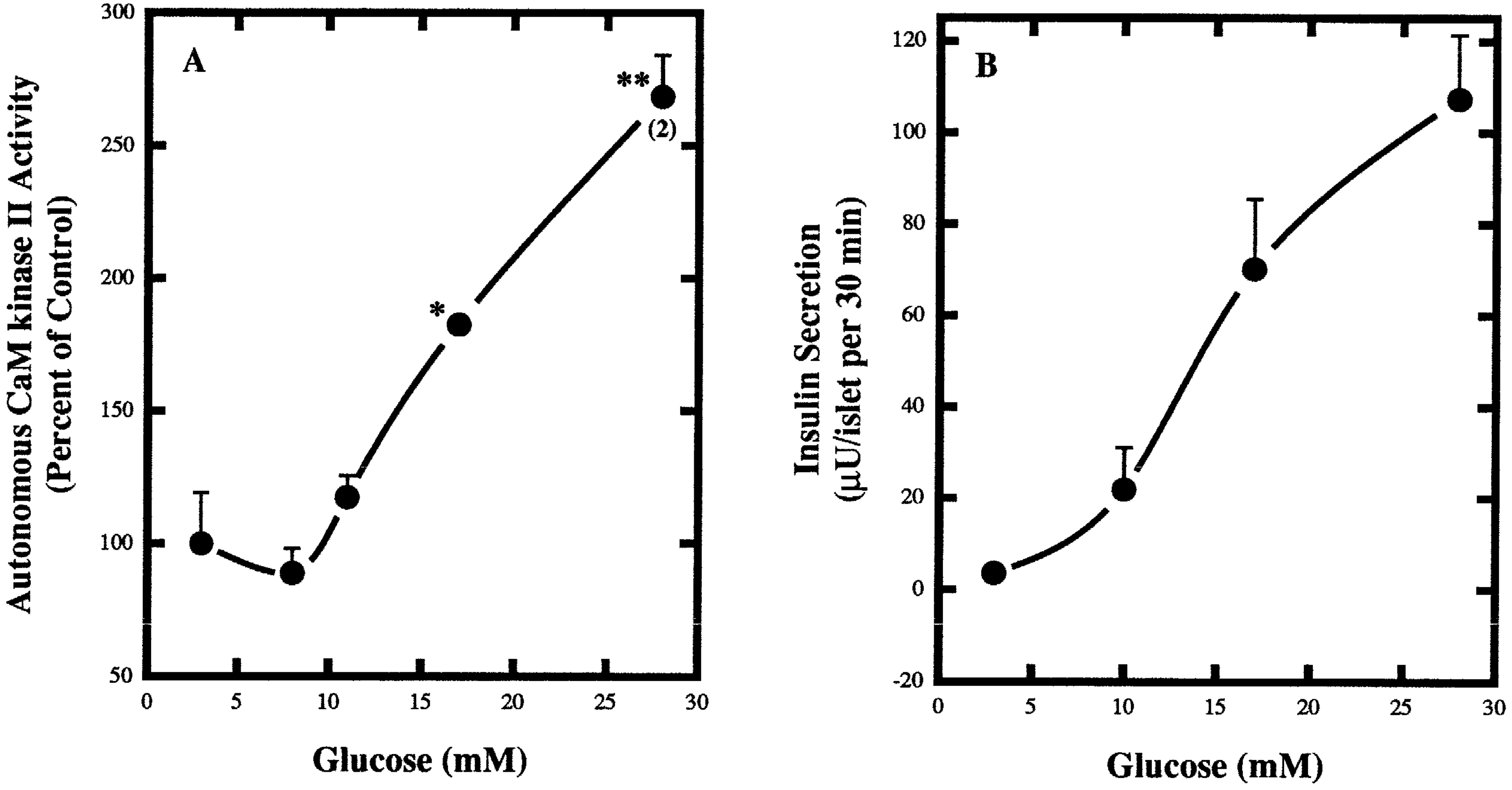

A
Autonomous CaM kinase II Activity
(Percent of Ca2+-Dependent Activity)
12
10
8
6
4
2
-5
0
5
10
15
20
25
Time (min)
(4)
***
**
(4)
(4)
*
(2)
(2)
(3)
(4)
(4)
(2)
B
Insulin Secretion
(μU/islet)
40
35
30
25
20
15
10
5
0
Time (min)
(1)
(5)
(6)
(7)
(2)

FIG. 13. **Dose-dependency of glucose in the induction of autonomous CaM kinase II activity and insulin secretion.** Islets (400 per tube) were incubated with glucose (3-28 mM) for 2.5 min at 37 °C. (A) Ca^{2+}-independent and Ca^{2+}-dependent CaM kinase II activities were determined and activation expressed as described in Experimental Procedures. (B) Islets (20 islets/tube) were incubated in the presence of glucose (3-28 mM) a described in Experimental Procedures. Results are of three determinations (n=3) except as indicated in parenthesis. * $p < 0.013$, ** $p < 0.001$.

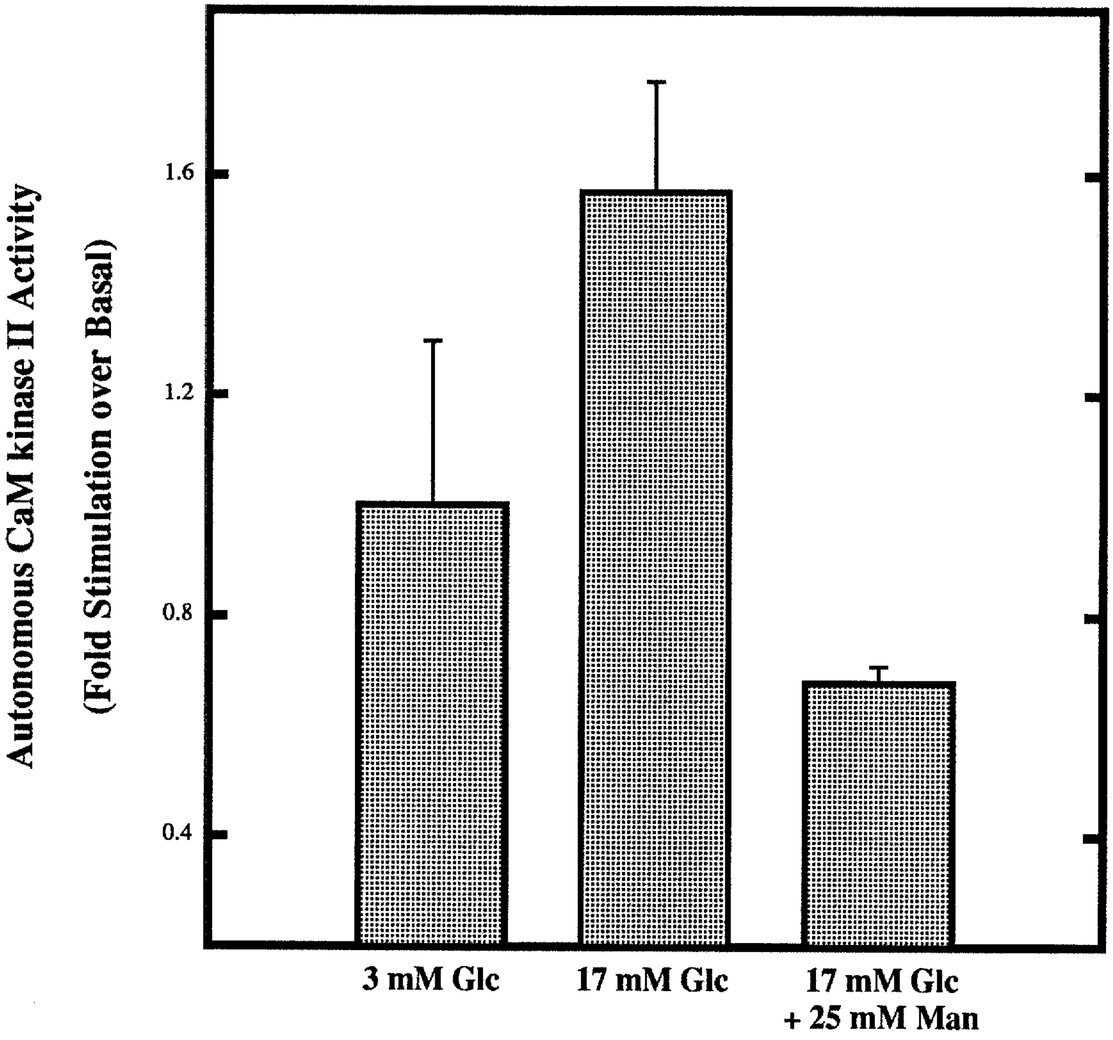

A
B
Autonomous CaM kinase II Activity
(Percent of Control)
300
250
200
150
100
50
**
(2)
*
Insulin Secretion
(µU/islet per 30 min)
120
100
80
60
40
20
0
-20
0
5
10
15
20
25
30
Glucose (mM)
Glucose (mM)

experiments, glucose (17 mM) was demonstrated to induce the 1.6 ± 0.2 fold activation of CaM kinase II determined by the appearance of autonomous kinase activity (Fig. 14). Although this was not statistically significant due to the variability of the controls in these experiments, this value does closely agree with the stimulation seen in the glucose dose-curve above (see Fig. 13A). Mannoheptulose (25 mM), when added in addition to glucose (17 mM), not only completely prevented activation of CaM kinase II, but appeared to have a depressing effect when compared to basal activity (68 ± 3% of basal), although this was not statistically significant (Fig. 14).

Inhibition of TPA/Ca^{2+}-Induced Insulin Release in Permeabilized Islets by KN-62—At this point, a paradox became apparent between data presented in this thesis and the literature. After publication of the studies examining the effects of KN-62 on insulin secretion from intact islets (71), it was observed by Li *et al.* that the inhibitor may have profound effects on β-cell Ca^{2+} channel activity in addition to its effects on CaM kinase II (72). This group similarly demonstrated a potent effect of KN-62 to suppress insulin secretion in hamster insulin tumor (HIT) cells induced by a nutrient mixture (10 mM glucose, 5 mM leucine, and 5 mM glutamine). However, Li *et al.* further demonstrated that these effects of KN-62 could be mimicked by both a calmodulin antagonist known to suppress Ca^{2+} channel activity in the β-cell and an L-type Ca^{2+} channel blocker (72). This group went on to show that KN-62 failed to inhibit Ca^{2+}-induced insulin secretion from permeabilized HIT cells, from which they concluded that CaM kinase II plays no role in insulin secretion. I have confirmed that KN-62 only has a modest effect on Ca^{2+}-induced insulin secretion from perifused electro-permeabilized islets (data not shown), eliminating a possible difference between the effect of KN-62 in clonal and primary β-cells.

It is noted, however, that fundamental differences exist between insulin secretion induced by Ca^{2+} and glucose in permeabilized and intact cells respectively. Ca^{2+}-

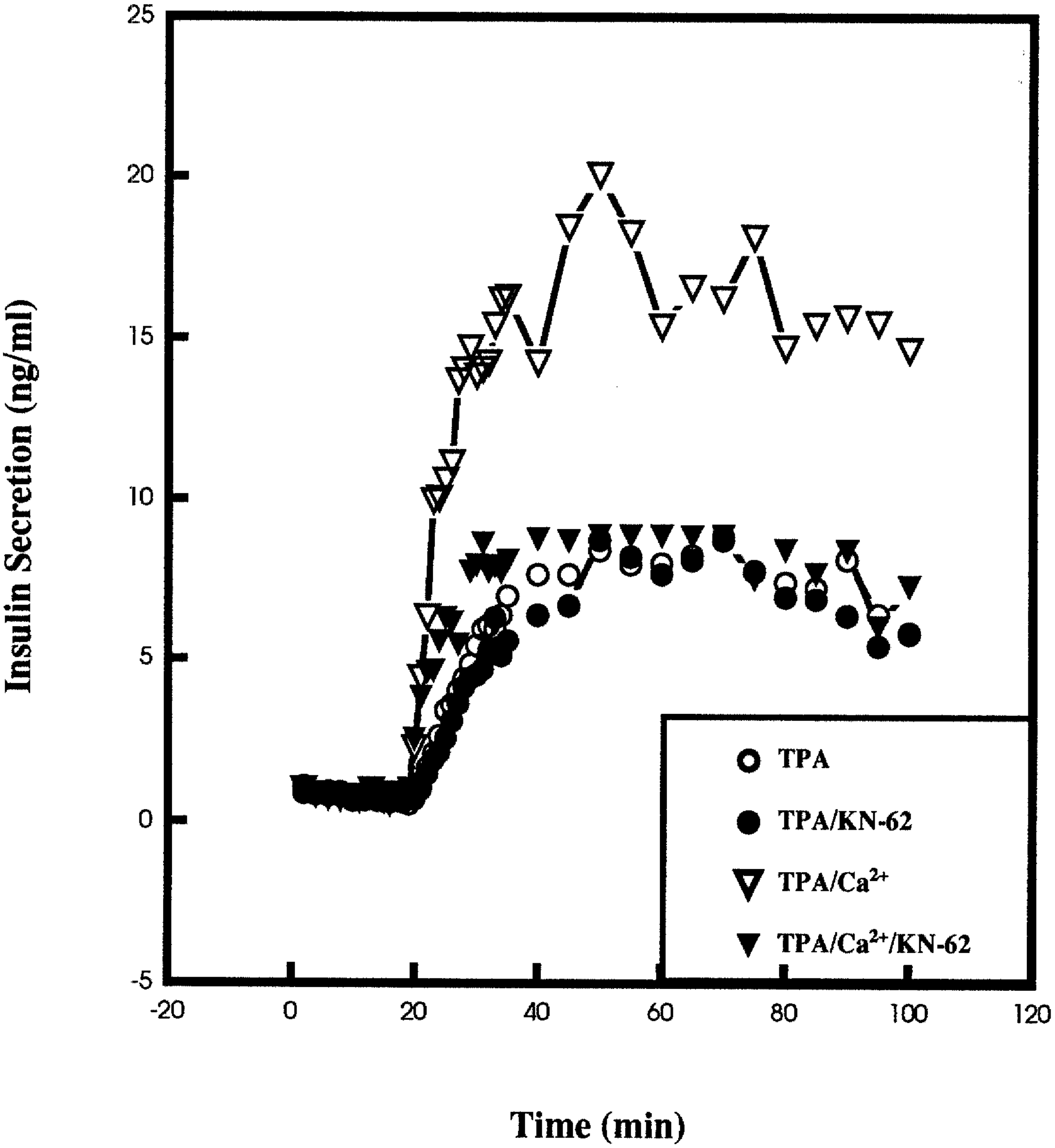
FIG. 14. **Inhibition of glucose-induced CaM kinase II activity by mannoheptulose.** Islets (400/tube) were preincubated for 10 min in basal (3 mM glucose) KRB medium and then incubated for 2.5 min in medium that contained either 3 mM glucose, 17 mM glucose, or 17 mM glucose and 25 mM mannoheptulose combined.


Autonomous CaM kinase II Activity
(Fold Stimulation over Basal)
1.6
1.2
0.8
0.4
3 mM Glc
17 mM Glc
17 mM Glc
+ 25 mM Man

induced secretion is modest compared to glucose and is transient such that it mimics only the first phase of secretion induced by glucose in intact islets. Biphasic secretion is recovered by the addition of activators of PKC and PKA. In light of the autonomous activity as a result of enzyme activation and its proposed role in prolonging the effect of Ca^{2+} transients, it was reasoned that the activation of CaM kinase II may be important in the mediation of second phase secretion. To test this hypothesis, the effect of KN-62 on insulin secretion induced by a combination of TPA and Ca^{2+} in permeabilized islet was examined. Islets were permeabilized as described in Experimental Procedures, then perifused with medium containing TPA (500 nM) or TPA and Ca^{2+} (12 μM) in the absence or presence of KN-62 (1 μM). For those islets exposed to inhibitor, KN-62 was introduced 5 minutes prior to stimulation. KN-62 completely suppressed insulin secretion induced by TPA and Ca^{2+} to the level of TPA alone (Fig. 15, n=1). Moreover, KN-62 had no effect on insulin secretion induced by Ca^{2+} (data not shown) or TPA alone (Fig. 15).

KN-93: Effects on Ca^{2+} Influx and Insulin Release—Due to the possible effects of KN-62 on Ca^{2+} channel activity in the β-cell, the effects of another inhibitor, KN-93 (2 -[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl) -N-methylbenzylamine) on insulin secretion were investigated in the intact islet. KN-93 effect on CaM kinase II has previously been investigated both *in vitro* and *in situ* in PC12h cells (73). By competing for the calmodulin binding site, it was shown to have a potent inhibitory effect on CaM kinase II phosphorylating activity (inhibitory constant of 0.37 μM) and autophosphorylation of both the α- and β-subunits of CaM kinase II without any significant effect on the catalytic activities of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), myosin light chain kinase (MLCK), and Ca^{2+}/calmodulin dependent phosphodiesterase (Ca^{2+}-PDE) (73).

FIG. 15. **Inhibition of Ca^{2+}/TPA- induced insulin release by KN-62 in electropermeabilized islets.** Islets were permeabilized by electroporation and perifused as described in Experimental Procedures. Islets were perifused in permeabilization buffer containing no Ca^{2+} for 10 minutes and then buffer containing either KN-62 (1 μM) or vehicle for 5 minutes. New buffer containing TPA (500 nM) or TPA and Ca^{2+} (12 μM) and either inhibitor or vehicle was subsequently introduced as indicated in the legend (n=1).


Insulin Secretion (ng/ml)
25
20
15
10
5
0
-5
-20
0
20
40
60
80
100
120
Time (min)
○ TPA
● TPA/KN-62
▽ TPA/Ca²⁺
▼ TPA/Ca²⁺/KN-62

In order to ascertain whether KN-93 shared a similar property to KN-62 to suppress Ca^{2+} channel activity, the effect of KN-93 to influence secretagogue-induced Ca^{2+} flux into RINm5F cells was assessed by fluorescent procedures employing the Ca^{2+}-selective fluoroprobe, fura-2/AM. Both K^+ and glyceraldehyde were shown to promote marked increases in intracellular Ca^{2+} as indicated by an increase in the ratio of the 380 nm and 340 nm excitation wavelengths (Figs. 17 and 18 respectively). K^+ elicited its classical rapid and transient Ca^{2+} spike (Fig. 16A), whereas the influx in response to glyceraldehyde was more prolonged (Fig. 17A). KN-93, however, failed to have any significant effect on Ca2+-influx induced by either of these agonists (Figs. 17B and 18B).

This observation presents the possibility of the use of the inhibitor to probe for the role of CaM kinase II in insulin release without effecting Ca^{2+} channels. Using a static secretion model as described in Experimental Procedures the effects of KN-93 were examined on insulin secretion induced by K^+ and glucose in intact islets. In a static incubation, KN-93 inhibited insulin secretion induced by K^+ by 73% and glucose by 77% (Fig. 18).

Fig. 16. **Effect of KN-93 on Ca^{2+} influx induced by K^+ in RINm5F Cells.** RINm5F cells were incubated in HEPES buffer containing 5 μM fura-2 for 45 minutes and then exposed to buffer with 40 mM KCl in the (A) absence or (B) presence of 75 μM KN-93 as described in Experimental Procedures.

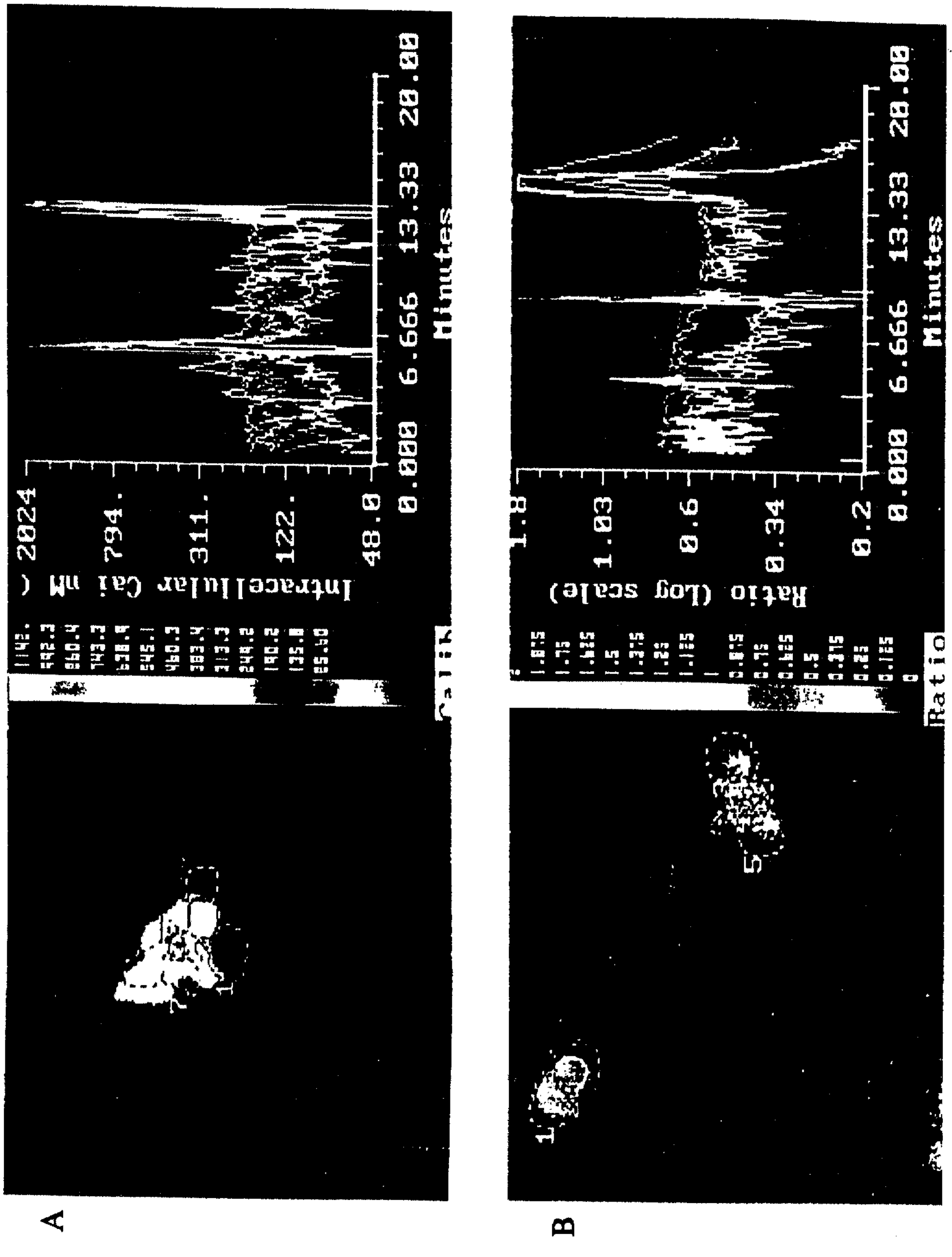
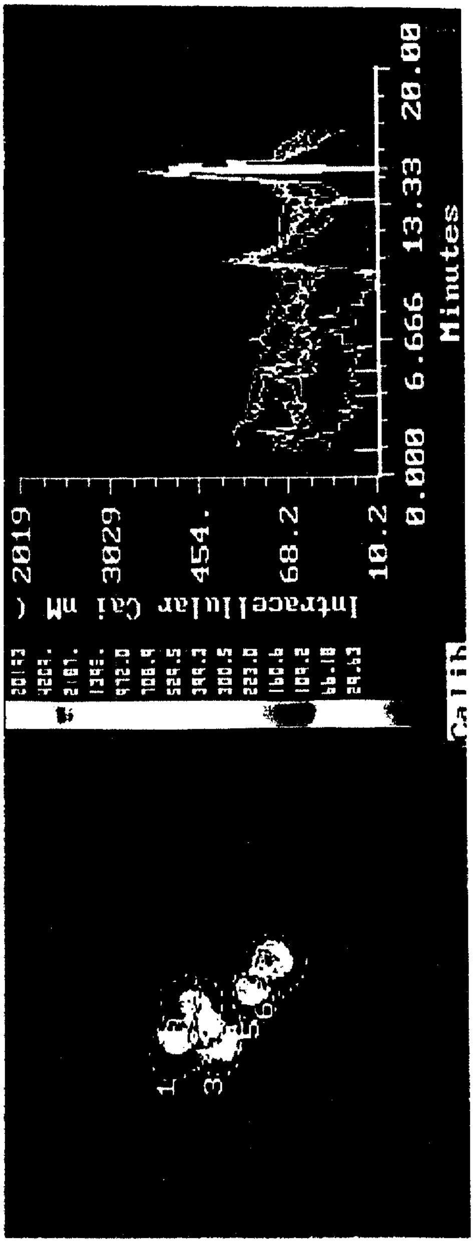

A
B
Calib
Intracellular Cai nM (
2024
794.
311.
122.
48.0
0.000
6.666
13.33
20.00
Minutes
Ratio
Ratio (Log scale)
1.8
1.03
0.6
0.34
0.2
1
5

FIG. 17. **Effect of KN-93 on Ca^{2+} influx induced by glyceraldehyde in RINm5F Cells.** RINm5F cells were incubated in HEPES buffer containing 5 μM fura-2 for 45 minutes and then exposed to buffer with 22 mM glyceraldehyde in the **(A)** absence or **(B)** presence of 75 μM KN-93 as described in Experimental Procedures.

A


1
3
5
6
Calib
Intracellular Cai nM (
2019
3029
454.
68.2
10.2
0.000
6.666
13.33
20.00
Minutes

B

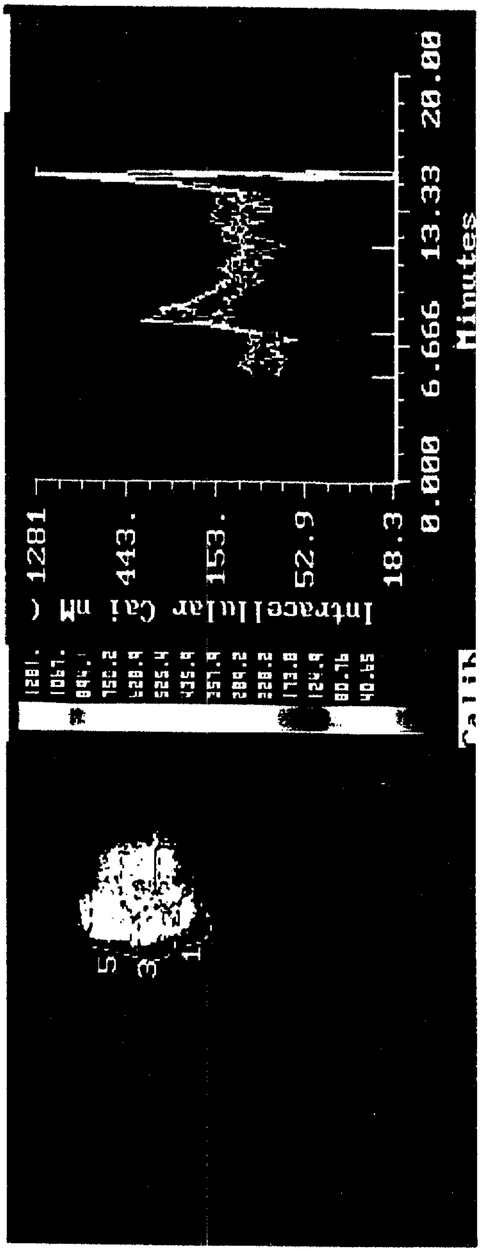
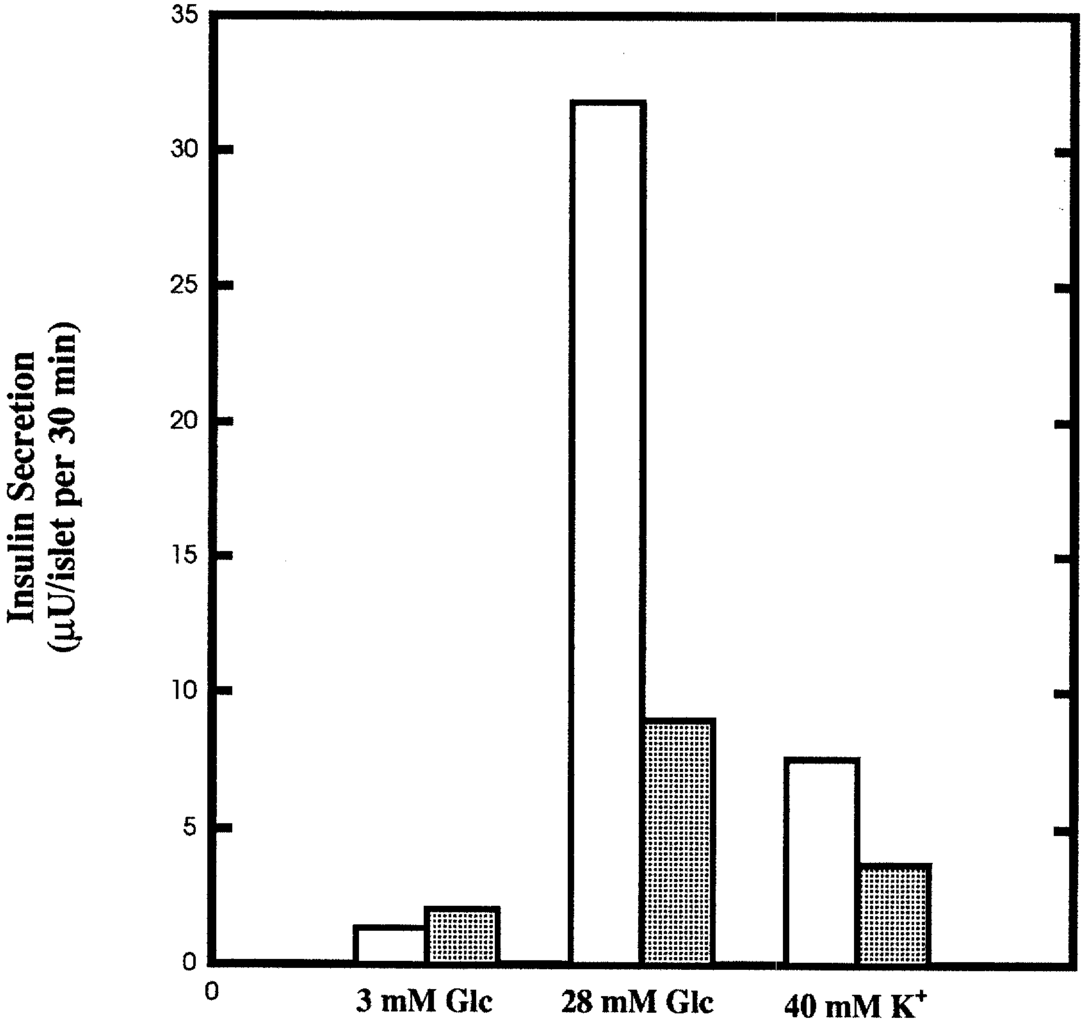

1
3
5
Calib
Intracellular Cai nM (
1281
443.
153.
52.9
18.3
0.000
6.666
13.33
20.00
Minutes

FIG. 18. **Effect of KN-93 on insulin secretion induced by glucose and K+ in islets.** Intact Islets (20 per tube) were incubated with either basal (3 mM glucose, 5 mM KCl), 28 mM Glucose or 40 mM KCl KRB medium in the absence or presence of 75 μM KN-93 (n=1).


Insulin Secretion
(μU/islet per 30 min)
35
30
25
20
15
10
5
0
0
3 mM Glc
28 mM Glc
40 mM K+

DISCUSSION

There is a general consensus concerning the early steps of insulin secretion in the β-cell. For example, it seems clear that glucose metabolism is necessary for the normal physiological release of insulin (74,75) and that this occurs dependently and primarily on the elevation of intracellular calcium via calcium influx (16). In contrast to these early steps, the link(s) between calcium and insulin secretion remains unclear. Since the initial observations of the parallel induction of insulin secretion and protein phosphorylation (27,76), the search has begun to identify possible protein kinases that may, as in other cells, coordinate a secretory response. Among these proteins in the β-cell are protein kinase C, glucokinase, myosin light chain kinase, protein kinase A, and the multifunctional Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II). Until the investigations in this thesis, there was little direct support for the involvement of CaM kinase II in the β-cell insulin producing machinery. Most data reported provided circumstantial evidence based on observed properties in the β-cell reminiscent of CaM kinase II. In fact, prior to the studies presented here, the β-cell had not been definitively proven to contain this enzyme. Proof of this was provided by immunoprecipitation which confirms the presence of the kinase in these cells. As a result of the recent availability of new techniques, antibodies and putative selective inhibitors for the enzyme, it has become possible to make a better evaluation.

The development of KN-62 appeared to promise a specific pharmacological means to probe for the role of CaM kinase II in the insulin secretion process (60,77). The reported selectivity of KN-62 was initially confirmed for the β-cell in experiments which demonstrated that KN-62 potently inhibited β-cell CaM kinase II 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 CaM kinase II activity. Inhibition of β-cell CaM kinase II was achieved within the concentration range effective to inhibit brain CaM kinase II (14). Confirmation of inhibitory capacity in β-cells was important because of the previous demonstration of tissue-specific distribution of CaM kinase II isozymes, which raised the possibility that susceptibility to inhibition by KN-62 could be different for the non-neuronal isozymes of CaM kinase II (22). Inhibition of CaM kinase II 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 CaM kinase II 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.

One should always use a degree of skeptical caution when interpreting results of experiments examining the effect of pharmacological compounds. This is especially true in the case of protein kinases, since many known inhibitors interact at the ATP-binding site. Nevertheless, the emergence and availability of an effective pharmacological inhibitor possessing specificity for an important enzyme has proven invaluable in the study of many cellular processes. However, inherent in the use of such pharmacological agents in biological systems are the inevitable non-specific perturbations of cellular processes other than the desired target. The utility of these agents is enhanced by knowledge of their biochemical mechanism, specificity, cellular toxicity, and capacity to reach the intended objective (*i.e.* membrane permeability).

Shortly after these initial investigations, Li *et al.* published data which casted serious doubt on the legitimacy of interpreting the early findings to suggest a correlation of KN-62 inhibition of insulin secretion with CaM kinase II activity (72). It was initially reasoned that some differences may exist between clonal and primary β-cells. Also there was concern that since this group only measured the first 5 minutes of insulin release, time had not elapsed sufficiently to allow the appearance of CaM kinase II effects. However, my demonstration that KN-62 only modestly effected Ca^{2+}-induced insulin secretion from perifused electro-permeabilized islets eliminated such possibilities.

Nevertheless, it would by extremely hasty to conclude from the above observations that CaM kinase II was not involved in insulin secretion. As was noted in the results, the dynamics of Ca^{2+}-induced insulin secretion from permeabilized islets are markedly different, being monophasic and reflecting first phase insulin secretion only (55,78). Thus other possibilities exist. First, the permeabilization process may disrupt the mechanics of the secretion process. Indeed, one group using digitonin permeabilized islets to measure Ca^{2+}/calmodulin-dependent protein kinase activity found that addition of Ca^{2+} alone to the buffer did not augment protein phosphorylation, but required the combined addition of Ca^{2+} and exogenous calmodulin (79). In fact, the addition of calmodulin to the permeabilized islets without the presence of exogenously added Ca^{2+}, stimulated insulin release. This group suggested that this was due to alterations of soluble-calmodulin concentrations, perhaps rendering it inaccessible to the kinase. Using electrical discharge, Pace *et al.* reported that islets permeabilized in this manner may, after a period of time, become depleted in ATP (80). Secondly, the monophasic nature of Ca^{2+}-induced insulin release indicates that other factors, in addition to Ca^{2+}, are required for complete biphasic secretion. This is likely to be a glucose metabolite and thus is consistent with current models of glucose-induced insulin secretion (81). Therefore, the possibility exists that the enzyme might have a less evident role in this process.

Striking evidence supporting the proposition that CaM kinase II is involved in the insulin machinery of the β-cell came from the clear demonstration of the autophosphorylation of CaM kinase II in islets stimulated with glucose and depolarizing concentrations of potassium and a positive correlation of the appearance of a Ca^{2+}-

independent enzyme activity with insulin secretion. Induction of the enzyme by such secretagogues is significant evidence for the activation of this enzyme by the same signals which elicit hormone secretion. CaM kinase II autophosphorylation induced by both glucose and potassium, as indicated by densitometry of autoradiographs, was very similar, implying that the mechanism involved in the kinase stimulation is similar for both secretagogues.

The definitive demonstration that the phosphorylation of CaM kinase II was the result of autophosphorylation would require identification, by phosphopeptide analysis, of the phosphoryaltion site as the autonomy site, *i.e.* Thr 286. This analysis was not attempted because of the low signal generated in the studies provided here and low amount of material provided by the islets. The production of a Ca^{2+}-independent activity is the hallmark of CaM kinase II autophosphorylation and had previously been used as a method to assay for the activation of CaM kinase II *in situ* in several cell systems. Depolarization in the presence of extracellular Ca^{2+} significantly increases Ca^{2+}-independent activity in intact synaptosomes (66), PC12 cells (50), GH3 cells (33), and acute hippocampal slices (82). From 15% to 50% of CaM kinase II can be converted in these cells to the autonomous state from a basal state in which less than 6% of the molecules were autonomous (29). In three of the above cell systems this activation was confirmed to be associated with the enhanced labeling of Thr 286 (33,50,66).

The assay using a peptide substrate representing the autoregulatory region in which the autophosphorylation occurs allowed a more accurate quantitative evaluation of the kinase activity. This further permitted a more convenient method for the correlation of enzyme activation with β-cell function, *i.e.* insulin secretion. CaM kinase II activation induced by glucose and potassium were essentially identical and the fold stimulation of each very similar. Furthermore, the time course of the generation of autonomous activity for both peaked at 2.5 min before declining at a steady rate. These observations further suggest that the mechanisms mediating the activation of CaM kinase II by each secretagogue is similar and is consistent with the proposed mechanism of glucose-induced insulin secretion involving cell depolarization by the closing of K^+ ATP-channels. The significance of the residual activity at 20 min in the case of glucose is not clear, but suggests that the activation of the enzyme by glucose is maintained longer. It is likely therefore that glucose-induced activation of CaM kinase II is the direct result of the opening of Ca^{2+} channels mediated by cell depolarization via the closing of K^+ ATP-channels. The demonstration that glucose-induced activation of CaM kinase II is inhibited by mannoheptulose indicates that this phenomenon is absolutely dependent on metabolism of the hexose, and therefore support a specific effect of glucose. Furthermore, since insulin secretion induced by glucose is similarly obliterated by co-incubation with mannoheptulose (66,83-85), the mechanisms involved in secretion and activation of CaM kinase II appear to overlap.

Quantification of the extent of CaM kinase II activation and insulin secretion induced by glucose were very similar. Both insulin secretion and enzyme activation were sigmoidally related with a threshold between 8 and 11 mM glucose and a leveling off at approximately 28 mM glucose. This close correlation argues for a role of CaM kinase II in the regulation of insulin secretion. The kinetics of insulin secretion induced by glucose and K^+ are however quite distinct. K^+ induces an initial burst of insulin secretion that reflects the first phase of glucose-induced insulin secretion (68,86). Secretion further correlates with concentrations of intracellular Ca^{2+} induced by K^+ (86). The insulin secretion response of K^+ is, however, transient and there does not appear to be any significant amount of insulin secretion corresponding to the second phase of glucose-induced secretion.

At this point a paradox becomes apparent between the literature and results described here. This enzyme was demonstrated not to participate in Ca^{2+}-stimulated secretion from permeabilized β-cells. In sharp contrast to this, the kinase is clearly activated by the primary physiological stimulator of insulin secretion, glucose, and does so in dose-dependent manner. Further evaluation of the kinase's role in this process is therefore necessary.

In light of the information about the possible nonspecific effects of KN-62, the effects of other agents with potent inhibitory effects on CaM kinase II were investigated. One of these, alloxan, was known to inhibit glucose-induced insulin secretion (41,84). Early studies reported that alloxan also inactivated an islet Ca^{2+}- and calmodulin -dependent protein kinase (41) in a way similar to brain CaM kinase II. This was primarily based on the ability of alloxan, when injected into live rats, to selectively inhibit Ca^{2+}- and calmodulin-dependent phosphorylation of two islet proteins of Mr ~ 54,000 and 57,000 without any effect on similar Ca^{2+}- and calmodulin-dependent activity in brain which had been identified as CaM kinase II (41). This was concluded to reflect the ability of alloxan to reach the intracellular target (β-cell CaM kinase II) rather than the existence of unique, non-susceptible forms of the kinase since alloxan was demonstrated to inhibit Ca^{2+}- and calmodulin-dependent protein kinase activity in cell free preparations of cerebral brain tissue and mammary acini (41). Evidence indicating this activity in islets as CaM kinase II led to investigations which indicated that the molecular basis for alloxan CaM kinase II inactivation is through the alkylation of a cysteine residue in its active site (48). Therefore, alloxan is now known to have inhibitory effects on CaM kinase II in parallel with its effects on glucose-induced insulin secretion. Interpretation of this data is now complicated by the demonstration that alloxan also potently inhibits glucokinase (61,62,84), considered to be the recognition enzyme for glucose in the β-cell (15).

However, the alloxan inhibition of insulin secretion induced by glyceraldehyde cannot be explained by its effects upon glucokinase since glyceraldehyde enters glycolysis at a point distal in the pathway to the phosphorylation of glucose. The same is true and is confirmed by the alloxan inhibition of K^+-induced insulin release, since K^+ should bypass glycolysis completely by directly increasing intracellular Ca^{2+} through cell depolarization. It is interesting to note that both the induction of insulin secretion as well as the percent of alloxan inhibition of the secretion was greater in the case of glyceraldehyde than for K^+. It would appear that factors resulting from the metabolism of glyceraldehyde are capable of potentiating the secretion response. It is possible that these same factors are also able to work in a manner that is synergistic with CaM kinase II activity.

KN-93 also inhibited glucose- and K^+-induced insulin secretion, albeit at a higher concentration than did KN-62 (75 μM vs. 10 μM). KN-93 had previously been shown to be selective for CaM kinase II *in vitro* without any effects on PKA, MLCK, PKC or Ca^{2+}-PDE (73). In contrast to the reported effects KN-62 might have to inhibit Ca^{2+}-channel activity, experiments using fura-2/AM indicated that KN-93 had no effect on increased intracellular Ca^{2+} concentration induced by either K^+ or glyceraldehyde in clonal β-cells. The reason KN-93 inhibits K^+-induced secretion from intact islets while KN-62 fails to inhibit Ca^{2+}-induced secretion from permeabilized cells is not known. One explanation, however, may be the alteration of the insulin secretory machinery by the permeabilization process itself. Nevertheless, it appears that KN-93, unlike KN-62, has a low affinity for the Ca^{2+}-channel and may represent a useful tool to probe for the role of CaM kinase II in β-cell function.

Perhaps the most intriguing result is the inhibition of insulin secretion by KN-62 from permeabilized islets induced by the combination of TPA and Ca^{2+}. KN-62 had only a modest effect on Ca^{2+}-induced secretion from these cells and no noticeable effect on TPA- induced secretion. Introduction of KN-62 inhibited TPA/Ca^{2+}-induced secretion near to the level of TPA alone. A plausible explanation for this would be the existence of cross-talk between the Ca^{2+}/calmodulin-dependent and PKC pathways.

Cross-talk between intracellular signaling systems is recognized as one of the major means for cells to regulate and orchestrate a variety of responses to differing stimuli. Evidence for bi-directional cross-talk already exists in the β-cell suggesting synergistic effects on the ATP-sensitive K^+ channel by glucose metabolism and PKA (87). Recent evidence suggests that glucose-induced insulin secretion may be controlled by the convergent and interdependent regulatory pathways involving changes in intracellular Ca^{2+}, cAMP and DAG (13). It is quite possible for such a relationship to exist between the Ca^{2+}-and PKC pathways in the β-cell with CaM kinase II providing a cellular link. Such a relationship might help explain many recently published observations. For example, Zawalich and coworkers have suggested that the Ca^{2+}-calmodulin dependent pathway was involved in the initiation of the secretory response, while PKC was responsible for the maintenance of secretion (88,89). This group showed that the concomitant use of the Ca^{2+} ionophore, A23187, and TPA was able to evoke a biphasic insulin response (88). It is also known that muscarinic receptor agonists that activate PKC are dependent upon the presence of glucose to stimulate insulin release (57). It has been further demonstrated that glucose can cause the activation of PLC (90-93), possibly through the intermediate action of its metabolite glyceraldehyde-3-phosphate. Furthermore, the muscarinic receptor agonist carbachol, which potently activates PIP_2 hydrolysis by PLC, has been shown to markedly increase glucose-induced insulin secretion (92). The effect of the combination of these two secretagogues was greater than additive which may indicate that PLC activation potentiates the effects of glucose stimulation by sensitizing the β-cell, thus shifting the insulin response curve. Alternatively, this effect may indicate a synergistic cross-talk between the two pathways.

Evidence for both negative and positive cross-talk between CaM kinase II and PKC has already been demonstrated in PC12 cells (51). Negative cross-talk was achieved by inhibiting the mobilization of intracellular Ca^{2+} stores. This was the result of inhibiting Ca^{2+} influx through voltage-sensitive Ca^{2+}-channels and by attenuating CaM kinase II stimulation during the simultaneous activation of the kinase and PKC by bradykinin-induced PLC activation. This group further reported that PKC also exhibited positive cross-talk with CaM kinase II. Concurrent activation of PKC by TPA allowed the potentiation of CaM kinase II activity when sub-maximally activated by ionomycin. This TPA treatment was found to increase the level of free cytosolic calmodulin and it was hypothesized that this enhanced activation of CaM kinase II by PKC may result from PKC-mediated phosphorylation of calmodulin-binding proteins, such as neuromodulin and MARCKS. These latter two proteins modulate the pool of free soluble calmodulin in the cell and phosphorylation of these can result in the subsequent increase in availability of previously bound calmodulin (94,95). Such a situation is possible in the β-cell since the presence of the MARCKS protein has been demonstrated as a substrate for PKC in these cells (57,96).

A model for the various interactions of these signal pathways has been forwarded by Rasmussen and associates (89). In this model, neurohumoral signals prime the β-cell for secretion, although if in sufficient quantity and in combination they can initiate insulin release by themselves. Elevation of plasma glucose to threshold levels provides the missing signal(s) necessary for the activation of two temporally and spatially distinct branches of a Ca^{2+}-messenger system to bring about the phosphorylation of two sets of proteins which are linked to two different phases of secretion. Glucose alone, however, at high enough concentrations can supply the missing factors normally provided by the neurohumoral potentiators. From a physiological standpoint, cross-talk in the β-cell makes sense and has enormous survival value. It may provide a mechanism to prevent inappropriate insulin secretion when blood glucose is low.

An alternative (or additional) role for CaM kinase II in the β-cell insulin producing machinery is the regulation of insulin gene expression. Insulin biosynthesis is an integral component of insulin secretion and is also dependent on glucose metabolism. (97). The magnitudes of both hormone release and biosynthesis are directly proportional to the sugar's intracellular metabolic rate (98). After β-cell stimulation by glucose, insulin biosynthesis increases 5 to 10 fold within a few minutes (98) which, although the significance of which is not clear, corresponds closely with the activation of CaM kinase II. It is noted that the β-cell releases only a small portion of its intracellular store of insulin under even maximal stimulation, however, these stores may be compartmentalized since newly synthesized insulin is preferentially secreted (98). Regulation of insulin biosynthesis is acted upon by glucose at both the level of transcription and translation (97,99), though the primary point of control appears to be in the amount of steady state mRNA through modulation of the rate of gene transcription and transcript stability (100,101). The mechanisms governing this are not well defined, but increased transcription is likely the result of the combination of a number of positive and negative regulatory factors binding at *cis*-acting elements in the 5'-flanking region of the insulin gene (99). It is of interest that the same cellular mediators are often implicated in both insulin biosynthesis and secretion.

Recent studies provide evidence that Ca^{2+} may be an important major regulator of insulin gene expression (28,102-104). Ca^{2+} channel blockers are shown to inhibit glucose-stimulated expression of a chimeric gene construct containing a portion of the rat insulin I promoter (-410 to +1) in neonatal islets (105) or insulin gene transcription in clonal βTC3 cells (106). Ca^{2+} has also been reported as a cellular signal for the induction of gene expression in other cell systems (*i.e.* GH3 and PC12 cells) (103,107-110).

The emergence of a model for CaM kinase II regulation of expression has already begun. Following over-expression of CaM kinase II in G/C rat pituitary cells , prolactin gene transcription was similarly increased (102). CaM kinase II may mediate such events through the phosphorylation of the known transcription factor factors, cAMP response element binding protein (CREB) (109), and CCAAT/enhancer binding protein (C/EBPβ) (103). Dash *et al.* demonstrated that purified bovine CREB is a substrate for both PKA and CaM kinase II (111). Moreover, transcription assays using a CREB-containing *c-fos* promoter indicated that phosphorylation of CREB by CaM kinase II increased gene transcription (109). Furthermore, CaM kinase II mediated prolactin gene expression by a mechanism distinct from PKA activation (103).

Such mechanisms may be utilized in the β-cell to induce insulin expression. Indeed, consensus sequences for the binding of CREB and C/EBPβ are conserved within a portion of the 5'-flanking region of the rat and human insulin genes (99,112) and mutation of the latter is shown to suppress gene expression (113,114). A further study has provided evidence that gene transcription in cultured β-cells (HIT) is dependent on Ca^{2+} and may be stimulated by CaM kinase II phosphorylation of CREB (115). Although it should be mentioned that this last study did not consider the possible effects of KN-62 on the channel activity. That Evidence supporting such a role of CaM kinase II in insulin secretion has been shown by a co-worker in preliminary experiments that indicate inhibition of insulin expression in βTC3 cells by KN-93[2].

The precise role of CaM kinase II in β-cell function is not yet clear. Evidence here strongly argues that CaM kinase II is involved in the β-cell insulin secreting apparatus. A theoretical representation of some of the ideas presented in this thesis is displayed in Fig. 19. It is my opinion that purely coincidental activation of the kinase by physiological secretagogues, in a manner that so closely correlates with the parameters of the insulin secretory pathway, is very unlikely. Rather, I propose that CaM kinase II plays a functional role in this process; the exact nature of which is unknown. Clues are provided by the inhibitor studies, but future investigations identifying the endogenous cellular substrates should offer a clearer picture into the identification of its role. Other studies of interest include: (1) examining the effects of activators of PKA (*e.g.* glucagon like peptide-1, forskolin, and cAMP) and PKC (*e.g.* carbachol, TPA, and OAG) to potentiate both the extent of CaM kinase II activation and insulin secretion; (2) the use of KN-93 with intact islets stimulated by various agonists in a perifusion model; (3) evaluation of ability of alloxan, when incubated with islets in the presence of agents causing rises in intracellular Ca^{2+} (*i.e.* K^+), to inhibit CaM kinase II activity in a manner that correlates quantitatively with insulin release; (4) examine the effects of over-expressed or truncated (constitutively active) CaM kinase II on insulin secretion and insulin gene expression; and (5) immunohistochemical staining of β-cells to identify the subcellular distribution of CaM kinase II.

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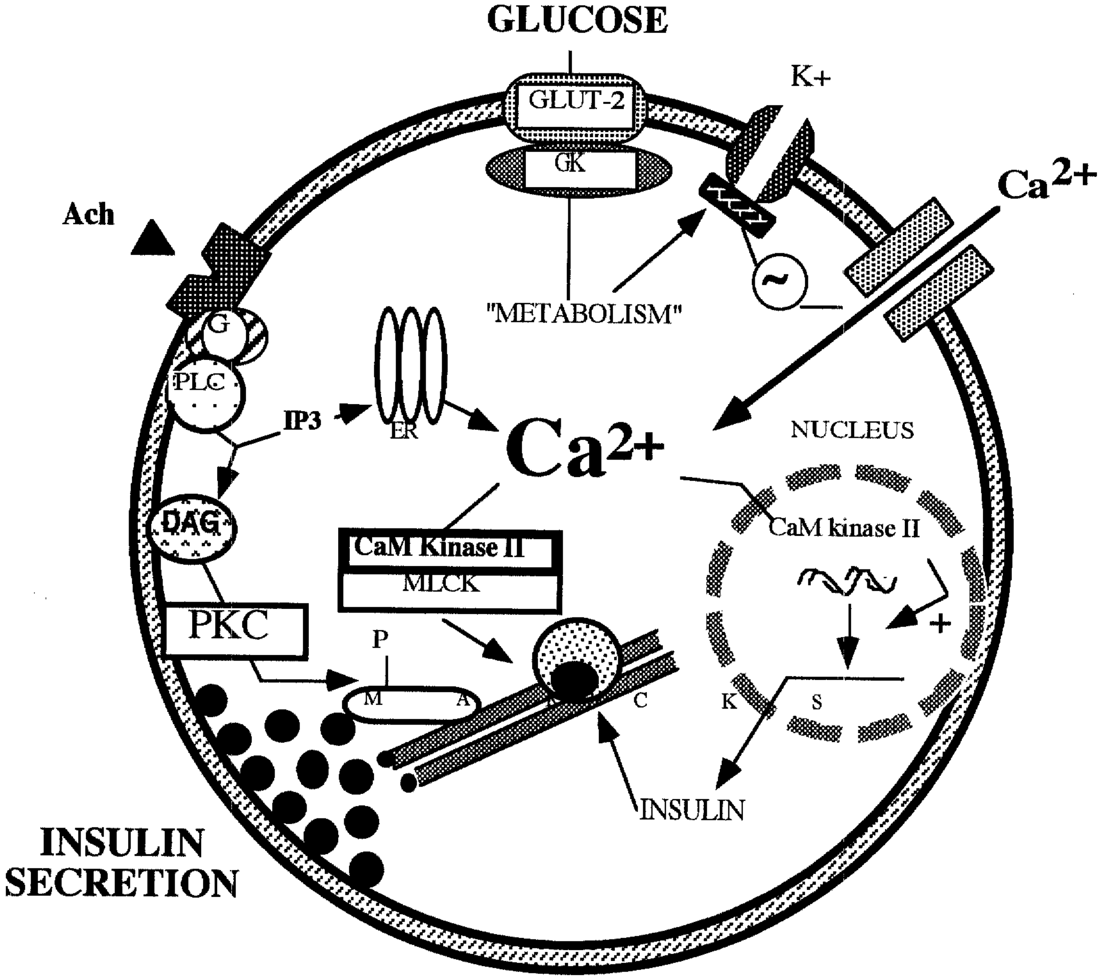
[2] L. C. Craig and R. A. Easom, unpublished observations.

FIG. 19. **Possible roles of CaM kinase II in the β-cell.** Abbreviations used are: GLUT-2, glucose transporter-2; GK, glucokinase; MLCK, myosin light chain kinase; MARCKS, myristylated alanine-rich C kinase (protein kinase C) substrate; PKC, protein kinase C; DAG, diacylglycerol; PLC, phospholipase C; G, G-protein; Ach, acetylcholine; IP_3, inositol trisphosphate; ER, endoplasmic reticulum; P, phosphate.


GLUCOSE
GLUT-2
GK
K+
Ca^{2+}
Ach
G
"METABOLISM"
PLC
IP3
ER
Ca^{2+}
NUCLEUS
DAG
CaM kinase II
CaM Kinase II
MLCK
PKC
P
M
A
C
K
S
+
INSULIN
INSULIN SECRETION

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