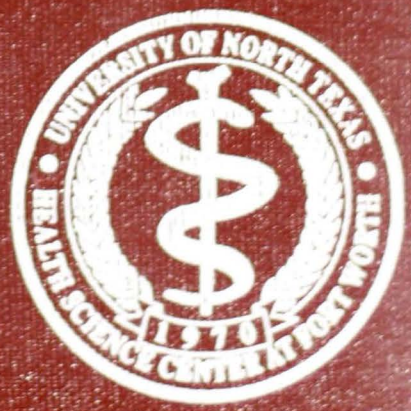
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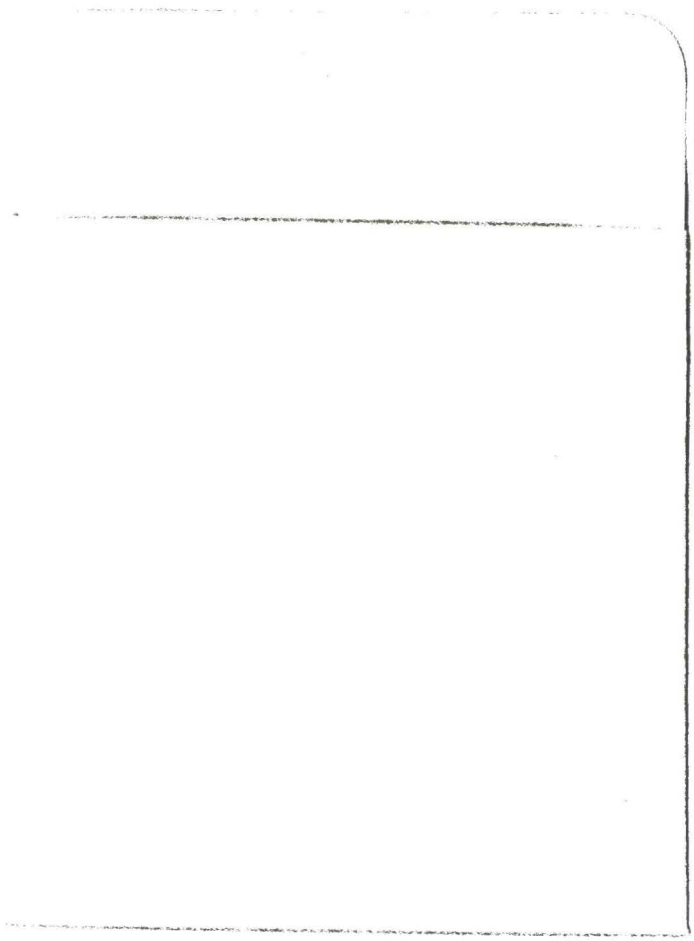

Dibas, Adnan I., Mechanism of Gramicidin D-Induced Insulin Secretion From βTC3 cells. Doctor of Philosophy (Biomedical Sciences), August, 1995, 190 pp., 5 tables, 38 illustrations, bibliography, 265 titles.

Gramicidin D, a sodium ionophore, was discovered to be a potent insulin secretagogue in the β-cell line βTC3 cells. Gramicidin D (1 μM) induced a 3.28-fold increase in insulin release relative to control, and when studied in a dynamic cell-perifusion system, was biphasic. Insulin secretion was accompanied by effects of gramicidin D to increase intracellular concentrations of Na^+ ($[Na^+]_i$) and Ca^{2+} ($[Ca^{2+}]_i$) in βTC3 cells as determined by dynamic single-cell video imaging techniques. Using similar techniques, gramicidin D had no effect on cellular pH.

The mechanism of gramicidin D-induced increase in $[Ca^{2+}]_i$ was dependent on the influx of extracellular Ca^{2+} and suggested to be mediated by a combination of membrane depolarization-induced activation of voltage-sensitive Ca^{2+} channels and the activation of a Na^+/Ca^{2+} exchanger in the reverse mode.

Gramicidin D-induced increase in $[Ca^{2+}]_i$ in the first phase correlated temporally with a profound (5.56-fold) activation of multifunctional Ca^{2+}/calmodulin-dependent protein kinase II. While these observations are consistent with the involvement of this enzyme in gramicidin D-induced insulin secretion, further observations suggested that the kinase may play only a modulatory role in insulin secretion. A similar activation of myosin light chain kinase was not detected.

In contrast to βTC3 cells, gramicidin D failed to induce insulin secretion from pancreatic islets. βTC3 cells and pancreatic islets exhibited distinct responses to ouabain, an inhibitor of the Na^+/K^+ ATPase, with respect to $[Ca^{2+}]_i$ and insulin secretion suggesting that different mechanisms controlling Na^+ homeostasis exist in these β-cell

preparations. Furthermore, Na^+/K^+ ATPase activity in βTC3 cell membranes was found to be approximately fifty percent that of primary β-cells.

Gramicidin D was identified as a secretagogue in βTC3 cells with a novel mechanism of action. The ability of this ionophore to induce insulin secretion from these cells and not primary β-cells is thought to be a function of different mechanisms of Na^+ homeostasis and documents a functional difference in this insulinoma cell line.

MECHANISM OF GRAMICIDIN D-INDUCED

INSULIN SECRETION FROM

βTC3 CELLS

Adnan I. Dibas, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

Committee Member

Chair, Department of Biochemistry and Molecular Biology

Dean, Graduate School of Biomedical Sciences

MECHANISM OF GRAMICIDIN D-INDUCED INSULIN SECRETION FROM βTC3 CELLS

DISSERTATION

Presented to the Graduate Council of the
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By

Adnan I. Dibas, B.S.

Fort Worth, Texas

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ABBREVIATIONS

BCECF	2',7'-biscarboxy-ethyl-5',(6')-carboxyfluorescein
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
Cam kinase II	Multifunctional Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine-3',5'-monophosphate
cGMP	Cyclic guanosine-3', 5'-monophosphate
DAG	Diacylglycerol
DCB	3,4-Dichlorobenzamil
DIDS	4,4'-Di-isothiocyano-2,2'-stilbene
DMB	2,4-Dimethylbenzamil
DMSO	Dimethylsulfoxide
EIPA	Ethylisopropyl amiloride
GD	Gramicidin D
12-HETE	12(S)-Hydroxy-eicosa-5, 8, 10, 14-tetraenoic acid
12-HPETE	12(S) Hydroperoxy-eicosa-5, 8, 10, 14-tetraenoic acid
IP_3	Inositol-1,4,5-trisphosphate

K_{ATP}	ATP-sensitive K channel
KIC	Ketoisocaproate
L-Arg	L-Arginine
L-His	L-Histidine
L-Leu	L-Leucine
L-Lys	L-Lysine
L-Orn	L-Ornithine
MLCK	Myosin light chain kinase
$[Na^+]_i$	Intracellular Na^+ concentration
NO	Nitric oxide
PC	Phosphatidyl choline
PDD	4 α-phorbol-12,13-Didecanoate
PHHI	Persistent Hyperinsulinemic Hypoglycemic of Infancy
pH_i	Intracellular pH
PKA	cAMP-dependent protein kinase
PKC	Calcium/phospholipid-dependent protein kinase
PLA_2	Phospholipase A_2
PLC	Phospholipase C

PMA	Phorbol myristate acetate
PS	Phosphatidyl serine
SBFI	Sodium benzofuran isophthalate
SITS	4-acetamido-4'-isothiocyanate-2,2'-stilbene sulphonic
STA	Staurosporine
TCA	Trichloroacetic acid
TPA	Tetradeconylphorbol acetate
Ver	Verapamil
VSCC	Voltage-sensitive Ca^{2+} channels
Wort	Wortmannin

INTRODUCTION

Diabetes mellitus is a heterogeneous disorder characterized by marked hyperglycemia which can be divided into 2 major groups: (i) insulin-dependent diabetes mellitus (IDDM, also known as Type I diabetes mellitus) and (ii) non insulin dependent diabetes mellitus (NIDDM, known as Type II diabetes mellitus). In Type I diabetes mellitus, β-cells of islets of Langerhans are destroyed by an autoimmune mechanism under the influence of both genetic and environmental factors (1). Type II diabetes mellitus can be viewed as a progressive disorder of β-cell function (2-4), but is further complicated by the association with obesity and insulin resistance. In this disease, insulin secreting β-cells no longer identify glucose as a secretagogue; however, they still secrete insulin in response to other secretagogues in the presence of glucose (L-Arg and glucagon)(5, 6). In this disease, glucose-induced increase in $[Ca^{2+}]_i$ is impaired (7). Hence, intense research has been directed to understand how glucose-metabolism increases $[Ca^{2+}]i$ in β-cells and how such events can be mimicked *in vitro* or *in vivo*. These efforts led to the discovery of sulfonylurea drugs which are currently used for treatment of Type II diabetes mellitus (8). However, research efforts are still in progress to dissect the mechanism of glucose-induced insulin secretion. Insulin release regulates the blood levels of glucose. The physiological importance of insulin release is due to the ability of insulin to promote glucose uptake at the peripheral tissues (9).

β-cells are found in the pancreas, an elongated gland that lies immediately beneath the stomach. The pancreas is composed of two different types of tissue. One type is the acini, which secrete digestive juices into the intestine. The other type is the islets of Langerhans, which secrete hormones directly into the blood. This secretion into

the blood is an endocrine function of the pancreas, whereas the secretion of digestive juices is an exocrine function.

The islets of Langerhans are aggregates of about 2000 cells. The islets contain four principal endocrine cell types. β-Cells constitute about eighty percent of the islet mass and secrete insulin (10). α-Cells secrete glucagon, which opposes the physiological effects of insulin in many responses (11). δ-Cells secrete somatostatin, which suppresses the secretion of glucagon and insulin and may serve important paracrine functions within the islet (12). PP-cells secrete pancreatic polypeptide, the physiological function of which is not understood (12).

The primary function of β-cells is to synthesize and secrete insulin in order to regulate the blood levels of glucose. Research reports have suggested that newly synthesized insulin destined for secretory granules traverses the rough endoplasmic reticulum (RER) to the Golgi complex. Insulin is believed to exit the RER in transport vesicles and be delivered to the *cis* region of the Golgi complex. Then, by unknown signal, insulin is diverted to the secretory granules where it is stored until secretion. Upon the receipt of specific signals, insulin secretory granules are transported to the plasma membranes for insulin exocytosis (19).

In the following section, analysis of the latest hypotheses that have been proposed to explain the exocytosis event is discussed.

Generally, exocytosis is defined as secretion from storage granules regulated by direct agonist action. There are multiple processes regulating secretion, including synthesis of the secretory products, packaging into granules and movement of granules to the sites at which they are primed for rapid secretion upon the receipt of the appropriate signal.

There are many steps involving secretion, but the movement of secretory granules and granule apposition with the plasma and fusion of the granules with the plasma membrane are important key steps.

Granule movement by cytoskeleton

Cytoskeletal polymers have been suggested to provide the force that moves the granules (13-14). Electron microscopic and immunocytochemical localization have shown that a dense network of actin filaments is situated between plasma membrane and secretory granules (15). If the cytoskeleton is involved in regulation of granule apposition with plasma membrane, two types of mechanism could be involved: cytoskeletal polymers could provide the force that moves the two elements together, or they could form a barrier that must be released before diffusion of granules to membrane (16-18). There are studies that support each type of mechanism. However, the exact mechanism involved in the regulation of granule apposition with plasma membrane is not known.

Granule fusion with plasma membrane

Granule membranes must fuse with the plasma membrane for normal exocytosis to occur because intragranule contents are secreted from the cell without secretion of granule lipids or membrane markers (19). *In vitro* reconstitution of regulated exocytosis is the goal of many research efforts. Davis and Lazarus (20) prepared mouse pancreatic islet granule and islet plasma membranes, then reconstituted insulin release. Reconstitution required both granules and plasma membrane and Ca^{2+} at an appropriate concentration (> 0.1 μM). Insulin release was enhanced greatly by the inclusion of ATP (5 μM) and glucose (8 mM) in the incubation. Islet membranes could not be replaced by plasma membranes prepared identically from rat pituitary or rat liver, proving islet membrane specificity.

Insulin secretagogues

Glucose and other carbohydrates

Non-glucose secretagogues

Insulin secretagogues can be classified into two classes; (1) glucose and other carbohydrates and (2) non-glucose secretagogues. β-cells of pancreatic islets can be induced to secrete insulin by a variety of stimuli. D-glucose is the primary stimulus for insulin release. Non-glucose carbohydrates, mannose, glyceraldehyde and dihyroxyacetone also stimulate insulin secretion from islets. However, mannitol, fructose and galactose do not stimulate insulin secretion (21-22).

Although the detailed mechanism(s) of glucose-induced insulin secretion is not fully elucidated, the consensus is that glucose metabolism increases $[ATP]_i$ which blocks the ATP-sensitive K^+ channel (K_{ATP}). The closure of K_{ATP} channel depolarizes the plasma membrane and promotes the influx of extracellular Ca^{2+}. An increase in $[Ca^{2+}]_i$ is suggested to be the key signal that triggers insulin release, but the detailed mechanisms by which increased $[Ca^{2+}]_i$ stimulates insulin secretion are not yet identified.

K_{ATP} channels are thought to be the link between nutrient (glucose) availability and insulin secretion from the islets. In the islets, K_{ATP} in the β-cell is predominantly closed in the presence of physiological concentrations of $[ATP]_i$ and opens as $[ATP]_i$ falls. Despite this, K_{ATP} is the major K-channel responsible for setting the basal level of membrane potential in β-cell and its modulation is thought to play an important role in the regulation of glucose-stimulated insulin secretion (112-115). Since the membrane resistance of the β-cell is very high, the closure of a few K_{ATP} channels has a profound effect on the membrane potential (116). An increase in intracellular glucose concentrations produces an increase in $[ATP]_i$. The consequential closure of K_{ATP} depolarizes the cell, enhancing stimulation of calcium influx and the initiation of insulin

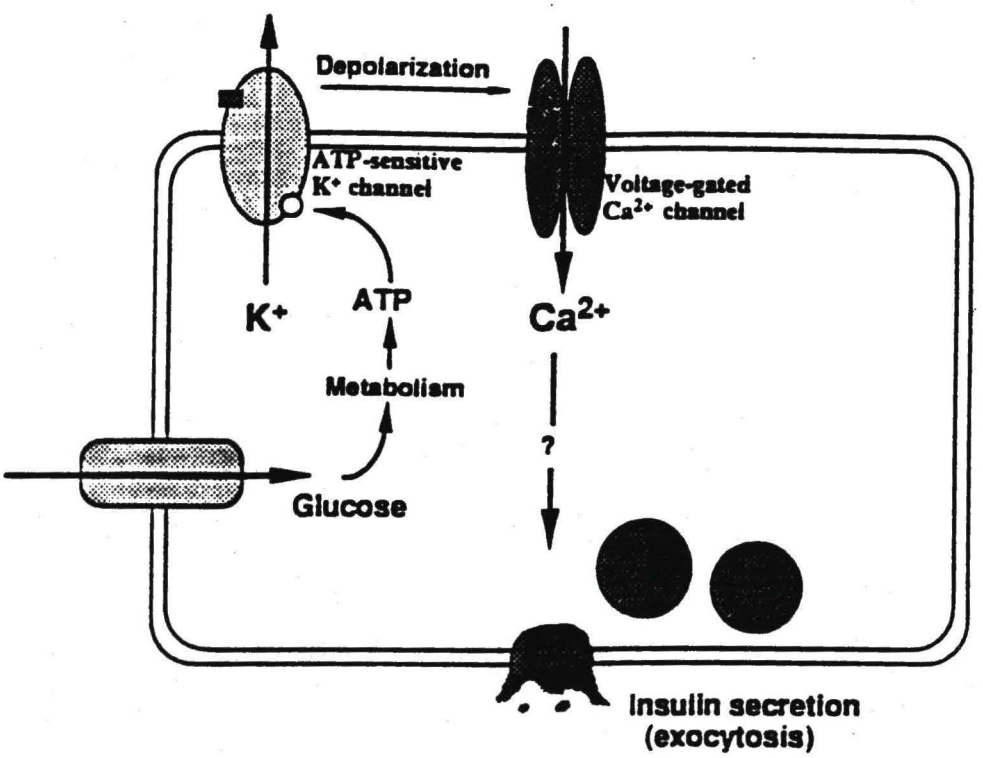
secretion. K_{ATP} channels are characterized in details below. In Fig. 1, a proposed model of the mechanism of glucose-induced insulin secretion from β-cells is depicted.

Besides the ability of glucose to increase $[Ca^{2+}]_i$, glucose-induced insulin release is accompanied by generation of other second messengers which play important roles in regulating insulin secretion. The second messengers generated include: inositol-1,4,5-trisphosphate (IP_3), diacylglycerol (DAG), arachidonic acid, cylic guanosine-3', 5'-monophosphate (cGMP) and cyclic adenosine-3', 5'-monophosphate (cAMP).

Ca^{2+}

Raising intracellular Ca^{2+} ($[Ca^{2+}]_i$) appears to be central to the insulin secreting pathway. Glucose-induced insulin secretion is absolutely dependent on extracellular Ca^{2+}, and all known insulinogenic stimuli affect Ca^{2+} fluxes in intact cells (23-24). Glucose enhances $^{45}Ca^{2+}$ uptake in intact islets (25-26). Some studies have suggested that the initial, rapid phase of glucose-induced insulin release is dependent on the mobilization of intracellular Ca^{2+} (27-28), whereas the second sustained phase of insulin release reflects an Ca^{2+} influx (29). Changes in $[Ca^{2+}]_i$ after glucose stimulation revealed that the response in increasing $[Ca^{2+}]_i$ was divided into three phases (30). The first phase was characterized by an initial decrease in $[Ca^{2+}]_i$, whereas the second phase was characterized by oscillating $[Ca^{2+}]_i$ in which $[Ca^{2+}]_i$ was spiking. In the third phase, $[Ca^{2+}]_i$ was maintained at a level higher than basal levels. Interestingly, $[Ca^{2+}]_i$ and insulin secretion oscillated in synchrony from a single pancreatic islet, therefore suggesting a direct correlation between changes in $[Ca^{2+}]_i$ and insulin release. The study was the first to demonstrate that sustained oscillations of $[Ca^{2+}]_i$ induced by a physiological stimulus entrain synchronous oscillation of the functional response.

Fig. 1 **A Proposed mechanism of glucose-induced insulin release from β-cell.**


Depolarization
ATP-sensitive
K+ channel
Voltage-gated
Ca2+ channel
K+
ATP
Ca2+
Metabolism
?
Glucose
Insulin secretion
(exocytosis)

The increase in $[Ca^{2+}]_i$ represents an important event in stimulus-secretion coupling in the pancreatic β-cells. There are several Ca^{2+}-activated proteins that have been suggested to play important roles in regulating insulin secretion. Virtually, every step involved in insulin exocytosis is regulated directly or indirectly by Ca^{2+}. These steps include translocation of insulin secretory granules, fusion of secretory granules and polymerization of microfilaments and microtubules (31-37). In addition, cAMP, IP_3 and arachidonic acid production in β-cells is suggested to be Ca^{2+}-dependent.

Also, the increase in $[Ca^{2+}]_i$ activates calmodulin which is an important regulatory subunit of several protein kinases. A Ca^{2+} and calmodulin-dependent protein kinase has been identified in pancreatic islets (39). Alterations in the activity of this kinase in islets correlate with glucose-induced insulin secretion (40-42).

Rasmussen *et al.*. have suggested that the first phase of hormonal secretion reflects in part activation of a Ca^{2+} and calmodulin-dependent protein kinase. The second phase of hormonal secretion was proposed to reflect activation of the Ca^{2+} and phospholipid-dependent protein kinase C by DAG (PKC) (38). In fact, analogs of DAG (phorbol esters such as tetradeconyl phorbol acetate (TPA)) stimulated glucose-induced insulin secretion from intact islets (43-46). Based on such observations, PKC was suggested to play an important regulatory role in insulin release.

Other kinases regulated by Ca^{2+} include myosin light chain kinase (MLCK) which was detected in islets. It is suggested that MLCK phosphorylation of myosin light chain activates polymerization of microfilaments required for the translocation of insulin secretory granules. However, a decisive role of regulating insulin secretion by this kinase is still not established (47). Experiments are currently being performed in the laboratory to verify a correlation between the activation of MLCK and insulin release.

In summary, the increase in $[Ca^{2+}]_i$ activates several intracellular processes required for insulin secretion, but the detailed mechanisms by which an increased $[Ca^{2+}]_i$ stimulates insulin release are yet to be discovered.

IP3

As suggested earlier, the increase in $[Ca^{2+}]_i$ can be achieved by the influx of extracellular Ca^{2+} and the mobilization of intracellular Ca^{2+}. One of the most important second messengers generated as a result of glucose metabolism is IP3 which mobilizes Ca^{2+} from the endoplasmic reticulum of insulin secreting cells (76). It was suggested that glucose-induced influx of extracellular Ca^{2+} activates phospholipase C (PLC) which hydrolyses PIP_2 and generates IP3 and DAG (76).

The effect of glucose on the accumulation of inositol phosphates was abolished by manoheptulose which suggested that this event was dependent on glucose metabolism and believed to be GTP-independent (77-78). Furthermore, other carbohydrates including mannose (20 mM) and D-glyceraldehyde (10 mM) promoted the accumulation of IP3 (79).

Other agonists that activate PLC and increase the generation of IP3 have been shown to potentiate glucose-induced insulin secretion. These include muscarinic agonists (carbachol and acetylcholine) and vasopressin (80).

In summary, the exact role of IP3 in glucose-induced insulin secretion is incompletely defined, but suggested to increase cytosolic levels of Ca^{2+} which are critical for insulin exocytosis.

DAG

Although glucose-induced activation of PLC resulted in the generation of DAG (48-50), DAG has been also shown to be synthesized in β-cells from glyceraldehyde (247). DAG is a potent activator of protein kinase C (PKC), an enzyme that is suggested to regulate insulin secretion. PKC has been reported in pancreatic islets (45). The enzyme exhibited maximal activity in the presence of Ca^{2+}, phosphatidyl serine (PS) and DAG. DAG analogs including phorbol myristate acetate (PMA) and tetradeconyl phorbol acetate (TPA) potentiated glucose- and hypoglycemic sulphonylurea, glipclazide-induced insulin secretion and therefore, suggesting a regulatory role for PKC in insulin release (51-52, 58).

The regulation of PKC activity has been currently elucidated in other cell types. A receptor for PKC was discovered at the plasma membrane and was named RACK protein (53). It is suggested that PKC is translocated to the plasma membrane and binds to its receptor. DAG and PS stabilize such interaction of PKC with its receptor. In summary, the translocation of PKC to the plasma membrane was postulated to be part of its mechanism of activation.

Glucose-dependent activation of PKC is still controversial. While some groups suggested that glucose stimulated PKC activation and translocation to plasma membrane (173), others were unable to detect any redistribution of PKC activity (174).

The role of PKC in regulating insulin secretion is not fully understood. However, the key events are mediated by PKC phosphorylation of a number of proteins. A key protein that was identified to be phosphorylated by PKC was the K_{ATP} which is the main channel that sets the membrane potential in β-cells (131). It is suggested that the phosphorylation of such channel leads to depolarization and increases Ca^{2+}-influx. The role of this channel in regulating insulin secretion will be discussed below.

Another protein that appeared to phosphorylated by PKC is the Na^+/H^+ exchanger which is suggested to regulate the intracellular pH of β-cells (54). The regulation of pH_i will be discussed in details in section 3. Other proteins that have been identified as endogenous substrates for PKC in rat islets had molecular weights of 11, 15, 20, 29, 35, 38 KDa (43, 51). However the physiological importance of such proteins is not known.

Arachidonic Acid

Glucose-induced increase in $[Ca^{2+}]_i$ is suggested to activate PLA_2 (55). PLA_2 hydrolyses phosphatidyl choline (PC) and generates arachidonate. Arachidonic acid may function as a mediator in signal transduction processes. Hokin has suggested that free arachidonic acid could have some primary role as a mediator in signal transduction which does not involve its conversion to metabolites (55) and Wolf *et al* . (56-57) has shown that arachidonic acid mobilized Ca^{2+} stored in the endoplasmic reticulum (E.R.) of digotonin-permeablized islets. The 12-lipoxygenase metabolites, 12-HETE2 and 12-HPETE failed to stimulate Ca^{2+} release from the E.R. (57). Furthermore, arachidonate-induced Ca^{2+} release was not affected by the addition of inhibitors of lipoxygenase and cycloxgenase pathways (58-61). Thus, Ca^{2+} mobilization effect of arachidonate is not attributed to its metabolites. In summary, arachidonic acid role in insulin secretion is not fully elucidated, but suggested to increase $[Ca^{2+}]_i$ in β-cells which is required for insulin release.

cGMP

As discussed in the previous section, glucose enhances PLA_2 activity which releases arachidonate. Arachidonate can mediate the activation of guanylate cyclase

which synthesizes cGMP (69-71). The role of cGMP in regulating insulin secretion is mediated by the activation of cGMP-dependent protein kinase (70). cGMP has been shown to antagonize the inhibitory effects of glucose-1,6-bisphosphate and increase glucose utilization (72, 73). While 8-Br-cGMP increased glucose-induced insulin secretion, it has no effect on glyceraldehyde or ketoisocaproate-induced insulin secretion (69). This observation confirmed that cGMP enhances glucose-induced insulin secretion, an event which occurs earlier in glucose metabolism.

In summary, cGMP role has been suggested to increase β-cell metabolism of glucose.

cAMP

As previously described, glucose-induced increase in $[Ca^{2+}]_i$ activates calmodulin. Calmodulin has been shown to activate adenylate cyclase (62, 66). cAMP has been reported to be neither a sufficient nor a necessary mediator of insulin secretion, but, rather a positive modulator of secretion (67). Forskolin, an activator of adenylate cyclase potentiated glucose-induced insulin secretion but had no effect on basal insulin secretion (68). However, the role exerted by cAMP is suggested to be mediated by cAMP-dependent protein kinase (PKA). PKA has been suggested to increase $[Ca^{2+}]_i$ by either inhibiting the Ca^{2+}-ATPase (which pumps Ca^{2+} outside the cell)(63) or by phosphorylating the L-type Ca^{2+} channel (the phosphorylation activates the channels which promote the influx of Ca^{2+})(64). Also, PKA is known to regulate certain glycolytic enzymes (65).

The pathways of production of second messengers and the their proposed functions as a result of glucose metabolism in β-cell are depicted in Fig. 2.

A number of amino acids including Leu, Arg, Lys, Orn, and His stimulate insulin secretion in the presence of glucose from islets (20, 80). Free fatty acid such as palmitate stimulate insulin secretion in the presence of glucose (80). Acetylcholine and vasopressin also stimulate insulin secretion in the presence of glucose (80). Sulphonylurea drugs such as tolbutamide and depolarizing concentrations of KCl also stimulate insulin secretion in the absence of glucose. A new secretagogue gramicidin D, was identified as a potent insulin secretagogue in the present study in βTC3 cells.

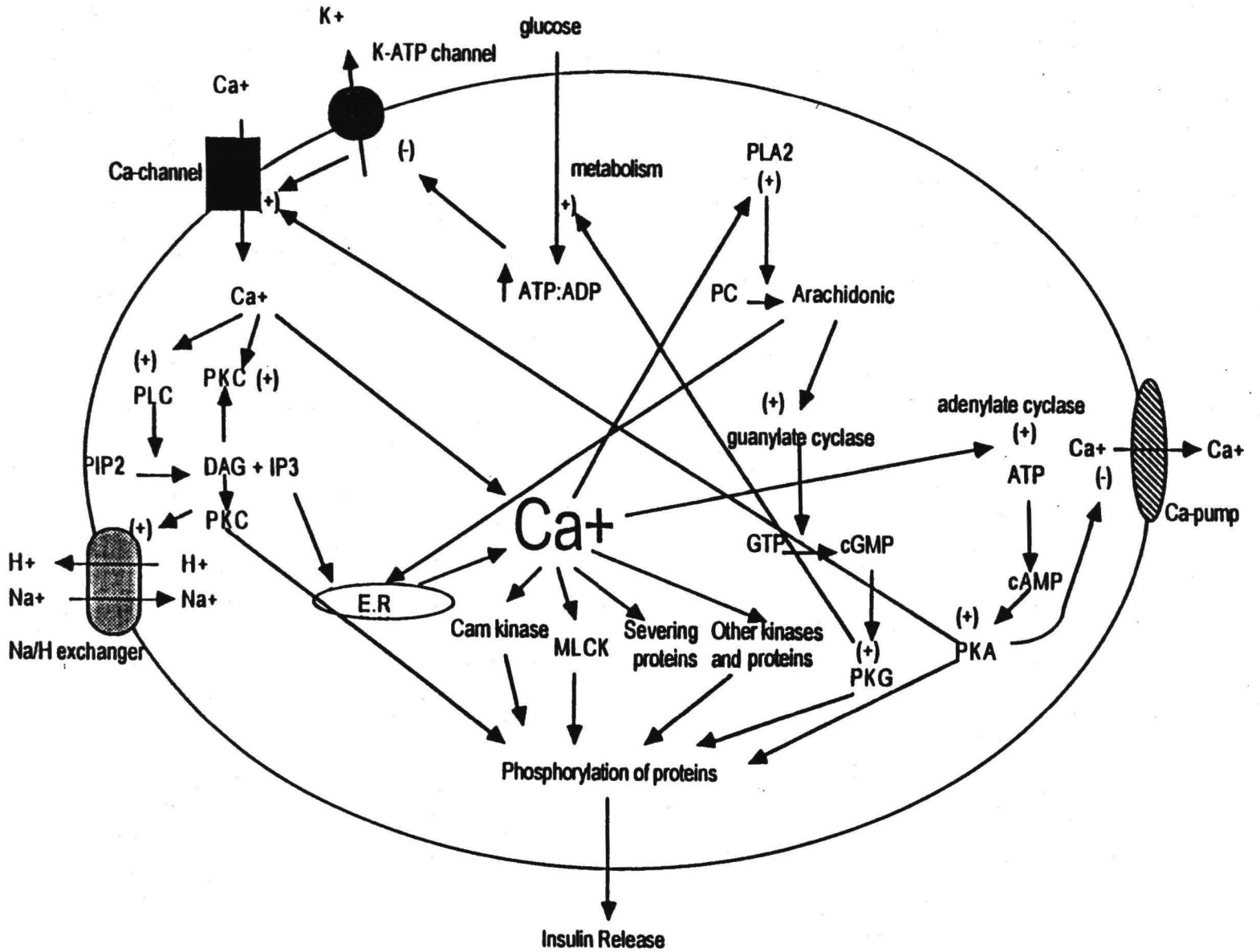
L-Leu

Mechanism of action

L-Leucine and its deamination product 2-ketoisocaproate (81) are stimulators of insulin secretion, while L-valine and its deamination product 2-ketoisovalerate (82-87) do not enhance insulin secretion. L-isoleucine does not stimulate insulin secretion, whereas its deamination product 2-ketocaproate is a potent stimulator of insulin secretion.

Two model substances have indicated an approach to elucidate the insulin potency of the aliphatic amino acids and 2-keto acids. These were the nonmetabolizable leucine analogue 2-endo-aminonorbornane-2-carboxylic acid (88-89) and the aromatic keto acid 3-phenylpyruvate (89). Neither substance provides metabolic fuel for the β-cells, although both agents induce insulin secretion. They increase the generation of intramitochondrial 2-ketoglutarate. 2-Endo-aminonorbornane-2-carboxylic acid achieves this by the activation of glutamate dehydrogenase and 3-phenylpyruvate achieves similar action by intramitochondrial transamination of L-glutamate and L-glutamine (90). These mechanisms emphasize the importance of intramitochondrial mechanisms for initiation of insulin secretion.

Fig. 2 **Pathways of production of second messengers and their proposed functions as a result of glucose metabolism in β-cell.**


K+
K-ATP channel
glucose
Ca+
Ca-channel
(-)
(+)
metabolism
(+)
PLA2
(+)
ATP:ADP
PC
Arachidonic
Ca+
(+)
PLC
PKC (+)
(+)
guanylate cyclase
adenylate cyclase
(+)
ATP
Ca+
(-)
Ca+
Ca-pump
PIP2
DAG + IP3
PKC
(+)
Ca+
GTP
cGMP
cAMP
H+
H+
Na+
Na+
Na/H exchanger
E.R
Cam kinase
MLCK
Severing proteins
Other kinases and proteins
(+)
PKG
(+)
PKA
Phosphorylation of proteins
Insulin Release

It has been found that L-Leu and 2-ketoisocaproate can increase intramitochondrial generation of 2-ketoglutarate. In contrast, L-Isoleucine, L-valine and 2-ketoisovalerate do not increase the intramitochondrial generation of 2-ketoglutarate. Therefore, it has been suggested that the key mechanism to explain the insulin secretory of L-Leu and its deamination product is the increased generation of 2-ketoglutarate, providing an additional feeding of acetyl-CoA into Krebs cycle and enhancing production of reducing equivalents.

Basic amino acids

Mechanism of stimulation of insulin secretion

The mechanisms by which basic amino acids influence β-cell function are not fully understood. Basic amino acids are transported into β-cells by the system Y transporter (91).

It has been shown that basic amino acids stimulate $^{86}Rb^{+}$ efflux and $^{45}Ca^{2+}$ influx from perifused rat islets in the absence or the presence of glucose (92). One group has suggested that the mechanism by which basic amino acids stimulate insulin secretion involves the activation of the efflux of K^{+} and influx of Ca^{2+} into β-cells (93). Metabolism of L-Arg into citrulline and nitric oxide is one major pathway of L-Arg metabolism in β-cells. Other pathways of basic amino acids include polyamine production and transamination reaction at the mitochondria (94).

The roles of nitric oxide (NO) and cGMP as key mediators of the L-Arg-induced insulin secretion are still controversial (95-96). By contrast, the role of polyamines in mediating basic amino acid-enhanced insulin secretion has been rejected (97).

Also, L-Arg stimulates transamination reactions at the mitochondria (98). Therefore, a possible mechanism of L-Arg is likely similar to that exerted by L-Leu and 2-ketoisocaproate. However, L-homoarginine, a nonmetabolizable analogue of L-Arg is not a substrate for transaminase enzyme, yet stimulates insulin secretion from islets. Therefore, transamination of L-Arg is not likely involved in L-Arg-enhanced insulin secretion. L-Arg did not stimulate IP_3 production in pancreatic β-cells which eliminates a phospholipase C system activation in the L-Arg-mediated mechanisms (99).

In general, the most current accepted hypothesis explaining the mechanism of basic amino acid enhanced insulin secretion suggests that the accumulation of positive charges leads to depolarizing the plasma membrane and subsequent gating of voltage-sensitive Ca^{2+} channels. However, such hypothesis does not explain the lack of effect of L-Arg on membrane potential and the inability of L-Arg to induce an increase in $[Ca^{2+}]_i$ in the absence of glucose (100-101). L-Arg does not promote any changes in membrane potential in the absence of glucose, but potentiates glucose-induced changes in membrane potential. Also, the proposed model does not provide an explanation for the ability of L-Arg to stimulate insulin secretion from electrically-permeablized islets (102). Furthermore, the model also does not explain the additive effect of L-Arg on K-induced insulin secretion, a mechanism is mediated by depolarizing the plasma membrane and promoting the influx of extracellular Ca^{2+}. Finally, the current hypothesis does not explain the inability of number of basic substances to stimulate insulin secretion (103). These include: 2-amino-3-guanidinopropionic acid, guanidinoactic acid, 3-guanidinopropionic acid, 1,5 diaminopentane and 6-aminohexanoic acid. Although all the mentioned substances stimulate $^{86}Rb^+$ efflux as L-Arg and L-Orn, however, none of them enhanced glucose-induced insulin secretion. In conclusion, the actual mechanism of basic amino acids stimulation of insulin secretion is not resolved yet.

Sulfonylurea drugs

Mechanism of stimulation of insulin secretion

Sulfonylurea drugs and depolarizing concentrations of KCl stimulate insulin secretion by inhibiting ATP-sensitive K channel. A potassium (K) channel that is inhibited by physiological (μM) concentrations of intracellular ATP ($[ATP]_i$) and that is opened by as $[ATP]_i$ decreases is known as ATP-sensitive K channel. Such channels were detected in pancreatic β-cells, skeletal muscle, neurons and cardiac muscle (104-108). The channel has unitary conductance in the range of 40-80 pS (measured under symmetrical high K^+ conditions (140 mM)). Such channels are termed by Ashcroft as Type 1 (109). Type 1 K_{ATP} channels are calcium and voltage-independent but, K^+-selective. They are half-maximally inhibited by $[ATP]_i$ in the range 10-100 μM. Other ATP-sensitive K-channels exist and they were designated as Type 2, 3, 4 and 5 (109). Such channels vary not only in their sensitivity to calcium and to inhibitory effects of $[ATP]_i$, but also in their selectivity for potassium and their susceptibility to pharmacological modulators.

The K_{ATP} channels are opened by number of drugs known as "K_{ATP} channel openers". There are five classes of K_{ATP} channel openers. They are: Benzopyran, pyridine, pyrimidine, benzothiadiazine and Butenoic acid (110). Also, some calmodulin antagonists may exert similar actions (111).

In both pancreatic β-cells and insulinoma cells, diazoxide (a member of benzothiadiazine family) stimulates the opening of K_{ATP}, inducing membrane hyperpolarization and inhibiting insulin secretion (117-120).

Sulfonylurea drugs, long used in the treatment of noninsulin-dependent diabetes mellitus, are known to stimulate insulin secretion by inhibiting the opened K_{ATP} in pancreatic β-cells. Sulfonylurea drugs also inhibit K_{ATP} in cardiac myocytes (122-123)

and skeletal muscles (108). The most potent of the sulfonylureas, glyburide (124), which possesses both benzoic and sulfonylurea groupings, is capable of inhibiting K_{ATP} by an interaction with site additional to that which recognizes the sulfonylurea moiety (125). However, its action is irreversible, due to inhibiting cAMP-dependent protein kinase (126).

Bray and Quast (127) and Schwanstecher and coworkers (128) have concluded that the K_{ATP} openers and the hypoglycemic agents bound to different sites on the K_{ATP} channel. The sites were not identical, but closely and negatively allosterically coupled.

The purified sulfonylurea-binding sites extracted from brain or pancreatic β-cells have similar molecular weights (140 KDa and 150 KDa, respectively)(130). K_{ATP} channels have been recently cloned from HIT cells (131). The open reading frame encodes proteins of 1582 amino acids with molecular masses of 177 KDa. K_{ATP} has three potential phosphorylation sites for protein kinase A and twenty potential phosphorylation sites for protein kinase C. There are nine phosphorylation sites in the nucleotide binding motifs. The importance of K_{ATP} channels is currently under more investigation due to their link to Familial persistent hyperinsulinemic hypoglycemia of infancy (PHHI), an autosomal recessive disorder characterized by unregulated insulin secretion. PHHI has been shown to be linked to chromosome 11p14-15.1. The newly cloned K_{ATP} gene was mapped to 11p15.1 by means of fluorescence *in situ* hybridization. Two separate K_{ATP} gene splice site mutations, which segregated with disease phenotype, were identified in affected individuals from nine different families. Both mutations resulted in aberrant processing of the RNA sequence and disruption of the putative second nucleotide binding domain of the K_{ATP} protein. Therefore, it was suggested that abnormal insulin secretion in PHHI was caused by mutations in the K_{ATP} gene (132).

GTP-binding proteins (G_i and G_o) have been demonstrated to regulate Type 1 K_{ATP}. $G_{\alpha i}$ has been shown to interact with K_{ATP} in cardiac myocytes (133), whereas $G_{\alpha o}$ has been shown to interact with K_{ATP} in skeletal muscle (134). In general, the interaction of G_α keeps the K_{ATP} channel in the open state.

As mentioned earlier, the closure of β-cell K_{ATP} depolarizes the plasma membranes and activates voltage-sensitive Ca^{2+} channel (VSCC) which promotes the influx of extracellular Ca^{2+}. VSCC are key channels that regulate insulin secretion and discussed in details in the next paragraph.

Voltage-sensitive Ca^{2+} channels (VSCC)

Ca^{2+} channels are structures that form functional pores in the plasma membranes. Ca^{2+} channels are normally closed; when opened, Ca^{2+} passively flows through the channels along the Ca^{2+} electrochemical gradient. Several million Ca ions per sec can enter cells through open Ca^{2+} channels. Ca^{2+} channels can be classified into two main classes according to mechanism of stimulation. The first type is known as voltage-sensitive Ca^{2+} channels (VSCC) which are stimulated by membrane depolarization. There are at least three types of VSCC; (1) L-type Ca^{2+} channels, (2) T-type Ca^{2+} channels and (3) N-type Ca^{2+} channels (135). The classification is based on their different sensitivities to voltage changes and pharmacological effectors (drugs). The second type of Ca^{2+} channels is Receptor-operated Ca^{2+} channels. Examples of this class are: nicotinic acetylcholine receptor, GABA receptors and glutamate receptors.

However, the key Ca^{2+} channels that are likely to mediate glucose-induced increase in $[Ca^{2+}]_i$ are the VSCC. Ca^{2+} currents have been reported in neonatal, adult rat β-cells, in mouse β-cells, in human β-cells and in the clonal insulin-secreting cell lines RINm5F, HIT 15T and βTC3 cells (136-144). Like other Ca^{2+} channels, β-cell Ca^{2+}

currents are inhibited by the transition metals Co^{2+}, Cd^{2+} and Mn^{+}. They are also sensitive to the dihydropyridines (nitrendipine (140) and nifedipine (139)) and increased by the Ca^{2+} channel agonist BAY K8644 (1 μM)(141-142). β-cell Ca^{2+} channels are also permeable to Ba^{2+} (1.5-2 times more permeant than Ca^{2+}). Also, when Ca^{2+} is reduced to micromolar levels, β-cell Ca^{2+} channels have been shown to permeate Na^{+} as well (140). Some studies have estimated the existence of 500 Ca^{2+} channels/β-cell (1 channel/1 μM^2)(141, 145). The fact that β-cell Ca^{2+} currents are long-lasting, show Ca^{2+}-dependent inactivation, sensitive to micromolar cadmium and are dihydropyridine-sensitive suggest that β-cells possess L-type Ca^{2+} channels. The presence of T-type Ca^{2+} channel is still controversial in rat β-cells (146). However, it was suggested that mouse β-cells lack T-type Ca^{2+} channel. The regulation of VSCC activities in β-cells is not well-established. Some reports have shown that dibutyryl cAMP activated Ca^{2+} currents, whereas others have shown its lack of effect (146-147). In addition, diacylglycerol, an activator of PKC stimulated Ca^{2+} currents in RINm5F cells. However, phorbol-12-myristate 13-acetate (PMA) was with no effect. It is possible that several isoforms of PKC exist in RINm5F cells (148). Also, tetradeconylphorbol-acetate (TPA), another potent activator of PKC stimulated Ca^{2+} currents in HIT cells, however, this effect may be an indirect effect induced by TPA due to inhibition of K_{ATP} channels and subsequent depolarization of plasma membranes (149). In conclusion, the physiological role of Ca^{2+} channels in β-cells is to mediate the influx of extracellular Ca^{2+}, as it has been shown that Ca^{2+} channels inhibitors inhibited extracellular Ca^{2+} influx as well as insulin secretion induced by glucose.

Regulation of pH_i in β-cell

Along with the research efforts dedicated to the understanding of glucose effects on $[Ca^{2+}]_i$ and K^+ permeability in β-cells, the effects of glucose and other secretagogues on intracellular pH in (pH_i) β-cell have been studied. It is well documented that nutrient metabolism in β-cells influences intracellular pH (pH_i)(150). There are at least three different ionic transporters that were suggested to regulate pH_i. The most common transporter, found virtually in all mammalian cells, is the Na^+/H^+ exchanger (151). The other two systems involved in regulating pH_i are the Na^+-dependent and -independent HCO_3^-/Cl^- exchangers (152). Experimental evidence has suggested the presence of Na^+/H^+ exchanger in β-cells (based on the ability of amiloride (a modest inhibitor of the exchanger) to affect pH_i)(153-156).

Inhibitors of the HCO_3^-/Cl^- exchanger; stilbene disulphonates, 4,4'-di-isothiocyano-2,2'-stilbene disulphonic (DIDS) and 4-acetamido-4'-isothiocyanate-2,2'-stilbene disulphonic (SITS) modified pH_i in pancreatic β-cells (157-159) and therefore, these observations suggested the presence of HCO_3^-/Cl^- exchanger in β-cells.

Several studies have demonstrated an effect for insulin secretagogues on pH_i in β-cells (153, 159, 162-168). While glucose increased pH_i in rat islets and HIT cells (166), 4-methyl-2-oxopentanoate, glyceraldehyde and high concentrations of K^+ were found to decrease pH_i (167). However, in *ob/ob* mouse β-cells, raising glucose concentration from 3 to 20 mM did not influence pH_i, although tolbutamide and carbachol caused a fall and a rise in pH_i respectively (168). Therefore, it was suggested that glucose regulation of pH_i was impaired in *ob/ob* mouse β-cells. However, it has been suggested that glucose-induced increase in pH_i in β-cells may be mediated by the Na^+/H^+ exchanger.

The involvement of a HCO_3^-/Cl^- in regulating glucose-induced secretion is still not elucidated (157, 159 and 161).

In an attempt to evaluate the role of the Na^+/H^+ exchanger in regulating glucose-induced insulin secretion, the effect of amiloride on insulin secretion was studied. Unfortunately, amiloride produced opposing results (157). Amiloride has been shown to stimulate basal insulin secretion, whereas it inhibited glucose-induced insulin secretion at high glucose concentrations. However, such inhibition was suggested to be the result of inhibiting glycolytic enzymes. Also, amiloride inhibits Ca^{2+}-activated K^+ channel, thus causing depolarizing of plasma membrane. Such action may explain the potentiation of basal insulin secretion (160). Therefore, the use of inhibitors of the Na^+/H^+ exchanger was proven unsuccessful and the role of such activity in regulating insulin release is still not elucidated. However, in other cell types, the Na^+/H^+ exchanger has been shown to be activated by phosphorylation by PKC (171) and tyrosine kinase (170) whereas, it was inhibited by phosphorylation by Cam kinase II (172).

In the pancreatic β-cells, it has been suggested that glucose-activation of the Na^+/H^+ exchanger was PKC-independent (169) and there is no evidence for the involvement of a tyrosine phosphorylation or phosphorylation by Cam kinase II in regulating pH_i in β-cells.

In conclusion, the regulation of pH_i in β-cells as a result of glucose metabolism is not fully understood.

Regulation of intracellular $Na^{\pm}$ levels in β-cells

In contrast to the numerous studies characterizing the effects of insulin secretagogues on $[Ca^{2+}]_i$ in β-cells, few studies attempted to characterize the effect of insulin secretagogues on $[Na^+]_i$.

The key channel for setting $[Na^+]_i$ in β-cells is the Na^+/K^+ ATPase (175). Normally, the Na^+/K^+ ATPase will promote the influx of 2 K^+ ions and the efflux of 3

Na^+ ions. In purified plasma membranes, it has been shown that glucose, glyceraldehyde and ketoisocaproate; known insulin secretagogues, had no effect on the activity of the Na^+/K^+ ATPase (176). However, using integrated flame photometry technique for measuring Na^+ in rat pancreatic islets, Na^+ content decreased by nearly 40 % when islets were exposed to 5 mM glucose (176) and no further reduction was seen with an additional increase in the concentration of glucose to 20 mM. The suppressing effect of glucose was mimicked by quinine (an inhibitor of K_{ATP} channel, 100 μM). This inhibitory effect was seen also after the inhibition of the Na^+/K^+ ATPase by ouabain. Also, glucose exerted similar action in single β-cells (177). However, acetycholine increased $[Na^+]_i$ in β-cells, a response was abolished by glucose (178). The cross-talk between glucose metabolism and reduction of $[Na^+]_i$ is not established, although it has been suggested that such mechanism involves a channel other than the Na^+/K^+ ATPase in mediating glucose action. Another pathway of regulating Na^+ levels involves the Na^+/Ca^{2+} exchanger. Under normal conditions, the Na^+/Ca^{2+} exchanger will promote the influx of Na^+ and efflux of Ca^{2+}. However, it can function in the reverse mode, i.e., promoting the efflux of Na^+ and the influx of Ca^{2+} (179-181). Whether such reverse mode of the Na^+/Ca^{2+} exchanger is mediating the observed glucose reduction of $[Na^+]_i$ is not known yet. In the following study, we used gramicidin D (D: named after the scientist Dubos Hutchkiss who reported its purification (182)), a sodium ionophore, to activate the reverse mode of the Na^+/Ca^{2+} exchanger.

Gramicidins are polypeptide antibiotics, produced by *Bacillus brevis* strain ATCC 8185. They are linear pentadecapeptides, consisting of alternating D- and L- amino acids. Their N-terminal and C-terminal parts are blocked by a formyl group and an ethanolamine group, respectively. Due to the absence of any charged or polar amino acids, these molecules are extremely hydrophobic. The natural mixture consists of 85 %

gramicidin A, the structure of which is: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$. The less abundant gramicidin species B and C differ from gramicidin A in the substitution of Trp^{11} by a phenylalanine or tyrosine residue, respectively. In 5-20 % of the molecules, the Val^1 is substituted by an isoleucine (182).

The gramicidins are synthesized by a multi-enzyme complex (183) and most likely are, together with the tyrocidine type of peptide antibiotics produced by *B. brevis*, involved in gene regulation during the shift from the vegetative phase to the sporulation phase of growth of the bacteria (184). Gramicidin can interact *in vivo* with the DNA-tyrocidine complex, which results in activation of overall RNA synthesis (185-188).

The gramicidin can form transmembrane channels through which water and small cations can pass the membrane (Na^+, K^+). After binding, gramicidin dissipates Na^+-, K^+-, pH- and membrane potential-gradients. In the channel conformation, the molecule is completely hydrophobic at the outside and it spans the membrane as a dimer. Since this conformation resembles that of membrane-spanning part of intrinsic membrane proteins, gramicidin has been a popular model for the study of such proteins (189).

<u>βTC3 cell-line</u>

In this study, the tumor β-cell line βTC3 was used for characterization of gramicidin D effects on insulin release. The maintenance of culture cells is less expensive than isolation of pancreatic islets. In contrast to primary β-cells, tumor cells can be cultured for several cell lines (> 20 passages). In addition, the tumor β-cell line consists only of β-cells, whereas studies utilizing pancreatic islets need special handling and purification procedures in order to isolate primary β-cells. Unfortunately, primary β-

cells have to be used for experiments on the same day of preparation because they desensitize to glucose, probably due to the removal of α-cells.

The βTC3 cell line that has been used in the current study was established from an insulinoma derived from transgenic mice carrying a hybrid insulin-promoted simian virus 40 tumor antigen gene (190). It has been shown that the βTC3 cell line maintained the features of differentiated beta cells for about 50 passages in culture. Furthermore, the cells produce both proinsulin I and proinsulin II and efficiently process each into mature insulin, in a manner comparable to normal beta cells in isolated islets. Electron microscopy reveals typical beta-cell type secretory granules, in which insulin is stored. Insulin secretion is inducible by glucose (190). In addition, βTC3 cells are widely used to understand the regulation of insulin exocytosis. The use of this cell line has helped scientists perform studies in attempt to understand the complex steps involved in insulin release pathway in the primary β-cell line (191-200).

Hypothesis

The hypothesis to be tested in this dissertation is that changes in intracellular Na^+ concentrations ($[Na^+]_i$) modulate intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), membrane potential and intracellular pH (pH_i) in β-cells. Such changes in ion-fluxes across the plasma membrane are likely to affect insulin release and are considered as key signals in triggering insulin exocytosis from the β-cell. In this dissertation, the effect of gramicidin D, a pore forming antibiotic, that permits the free passage of Na^+ across the plasma membrane, to induce and modulate insulin release from β-cells has been studied. This experimental tool was chosen to permit an evaluation of the effect of Na+ influx on membrane depolarization, and the activation of Ca^{2+} influx mechanisms such as voltage-sensitive Ca^{2+} channels and a Na^+/Ca^{2+} exchange. Gramicidin D is considered as a

stimulus that will provide an integrated understanding about the cross-talk among changes in $[Na^+]_i$, $[Ca^{2+}]_i$, membrane potential (and pH_i) in β-cells and the subsequent effects on insulin secretion in β-cells. This study will be the first to provide an integrated understanding linking the different pathways of ion-fluxes in β-cells and providing analyses of the effects of such modulation on insulin release from β-cells.

Purpose of study

The main objective of this study is to characterize the effects of increasing $[Na^+]_i$ induced by gramicidin D on membrane potential, $[Ca^{2+}]_i$, pH_i and insulin release from β-cells. A strategy to be used in this study will be to compare the effects of gramicidin D to those produced by established insulin secretagogues in β-cells. Initial experiments identified gramicidin D as a potent insulin secretagogue in βTC3 cell. Therefore, the detailed understanding of gramicidin D-induced effects on ion-fluxes in β-cells will provide a detailed understanding of the mechanism by which it induces insulin secretion. The knowledge of its mechanism of action will establish new areas of research that will attempt to mimic its effects in β-cells as has been successfully done for other insulin secretagogues. The overall goal of the current study is to provide a better comprehension of the pathways of insulin secretion from β-cells.

METHODOLOGY

Materials

Male Wistar rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained on Tekland Rodent Diet (Indianapolis, IN) ad libtium for 7-10 days prior to use. CMRL-1066, RPMI-1640, glutamine, penicillin, streptomycin and fetal bovine serum were purchased from Life Technologies Inc.(Gaithersburg, MD). Hank's balanced salt solution was from Whittaker Bioproducts (Walkersville, MD). Ficoll, 12-*O*-tetradeconylphorbol-13-acetate (TPA), ATP (disodium salt), gramicidin D, L-Arg, ketoisocaproate, ouabain, and N-methyl-D-glucamine were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase P was purchased from Boehringer Mannheim (Indianapolis, IN), and glucose (Dextrose) was from the National Bureau of Standards (Gaithersburg, MD). [γ-^{32}P]ATP was purchased from Dupont NEN (Boston, MA). Autocamtide-2, sequence KKALRRQETVDAL was synthesized by Bio-Synthesis, Inc. (Lewisville, TX). Cell permeable, acetomethoxy (AM) derivatives of fura-2, sodium benzofuran isophthalate (SBFI-AM) and 2',7'-biscarboxy-ethyl-5',(6')-carboxyfluorescein-AM (BCECF-AM) were purchased from Molecular Probes (Eugene, OR). All other reagents were of the finest grade commercially available.

Cell Culture

βTC3 cells obtained from Dr. Shimon Efrat (Albert Einstein College of Medicine, NY)(passages 33-51), were maintained in RMPI-1640 medium supplemented with 10 % fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and 2 mM L-glutamine. For secretion studies, cells were subcultured into 12-well plates and used at

50-70 % confluency. For imaging experiments, cells were subcultured onto 25 mm glass coverslips 1-3 days prior to experimentation. Cells were counted using a hematocytometer.

Isolation of rat pancreatic islets

Pancreatic islets were isolated from the pancreati of male Wistar rats (200-300 g) with minor modification of the method of Johnson *et al.* (201) The excised pancreatic tissue was incubated with 20 ml of chilled Hank's balanced salt solution (HBSS) (pH 7.25) containing collagenase (0.012 %) at 38.5 ^{o}C for 16 minutes with occasional vigorous shaking. The digestion was stopped by adding chilled HBSS containing 0.5 % bovine serum albumin. The final sediment was resuspended in 4 ml of 25 % Ficoll in HBSS and overlaid with 2 ml of each 23 %, 20.5 %, and 11 % Ficoll (wt/vol). After centrifugation at 700 x g for 15 minutes at 25 ^{o}C, the islets were harvested from the 11 %-20.5 % Ficoll interface with a siliconized glass pipette. The Ficoll was removed by washing the islets with HBSS containing 0.5 % BSA and centrifuging at 700 x g for 2 minutes at room temperature. Following isolation, islets were cultured in CMRL-1066 containing 5.5 mM glucose and supplemented with 2 % L-glutamine, 10 % heat-inactivated fetal bovine serum, 50 units/ml streptomycin and 100 μg/ml penicillin under an atmosphere of 95 % air, 5 % CO_2 until use on the same day.

Dispersion of pancreatic islets

For the purpose of imaging studies of individual islet β-cells, islets were dispersed and cultured onto poly L-Lys treated coverslips.

Preparation of poly L-Lys coated coverslips

The preparation of coated coverslips was performed as described by Sigma Chem. Co. (St. Louis). Poly L-Lys (5 mg, molecular weight 47,000) was dissolved in 50 ml of distilled water. The poly L-Lys solution was sterilized by filteration through 0.2 μM filter (Millipore). One hundred microliters (100 μl) of the filtered poly L-Lys was added to each coverslip (25 mm) which were then incubated for 5 minutes at room temperature. Finally, the coverslips were washed with 5 ml of sterile water to remove excess poly L-Lys, left to dry and were stored until use in a sterile petri-dish.

Dispase digestion

Islets were dispersed by dispase digestion as described by McDaniel *et al.* (202). Islets (1000) obtained from the pancreati of two rats, were incubated with 5 ml of Versene solution (0.53 mM EDTA, 137 mM NaCl, 8 mM Na_2HPO_4, and 2.7 mM KCl) for 7 minutes at room temperature to remove Mg^{2+} from medium (Mg^{2+} inactivates dispase enzyme). Islets were then sedimented to remove EDTA and were resuspended in 4 ml of solution containing reconstituted dispase (5 mg/20 ml HBSS (Boehringer Mannheim)). Digestion at room temperature for 5 minutes released single cells from islets which were harvested into a sterile tube and 2 ml of RPMI-1640 was added to protect cells from further digestion. Three milliliters (3 ml) of dispase solution was then added to the undigested tissue and digestion continued for a further 5 minutes. The batches of single cells were combined and then pelleted by centrifugation at 1,000 rpm for 2 minutes. The cells were then resuspended into 1-2 ml of RPMI-1640 and aliquots (100 μl) were added to each coverslip. Cells were incubated at 37 ^{o}C (air/CO_2:95:5 %) and were used after 24-48 hours.

Intracellular cation measurements

All measurements were conducted using a Nikon Diaphot microscope (Tokyo, Japan), utilizing a dynamic single-cell video imaging technique using Image-1FL Quantitative Fluorescence System (Universal Imaging, West Chester, PA).

$[Na^+]_i$ measurements

βTC3 cells were loaded with 5 μM SBFI/AM in the presence of 0.02 % pluronic acid (to enhance uptake) in KRB medium (KRB; 25 mM Hepes, pH 7.4, 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 % BSA) for 3 hours at 37 °C. Cells were washed twice with 2 ml KRB to remove excess probe and $[Na^+]_i$ measurements were made by monitoring the ratio of SBFI fluorescence at excitation wavelengths of 340 nm and 380 nm as described previously (177).

The absorption shift of SBFI dye that occurs on binding of Na^+ can be determined by measuring the excitation at 340 nm and 380 nm, while monitoring the emission at 510 nm. Spectral shifts that results from binding allow this indicator to be used ratiometrically, thereby making the measurements of Na^+ essentially independent of the extent of dye leaking, cell thickness, cell volume, photobleaching, or dye loading (210). To facilitate cell loading, ester forms of SBFI are commonly used (SBFI-AM). These esters passively cross the plasma membrane, and once inside the cell, are cleaved to cell-impermeant products by intracellular esterases. For this reason, SBFI-AM dye was used in all of the described experiments.

On Na^+ binding to SBFI, the fluorescence of the dye at 340 nm increases, whereas the fluorescence at 380 nm decreases. Therefore, by measuring the ratio of 340:380, an increase in the ratio indicates an increase in $[Na^+]_i$, whereas a decrease in the fluorescence ratio corresponds to a decrease in the intracellular $[Na^+]_i$.

$[Ca^{2+}]_i$ measurements

βTC3 cells or single islet β-cells were loaded with 0.5 μM fura-2/AM for 30 minutes at 37 °C in KRB medium. Cytosolic Ca^{2+} was calculated from the ratio (R) of fura fluorescence at excitation wavelengths of 340 nm and 380 nm according to equations described by Grykiewicz et al (203). The equation used was as follows;

$$[Ca^{2+}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times F_o/F_1.$$

R_{max} is the value of R at saturating $[Ca^{2+}]_i$, whereas R_{min} is the value of R at low $[Ca^{2+}]_i$. F_o/F_1 is the ratio of fluorescence at 380 nm in low $[Ca^{2+}]_i$ to that in high $[Ca^{2+}]_i$. K_d is the dissociation constant of fura-2 and is equal to 224 nM. Condition of saturating $[Ca^{2+}]_i$ were achieved by adding the Ca^{2+} ionophore A23187 (1 μM) whereas, conditions of low $[Ca^{2+}]_i$ were obtained by adding EGTA (4-5 mM).

Unlike SBFI loading period which takes 3 hours, loading of βTC3 cells with fura takes 30 minutes (226). Fura-2 dye permits excitation measurements at the same excitation wavlenghts of SBFI. On Ca^{2+} binding, the fluorescence of fura-2 at 340 nm increases whereas, the fluorescence at 380 nm decreases. Thus, by measuring the ratio of 340:380, an increase in the ratio indicates an increase in $[Ca^{2+}]_i$ while a decrease indicates a decrease in $[Ca^{2+}]_i$. In addition, this ratio method makes the measurement of Ca^{2+} concentration essentially independent of the extent of dye loading, cell thickness, cell volume, photobleaching and dye leakage, a characteristic that is similar to Na^+-measurements.

pH_i Measurement

Cells were loaded with 1 μM BCECF-AM in KRB for 30 minutes at 37 °C. pH_i was determined from the ratio of BCECF fluorescence at 505 nm and 470 nm as described previously (169). BCECF-AM, freely crosses cell membranes, thus facilitates

loading. Once inside the cell, BCECF-AM is hydrolyzed by intracellular esterases to release BCECF, which is retained by the cell. The dye is typically used as a dual-excitation wavelength of 505 nm and 470 nm. Therefore, signal errors caused by variations in concentrations, path length, leakage and photobleaching are canceled using this ratio method. Emission of BCECF is usually at 540 nm.

Assay of membrane potential

A suspension of βTC3 cells created by Trypsin/EDTA treatment of cells from a culture flask, was suspended in KRB (no BSA) and incubated at 37 ^{o}C. βTC3 cells (2 x 10^5) were loaded with Oxonol VI (1 μM) until levels of fluorescence stabilized (3-7 minutes). Secretagogues were then added to cells with brief stirring. Fluorescence of the dye at excitation 590 nm and emission at 660 nm was followed using a Shimadzu spectrofluorophotometer model no. FR5000U (Shimadzu Scientific Instruments, Columbia, MD). An increase in the fluorescence indicates membrane hyperpolarization whereas a decrease in fluorescence indicates membrane depolarization (204).

Insulin secretion

In the these studies, two models of insulin secretion were used. The first model was the **static model**. Usually, βTC3 cells were cultured in wells whereas, isolated islets were placed in siliconized tubes. The stimulation started with replacement of basal medium with fresh medium containing secretagogue. Finally, the medium was collected and assayed for insulin. Therefore, this model provides a cumulative measurement of insulin secretion over a period of time.

The second model used is the **perifusion model**. In this model, βTC3 cells or islets were placed on membrane filters in perifusion chambers. The stimulation started

with the replacement of basal perifusion medium with fresh medium containing the secretagogue. Unlike the static model, this model provided analyses of the insulin secretion pattern over a period of time studied.

Static model

βTC3 cells were washed twice in KRB and preincubated in the same medium for 60 minutes under an atmosphere of 95 % O_2/5 % CO_2. This medium was then replaced with fresh medium containing gramicidin D (1 μM) or vehicle (dimethylsulfoxide, DMSO) as control. Where studied, inhibitors were added simultaneously with gramicidin D. After a further 60 minutes incubation, the medium was removed, briefly centrifuged to sediment dislodged cells and the insulin content of the supernatant was determined by double antibody radioimmunoassay (144). All incubation volumes were 1 ml and were conducted at 37 °C.

Pancreatic islets (20/tube) into 12x75-mm siliconized borosilicate tubes were preincubated for 30 minutes at 37 °C with gentle shaking in KRB basal containing 3 mM glucose and 0.1 % BSA (200 μl) under an atmosphere of O_2/CO_2 (95:5 %). The medium was replaced with fresh KRB basal medium alone or supplemented with gramicidin D (1 μM) and the incubation continued for 30 minutes. The incubation was terminated by the removal of the medium and its insulin content was determined as described above.

Perifusion model

A suspension of βTC3 cells created by trypsin/EDTA treatment of cells from a culture flask, was pelleted. The cells were allowed to recover by incubation in suspension in RPMI-1640 medium for 3 hours. The cells (5-10 x 10^6) were then washed with basal KRB and placed on 8 μM-mesh nylon filters and placed in a Swinnex perifusion chamber

(Millipore). βTC3 cells were perifused with basal KRB for 15 minutes at a flow rate of 0.5 ml/minute and samples were collected every 2.5 minutes. In case of inhibitor studies, the drug of interest was introduced in the last 5 minutes of basal perifusion. At time 15 minutes, perifusion was continued in KRB containing gramicidin D with or without inhibitor for 60 minutes at the same flow rate. During this phase, samples were collected every two minutes. Finally, the perifusion medium was changed back to basal KRB, and cells were perifused for a further 15 minutes. Samples were collected every 5 minutes in this phase.

Conditions for perifusion of islets were essentially the same as described with cells with the following modifications. Pancreatic islets (100) were perifused with KRB containing basal glucose (3 mM) for 30 minutes at a flow rate 1 ml/minute at 37 ^{o}C and samples were collected every 5 minutes. Following the 30 minutes, islets were perifused with KRB containing gramicidin D (1 μM) for 45 minutes at 37 ^{o}C. During this phase, samples were collected every 1 minute. Finally, the perifusion was changed to basal medium and islets were perifused for another 15 minutes and samples were collected every 5 minutes. Insulin content of samples was determined by radioimmunoassay.

Assay of Ca^{2+}/Calmodulin-dependent protein kinase (Cam kinase II)

Cam kinase II was assayed by modification of a method previously described by Schulman *et al.*. (205). The method was developed to assay the Ca^{2+}-independent form of the enzyme. As $[Ca^{2+}]_i$ increases, Cam kinase II undergoes autophosphorylation and is converted to a Ca^{2+}-independent form. Therefore, the assay is performed in the presence and absence of Ca^{2+}. The activity detected in the absence of Ca^{2+} represents the proportion of Cam kinase II that underwent autophosphorylation.

Cells to be used in these experiments, were grown in petri-dishes (35 mm, Costar) to 70-80 % confluency. On the day of the experiment, cells were washed with KRB medium containing no glucose and preincubated for 1 hour at 37 °C. Stimulation was initiated by the removal of the bathing medium and replacement with KRB containing vehicle alone (dimethylsulfoxide) or gramicidin D (1 μM). Where studied, inhibitors were added simultaneously with gramicidin D. Incubations were continued for the required time (0-20 minutes) at 37 °C in a shaking bath. To terminate the incubation, medium was discarded and cells were washed with one ml of ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 10 mM sodium pyrophosphate, 0.4 mM ammonium molybdate and 100 μg/ml leupeptin). One hundred microliters (100 μl) of fresh homogenization was added and cells were immediately homogenized by sonication (10 pulses, setting 3, 30 % duty cycle). The resultant homogenate was used for the assay of Cam Kinase II activity.

Cam kinase II was assayed in a reaction mixture containing 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin (fraction V), 10 μM autocamtide-2, 20 μM ATP (specific activity, 40 Ci/mmole) 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. The total reaction volume was 50 μl. The assay was initiated by the addition of 10 μl of cell homogenate (approximately 1-3 mg/ml) and continued for 30 seconds at 30 °C. The reaction was terminated by the addition of ice-cold trichloroacetic acid (25 μl, 15 %). Tubes were placed on ice for 20 minutes to precipitate large proteins, which were then sedimented by centrifugation for 1 minute at 12,000 x g (Sorvall Microspin).

Thirty-five microliters (35 μl) of the resulting supernatant was spotted onto 5-cm by 2-cm strips of phosphocellulose paper (Whatman P-81). Strips were washed 5 times in 500 ml of distilled H_2O, dried at 110 °C for 10 minutes and $^{32}P_i$ incorporation into autocamtide-

2 was determined by Cerenkov radiation (Beckman Instruments). In the described experiments, $^{32}P_i$ incorporation into autocamtide-2 in the absence of Ca^{2+}/calmodulin (autonomous CaM kinase II) is expressed as a percentage of incorporation in the presence of these cofactors (Ca^{2+}/calmodulin-dependent Cam kinase II activity).

Assay of myosin light chain phosphorylation

Solubilization of myosin light chain

βTC3 cells were subcultured into 60 mm petri-dishes and used at 70-80 % confluency. Cells were starved in serum-free medium overnight. On the day of experiment, cells were washed twice in KRB and incubated for 30-45 minutes at 37 ^{o}C. Cells were stimulated with fresh KRB containing secretagogue for 0-15 minutes. To terminate the reaction, cells were washed with ice-cold KRB (basal) and Trichloroacetic acid (TCA) and DTT to final concentrations of 20 % and 2 mM respectively, were added. Cells were then scraped from the dishes and transferred to tubes and kept on ice for 10-15 minutes (to precipitate proteins). The tubes were then centrifuged at 4 ^{o}C for 1 minute (10,000 x g, Sorvall Microspin). The supernatant was discarded and the pellet was washed with ether (4X, 5 minutes) to extract TCA. The pellets were allowed to dry at room temperature for 45-60 minutes. The pellets were suspended in 50-70 µl of urea sample buffer (2 ml of urea sample buffer consists of; 1.83 ml of 8 M urea, 167 µl of urea gel buffer (241 mM Tris-base and 266 mM glycine), 40 µl of 0.5 M DTT, 100 µl of saturated sucrose and 40 µl of 0.2 % bromophenol blue). Pellets were sonicated (10 pulses, setting 3, 30 % duty cycle) on ice and the extraction was allowed to continue by vortexing for 1.5-2 hours at room temperature. The protein concentration was determined by Bradford assay (see section 11).

Glycerol/urea/gel electrophoresis

Urea extracts of cells homogenates were subjected to urea/glycerol electrophoresis to permit the separation of differently charged species of MLC_{20}. Glycerol/urea/electrophoresis was performed as previously described by Elson *et al* . (206). Phosphorylated MLC_{20} were separated in 1.5 mm-thick mini-gels containing 40 % glycerol, 10 % acrylamide (with 5 % bisacrylamide), 24 mM Tris, 26 mM glycine (pH 8.6). Before loading the samples, the gel was pre-electrophoresed for 1 hour at 400 volts with 2 mM of both DTT and thioglycolic acid added to the upper reservoir. Samples (70-90 µg/lane) were run for 90 minutes at 400 volts and transferred to 0.2 µM nitrocellulose (S&S Inc.) for 60-70 minutes at 18 volts (4 ^{o}C). The nitrocellulose was dried at room temperature and immunodetection of phosphorylated forms of MLC_{20} was performed as described below.

Western blotting

Immunoblotting was performed as described by Tropix Inc. The nitrocellulose membrane was washed twice in 1X PBS (0.058 M Na_2HPO_4 and 0.017 M NaH_2PO_4) and blocked for 2 hours (I-block, Tropix Inc.). After blocking, the membrane was briefly washed twice in 1X PBS for 10 minutes followed by incubation with the first antibody (rabbit anti bovine tracheal myosin light chain (1:1000), Stull *et al* (263)) for 2 hours with continuous shaking at room temperature). The first antibody was then removed and the membrane was washed for 10 minutes before adding the second antibody (goat anti-rabbit, 1:20,000). After 30 minutes incubation, the membrane was washed for 15 minutes. Detection of MLC_{20} was performed with Tropix chemiluminescent detection reagents (using CSPD as a chemiluminescent substrate). The x-ray film was quantified by densitometry.

Assay of plasma $Na^{\pm}/K^{\pm}$ ATPase activity

The activity of Na^+/K^+ ATPase was followed by measuring the concentration of released inorganic phosphate (P_i) by modification of the metohd described by Kyaw *et al.* (207).

Preparation of P_i standard curve

KH_2PO_4 (7.05 mM, dissolved in H_2O) was used for preparation of various concentrations of P_i (0.05-0.6 µMole P_i (10-120 µM KH_2PO_4)). To 2 ml volume sample, 1.425 ml of Triton-X (2.5 %, in H_2O), 75 µl perchloric acid (60 %) and 1.5 ml of ammonium molybdate (2.5 % in 3N H_2SO_4) were added and absorbance was measured spectrophometrically at 375 nm after 10 minutes. The blank was similar to sample preparation except replacing sample with an equivalent volume of distilled H_2O.

Preparation of βTC3 cell/islet plasma membrane homogenates

Plasma membrane proteins were prepared using the method of Levin *et al.* (208). βTC3 cells (10×10^6) or islets (800) were washed twice with homogenization buffer (0.25 M sucrose and 1 mM Tris-EDTA, pH 7.4) and then sonicated (10 pulses, setting 3, 30 % duty cycle) in the same homogenization buffer (750 µl) for 10 seconds. The homogenates were centrifuged at 35,000 x g for 30 min at 4 ^oC. The pellets were resuspended in ice-cold buffer Tris-EDTA (1 mM, pH 7.4) and used as a source of the Na^+/K^+ ATPase on the same day or stored at -70 ^oC until use.

$Na^{\pm}/K^{\pm}$ ATPase Enzyme analysis

The activity of the Na^+/K^+ ATPase was defined as the difference in inorganic phosphate liberated from ATP in the presence and absence of Na^+ and K^+. The

incubation medium contained 0.5 mM EDTA, 21 mM gly-gly, 21 mM histidine, 3.6 mM $MgCl_2$ with or without 100 mM NaCl and 20 mM KCl. Membrane aliquots from islets or βTC3 cells (50 µl (20 µg)) was added to 0.5 ml of the incubation medium, mixed and incubated at 37 °C for 10 minutes. The assay was initiated by adding 40 µl of Tris-ATP (final concentration 3 mM) to all tubes and the incubation continued in a 37 °C shaker water bath for 30 minutes. The reaction was stopped by immersing the tubes in ice bath and adding perchloric acid to a final concentration of 10 %. Samples were centrifuged at 1100 x g for 10 minutes and the supernatants were assayed spectrophotmetrically for inorganic phosphate as described in section 10.1.

Protein determination

Protein concentration was determined by Bradford assay (Bio-Rad) using bovine serum albumin as standard (209).

Statistical Treatment of Data

Statistical significance was assessed by Student's *t* test.

RESULTS

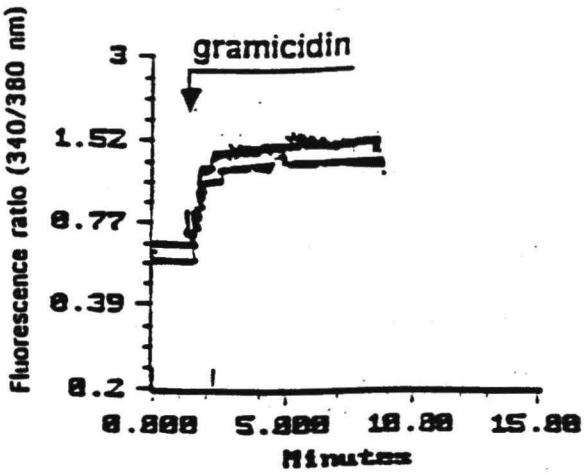
Effect of gramicidin D and other insulin secretagogues on $[Na^+]_i$ in βTC3 cells

The main objective of this study was to characterize the effect of the modulation of ion-fluxes induced by gramicidin D on insulin secretion from βTC3 cells. Gramicidin D is a pore-forming peptide specific for monovalent cations, but with greatest affinity for Na^+. Indeed, gramicidin D has been utilized as a Na^+ ionophore in numerous cell types (177, 210 and 212). Thus, the effect of gramicidin D on $[Na^+]_i$ in βTC3 cells was studied. A common fluorescent dye used for the measurement of $[Na^+]_i$ is SBFI (210). SBFI has been used successfully for $[Na^+]_i$ measurements in β-cells (177), fibroblasts and lymphocytes (210), colonic crypts (211), rabbit gastric gland cells (212), human platelets (213), MDCK cells (214) and guinea pig myocytes (215).

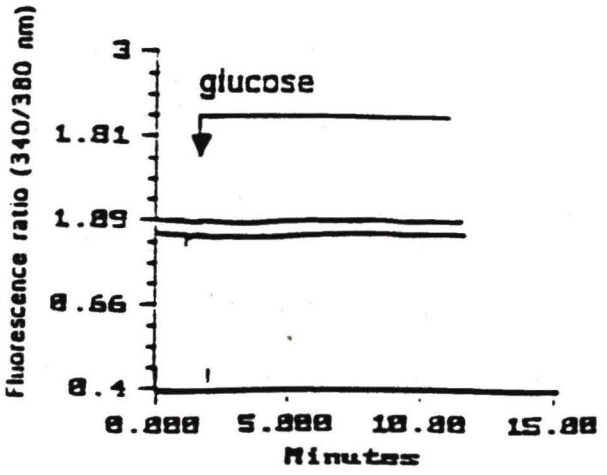
βTC3 cells were loaded with SBFI-AM as described in Methods (section 5.1). As $[Na^+]_i$ increases, the fluorescence ratio of 340/380 nm increases. In βTC3 cells, it was found that a minimum of three hours was needed to load sufficient amounts of SBFI-AM. Pluronic F-127 was added to facilitate the loading of the SBFI-AM dye as demonstrated previously (210). The exposure of βTC3 cells to gramicidin D (1 μM) promoted a rapid increase in the fluorescence ratio of SBFI excited at 340:380 nm indicative of increased $[Na^+]_i$ (Fig. 3A). The increase in $[Na^+]_i$ was monophasic and remained elevated for at least 30 minutes. This observation was in agreement with the reported effects of gramicidin D on $[Na^+]_i$ in lymphocytes and fibroblasts (210) and was likely the result of the pore-forming antibiotic, gramicidin D, to equilibrate cytosolic and extracellular $[Na^+]$ (Na^+_i and Na^+_o). This resulted in the net influx of Na^+ into the cell under normal physiological conditions where $Na^+_o > Na^+_i$.

Fig. 3 Effect of gramicidin D on $[Na^+]_i$ βTC3 cells; comparison with known insulin secretagogues.

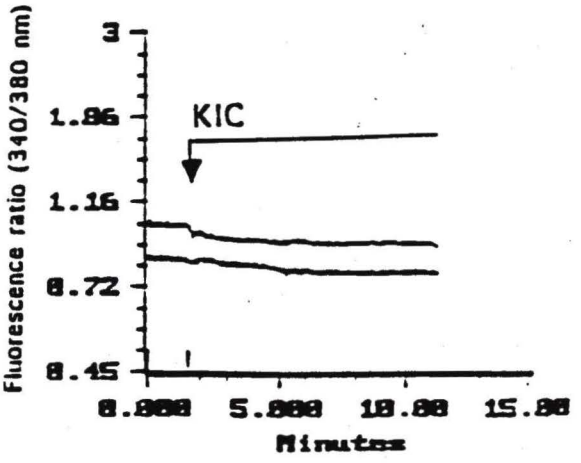
βTC3 cells were loaded with the Na^+-sensitive fluorescent dye SBFI/AM (5 μM) and exposed to: (A) gramicidin D (1 μM), (B) glucose, (20 mM), (C) KIC (ketoisocaproate, 20 mM), (D) KCl (40 mM) and (E) L-Arg (L-Arginine, 20 mM) at the time indicated by an arrow.


gramicidin
Fluorescence ratio (340/380 nm)
3
1.52
0.77
0.39
0.2
0.000
5.000
10.00
15.00
Minutes

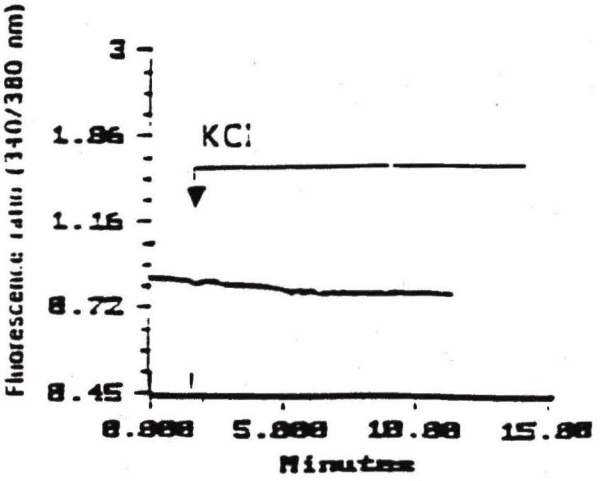
A


glucose
Fluorescence ratio (340/380 nm)
3
1.81
1.89
0.66
0.4
0.000
5.000
10.00
15.00
Minutes

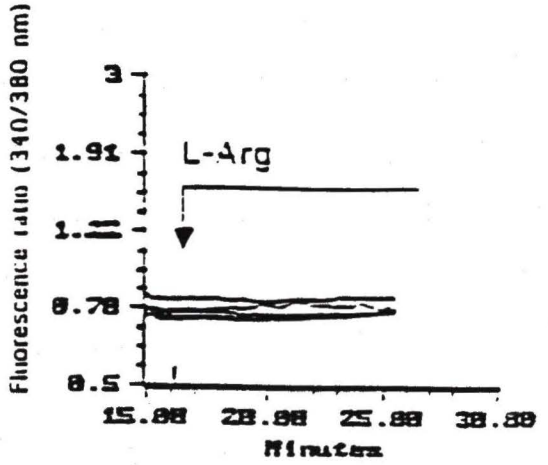
B


KIC
Fluorescence ratio (340/380 nm)
3
1.86
1.16
0.72
0.45
0.000
5.000
10.00
15.00
Minutes

C


KCl
3
1.86
1.16
0.72
0.45
0.000
5.000
10.00
15.00
Minutes

D


L-Arg
Fluorescence ratio (340/380 nm)
3
1.91
0.78
0.5
15.00
20.00
25.00
30.00
Minutes

E

By contrast, other insulin secretagogues including glucose (20 mM), ketoisocaproate (KIC)(20 mM), KCl (40 mM) and L-Arg (20 mM) had no effect on $[Na^+]_i$ (Figs. 3B-E). In the case of cells stimulated with either KCl or KIC, a modest decrease in $[Na^+]_i$ was occasionally observed but the response was not significant or reproducible. These results suggest that gramicidin D, unlike other insulin secretagogues, had a profound effect to increase $[Na^+]_i$ in βTC3 cells as expected.

Effect of gramicidin D on $[Ca^{2+}]_i$ in βTC3 cells

The disruption of the established Na^+ gradient across the plasma membrane of a resting β-cell has previously been shown to result in Ca^{2+} influx and elevation of $[Ca^{2+}]_i$ (181). Therefore, it was predicted that the influx of Na^+ would cause a rise in $[Ca^{2+}]_i$ in βTC3 cells. In order to evaluate the effect of gramicidin D and other insulin secretagogues on $[Ca^{2+}]_i$, βTC3 cells were loaded with the Ca^{2+}-selective fluorescence probe, fura-2, as described in Methods (section 5.2). Fura-2 has been used successfully used to measure $[Ca^{2+}]_i$ in hamster insulin tumor (HIT) cells (216-218), RINm5F cells (149, 219-220), primary β-cells (221-226) and βTC3 cells (195).

The experiments in the following studies were divided into three parts. The first part probed the effect of known insulin secretagogues on $[Ca^{2+}]i$ in βTC3 cells to establish methodology. The second part evaluated the effect of gramicidin D on $[Ca^{2+}]_i$ and compared its effects with those generated by known insulin secretagogues. The final part assessed the potential mechanisms of gramicidin D-induced increase in $[Ca^{2+}]_i$.

Conceptually, there are two major pathways of raising $[Ca^{2+}]_i$ in mammalian cells, including β-cells. The first involves the influx of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels (VSCC)(see Introduction) and the second pathway involves the mobilization of intracellular Ca^{2+} from the intracellular pools including the

endoplasmic reticulum. These pools can be differentially accessed by insulin secretagogues. Glucose induces both extracellular Ca^{2+} influx and the mobilization of intracellular Ca^{2+} (76). By contrast, depolarizing concentrations of KCl induce an increase in $[Ca^{2+}]_i$ by activation of the VSCC (221) and no there is no evidence of the involvement of mobilization of intracellular Ca^{2+} with this response (based on absence of IP_3 production (221)). The muscarinic agonist carbachol, acts primarily by mobilizing intracellular Ca^{2+} in β-cells (195). The three insulin secretagogues therefore, have different mechanisms of action in raising $[Ca^{2+}]_i$ and were chosen as positive controls in the following experiments.

Effect of known insulin secretagogues on $[Ca^{2+}]_i$ in βTC3 cells

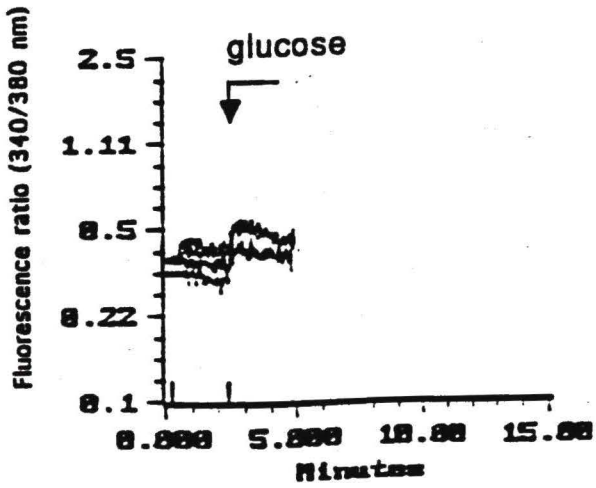
In initial experiments, βTC3 cells pre-loaded with fura-2, were stimulated with glucose (20 mM). As shown in Fig. 4A, glucose induced a modest monophasic increase in $[Ca^{2+}]_i$, an observation that was identical to that reported by Wolf *et al.* (195).

Next, depolarizing concentrations of KCl (40 mM) were tested. As shown in Fig. 4B, KCl induced a profound biphasic increase in $[Ca^{2+}]_i$. The response was characterized by a sharp increase in $[Ca^{2+}]_i$ that peaked at 30 seconds-1 minute, followed by a second sustained phase, in which $[Ca^{2+}]_i$ was maintained above basal levels.

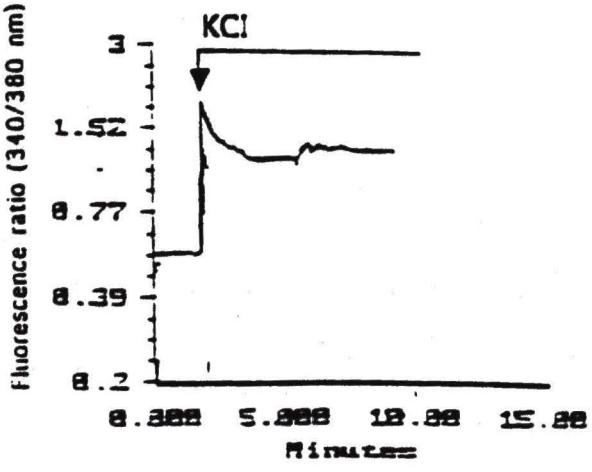
A similar biphasic response was reported by different groups characterizing the effects of KCl on $[Ca^{2+}]_i$ in β-cells (226). Finally, the effect of carbachol on $[Ca^{2+}]_i$ was evaluated. As shown in Fig. 4C, carbachol (500 μM) induced a transient increase in $[Ca^{2+}]_i$ which declined rapidly to basal levels. These findings were also similar to those reported by Wolf *et al.* (195).

Fig. 4 Effects of insulin secretagogues on $[Ca^{2+}]_i$ in βTC3 cells.

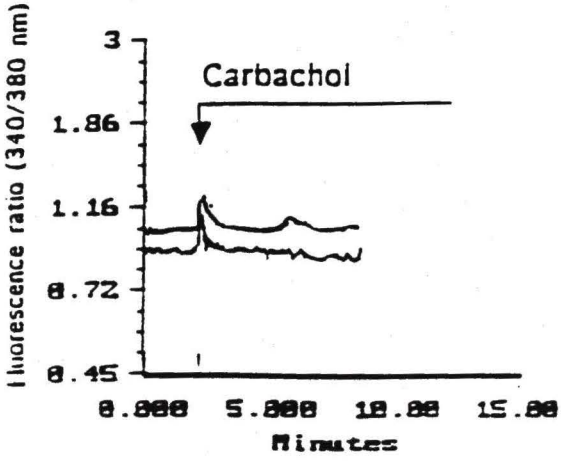
βTC3 cells were loaded with 0.5 μM fura-2 as described in Methods and exposed to: (A) glucose (20 mM), (B) KCl (40 mM) and (C) carbachol (500 μM) at the time indicated. An increase in 340/380 nm excitation ratio is indicative of an increase in Ca^{2+} concentration ($[Ca^{2+}]_i$).


glucose
Fluorescence ratio (340/380 nm)
2.5
1.11
0.5
0.22
0.1
0.000
5.000
10.00
15.00
Minutes

A


KCl
Fluorescence ratio (340/380 nm)
3
1.51
0.77
0.39
0.2
0.000
5.000
10.00
15.00
Minutes

B


Carbachol
Fluorescence ratio (340/380 nm)
3
1.86
1.16
0.72
0.45
0.000
5.000
10.00
15.00
Minutes

C

In summary, the findings of above experiments reproduced the published effects by the three insulin secretagogues. Therefore, Ca^{2+}-measurements using fura-2 in βTC3 cells were proven successful in my system.

Effect of gramicidin D on $[Ca^{2+}]_i$ in βTC3 cells.

Next, the effect of gramicidin D (1 μM) on $[Ca^{2+}]_i$ in βTC3 cells was studied. As shown in Fig. 5A, gramicidin D induced a profound, biphasic increase in $[Ca^{2+}]_i$. This response was characterized by an initial increase that peaked at 1-2 minutes, followed by a second phase in which $[Ca^{2+}]_i$ was maintained at an elevated level relative to the basal concentration in cells prior to stimulation. This response was observed in all cells studied (a total of 40 cells). When a typical response was quantitated, $[Ca^{2+}]_i$ of ionophore-treated cell was determined to be 518 ± 64 nM (mean ± SD, n =2) at the first phase peak and 168 ± 46 nM in the sustained second phase, relative to 67 ± 37 nM in a resting cell.

The response in $[Ca^{2+}]_i$ induced by gramicidin D was quantitatively similar to that induced by KCl. However, further comparison of the profile of increased $[Ca^{2+}]_i$ by KCl and gramicidin D revealed a significant difference. In the case of gramicidin D, the initial peak was characterized by a gradual increase and decrease in $[Ca^{2+}]_i$, and was in contrast to the sharp incline/decline in $[Ca^{2+}]_i$ observed in cells stimulated by KCl. This difference suggested the involvement of distinct mechanism(s) in gramicidin D-induced increase in $[Ca^{2+}]_i$ relative to KCl. Experiments were therefore designed to identify such different mechanisms.

The first strategy of these experiments was to test the ability of insulin secretagogues to modulate gramicidin D-induced increase of $[Ca^{2+}]_i$. βTC3 cells were treated with a maximally effective concentration of gramicidin D (1 μM) in the presence of glucose, KCl and carbachol (Figs. 5B, C and D). As shown in Fig. 5B, the

simultaneous addition of glucose (20 mM) and gramicidin D (1 μM) produced a biphasic Ca^{2+}-response that was essentially identical to that produced in the absence of glucose (see Fig. 5A). Furthermore, the addition of glucose during the second phase of gramicidin D-induced increase in $[Ca^{2+}]_i$ produced no further increase or decrease in $[Ca^{2+}]_i$. The failure of glucose to modify gramicidin D-induced increase of $[Ca^{2+}]_i$ suggests that glucose and gramicidin D have a common component in the mechanism(s) of raising $[Ca^{2+}]_i$. However, since the effect of gramicidin D was quantitatively larger than that induced by glucose, it is possible that small changes induced by glucose were 'masked' by the presence of high levels of $[Ca^{2+}]_i$ induced by the ionophore.

The simultaneous addition of KCl (40 mM) and gramicidin D (1 μM) produced a monophasic increase in $[Ca^{2+}]_i$ that was qualitatively distinct from either agent alone (Fig. 5C). This combination, produced a monophasic increase in $[Ca^{2+}]_i$ that contrasted with the biphasic response induced by either stimulus alone. The response was characterized by a sharp increase in $[Ca^{2+}]_i$ that reflected the effect induced by KCl, but there was no decline to lower levels as previously observed with either KCl and gramicidin D when added individually. This observation suggested that gramicidin D and KCl increased $[Ca^{2+}]_i$ by different mechanisms which are additive when added simultaneously. The addition of concentrations of KCl greater than 40 mM failed to produce a similar monophasic response implying a full activation of Ca^{2+} influx by VSCC. Finally, the effect of carbachol on gramicidin D-induced increase in $[Ca^{2+}]i$ was tested. As shown in Fig. 5D, the addition of carbachol (500 μM) during the second phase of gramicidin D-induced increase in $[Ca^{2+}]_i$ produced a transient increase in $[Ca^{2+}]_i$ which declined rapidly to lower levels of $[Ca^{2+}]_i$ observed prior to muscarinic stimulation. This effect was quantitatively similar to that elicited by carbachol when added alone (Fig. 4C). This observation suggests that the intracellular pools of Ca^{2+}

(IP_3-sensitive pools) were intact and that they had not been drained upon the exposure of βTC3 cells to gramicidin D. This further implies that the mechanism of gramicidin D-induced increase in $[Ca^{2+}]_i$ was independent of the mobilization of Ca^{2+} from the IP_3-sensitive pool, but rather the result of Ca^{2+} influx from the extracellular medium.

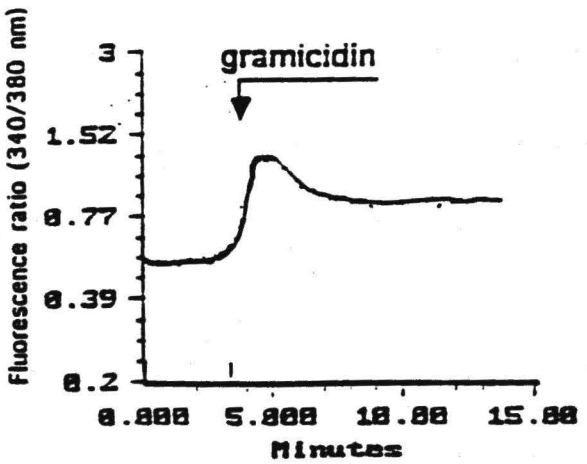
To test this hypothesis, another experiment was performed to probe the source of Ca^{2+} in gramicidin D-induced increase of [Ca $^{2+}$]i. EGTA (2 mM) at a concentration that had been previously shown to chelate extracellular Ca^{2+} without affecting the intracellular pool of Ca^{2+} (203) was added to the basal KRB medium, and the effect of gramicidin D on $[Ca^{2+}]_i$ was evaluated. As demonstrated in Fig. 6, gramicidin D failed to increase $[Ca^{2+}]_i$ under these conditions suggesting that the source of Ca^{2+} was solely extracellular. In summary, the described experiments demonstrated, for the first time, that gramicidin D was capable of increasing $[Ca^{2+}]_i$ in β-cells via a mechanism that involves the influx of extracellular Ca^{2+}. Some of these observations were recently published in Biochemical Biophysical Research Communications (144).

Mechanism of gramicidin D-induced increase in $[Ca^{2+}]_i$

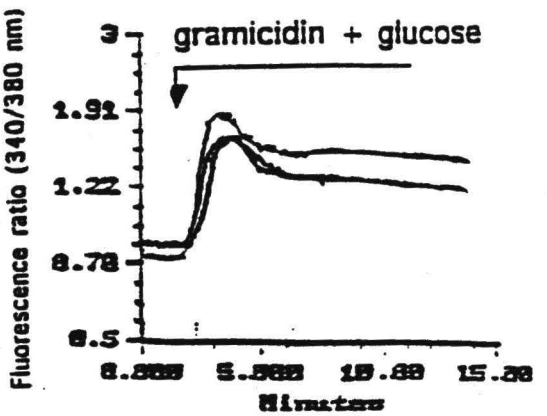
Having established the source of Ca^{2+} that resulted in an increase in $[Ca^{2+}]_i$ after gramicidin D treatment, it was important to identify the mechanism(s) involved in mediating extracellular Ca^{2+}-influx. There are at least two known pathways that are capable of promoting extracellular Ca^{2+}-influx in β-cell under physiological conditions. The first pathway is known to involve VSCC. The ability of gramicidin D to promote Na^+ influx was anticipated to cause the accumulation of positive charges intracellularly, thereby causing plasma membrane depolarization. This depolarization would be then expected to activate VSCC.

Fig. 5 Effect of gramicidin D on $[Ca^{2+}]_i$ in βTC3 cells. Modulation by insulin secretagogues.

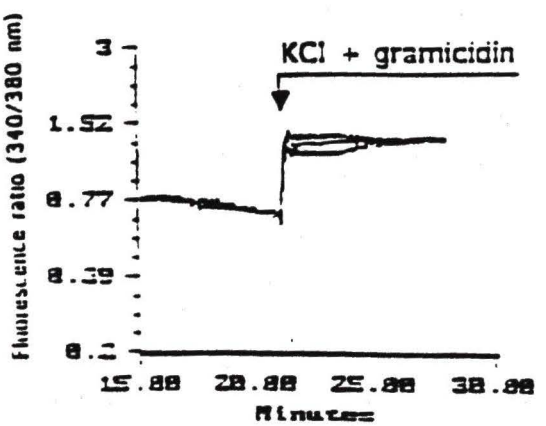
βTC3 cells preloaded with fura-2 as described in Methods were exposed to: (A) gramicidin D (1 μM), (B) gramicidin D (1 μM) and glucose, (C) a combination of gramicidin D (1 μM) and KCl (40 mM), and (D) gramicidin D (1 μM) followed by carbachol addition (500 μM). Arrows indicate the time of addition of each stimulus.


gramicidin
Fluorescence ratio (340/380 nm)
3
1.52
0.77
0.39
0.2
0.000
5.000
10.00
15.00
Minutes

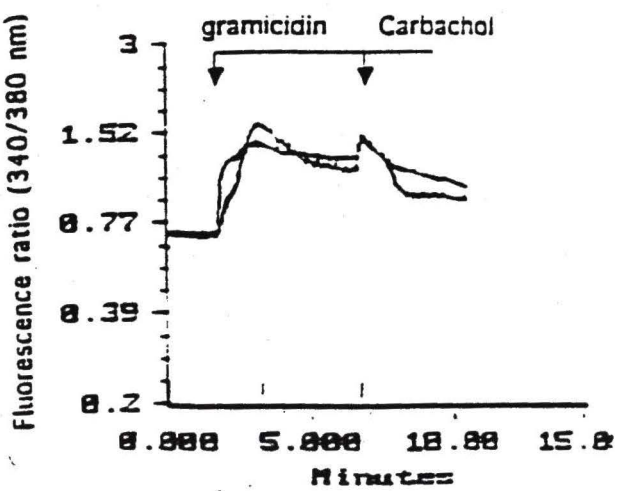
A


gramicidin + glucose
Fluorescence ratio (340/380 nm)
3
1.91
1.22
0.78
0.5
Minutes

B


KCl + gramicidin
3
1.52
0.77
15.00
20.00
25.00
30.00
Minutes

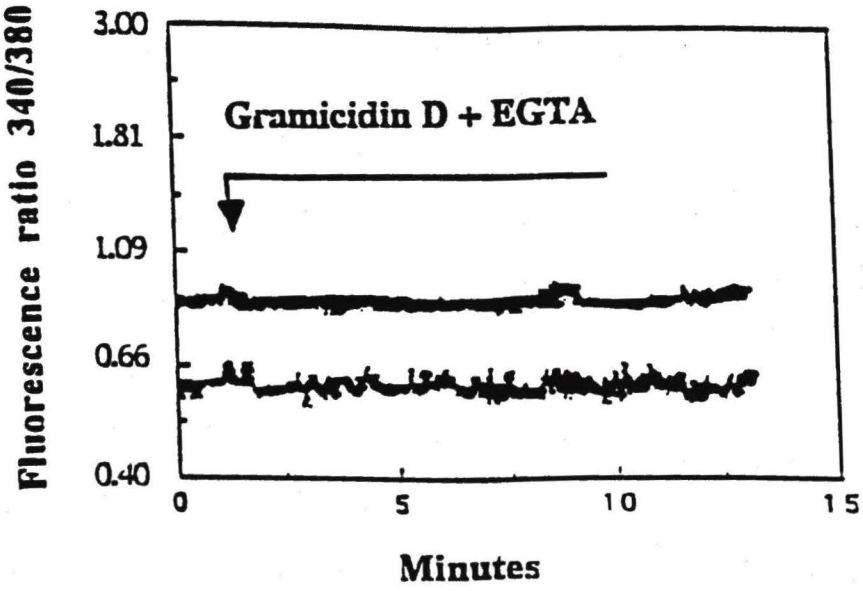
C


gramicidin
Carbachol
Fluorescence ratio (340/380 nm)
3
1.52
0.77
0.39
0.2
0.000
5.000
10.00
Minutes

D

Fig. 6 Effect of the removal of extracellular Ca^{2+} on gramicidin D-induced increase in $[Ca^{2+}]_i$ in βTC3 cells.

βTC3 cells were loaded with 0.5 μM fura-2 as described in Methods and exposed to EGTA (2 mM) for 2 minutes prior to gramicidin D (1 μM).


Fluorescence ratio 340/380
3.00
1.81
1.09
0.66
0.40
Gramicidin D + EGTA
0
5
10
15
Minutes

A second mechanism is mediated via a Na^+/Ca^{2+} exchanger. In cells expressing this protein, the exchanger promotes Na^+ efflux in exchange for Ca^{2+}. The elevation of $[Na^+]_i$ by gramicidin D, as observed in section 1 of Results, was reasoned to activate a Na^+/Ca^{2+} exchanger. The exchanger was anticipated to promote the efflux of intracellular Na^+ while promoting the influx of extracellular Ca^{2+}. The experiments in this section were designed to address the possible involvement of either mechanism in gramicidin D-induced increase in $[Ca^{2+}]_i$. The principal strategy used was to evaluate the effect of putative inhibitors of both mechanisms on the observed increase in $[Ca^{2+}]_i$ induced by gramicidin D.

Testing the effect of VSCC inhibitors on KCl and gramicidin D-induced increase in $[Ca^{2+}]_i$

In the initial experiments, the effects of verapamil, nifedipine and KN-62, potent inhibitors of the VSCC, on KCl-induced increase in $[Ca^{2+}]_i$ were tested. Depolarizing concentrations of KCl are thought to promote an increase in $[Ca^{2+}]_i$ exclusively via the activation of VSCC. Therefore, it was predicted that these inhibitors would profoundly inhibit KCl-induced increase in $[Ca^{2+}]_i$. A further aim of these experiments was to determine the condition(s) needed to totally inhibit VSCC. It was reasoned that this would help to repeat such condition on gramicidin D-induced increase in $[Ca^{2+}]_i$ to test if additional mechanisms of extracellular Ca^{2+}-influx exist in βTC3 cells activated by gramicidin D.

There were two phases of Ca^{2+}-response induced by gramicidin D and KCl. The experiments were designed to test the effect inhibitors on each phase. Therefore, the inhibitor was added during the second phase where it was anticipated that VSCC would exist in an active state, as well as a simultaneous addition with stimuli where it was

anticipated that VSCC would be in the inactive state at the time of addition. An alternative approach for the latter condition, was the pretreatment of cells with the inhibitor.

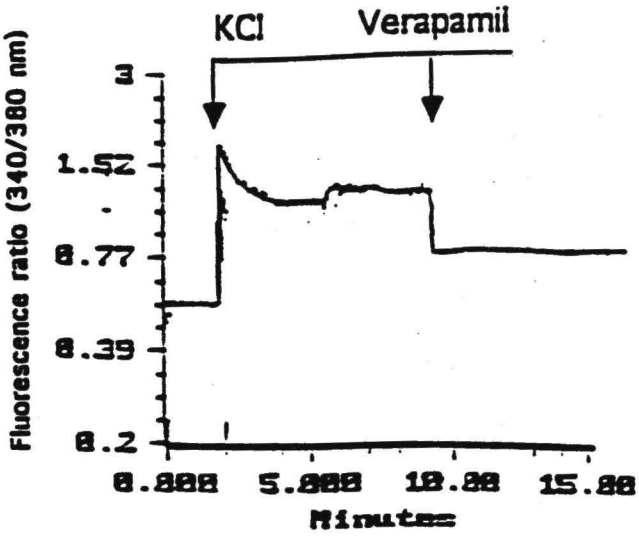
As shown in Fig. 7A, the addition of verapamil (20 μM) during the second phase of KCl-induced increase $[Ca^{2+}]_i$, suppressed the increase in $[Ca^{2+}]i$ by 72 %. Similar effects were produced by nifedipine (20 μM)(Fig. 7B). Nifedipine inhibited KCl-induced increase in $[Ca^{2+}]_i$ by approximately 80 %. Also, KN-62 inhibited KCl-induced increase $[Ca^{2+}]_i$. The ability of all three drugs to suppress the increase in $[Ca^{2+}]_i$ in the second phase suggested the involvement of VSCC in mediating KCl-induced Ca^{2+}-influx. However, the inhibition was not complete which may reflect the inability of the inhibitors to totally suppress an activated VSCC as demonstrated previously (135).

In order to evaluate the role of VSCC in the first phase, cells were pretreated with nifedipine prior to exposure to KCl. As shown in Fig. 7C, such treatment completely abolished KCl-induced increase $[Ca^{2+}]_i$ suggesting that both phases of this response were dependent on Ca^{2+}-influx via VSCC. The same condition of pretreatment was tested with verapamil, but was unsuccessful due to the consistent detachment of cells.

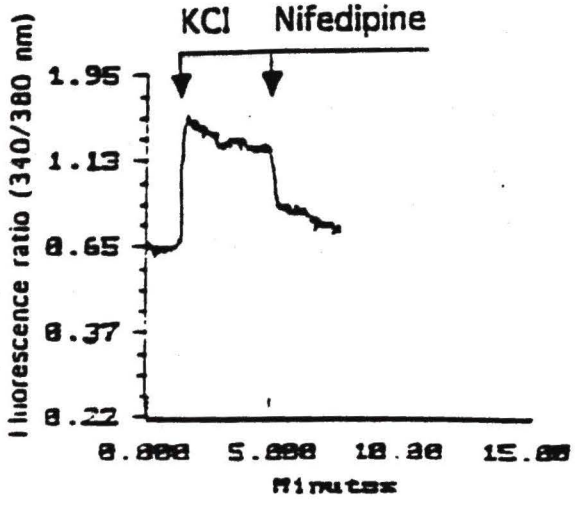
A similar strategy was used to evaluate the involvement of VSCC channels in the Ca^{2+}-response induced by gramicidin D. As shown in Fig. 8A, verapamil (20 μM) suppressed gramicidin D-induced increase in $[Ca^{2+}]_i$ by approximately 30 % when added during the second phase. However, when verapamil was added simultaneously with gramicidin D, an increase in $[Ca^{2+}]_i$ by gramicidin D was observed (Fig. 8B). Under these conditions, both the first phase and the second phase of gramicidin D-induced increase in $[Ca^{2+}]_i$ were markedly suppressed such that the response was now essentially monophasic. These results suggested that VSCC were likely involved in both phases of the observed Ca^{2+}-response.

Fig. 7 Effects of VSCC inhibitors on KCl-induced increase in $[Ca^{2+}]_i$ in βTC3 cells.

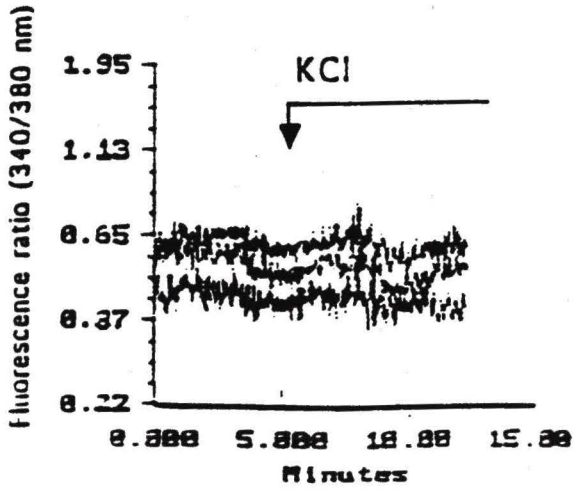
βTC3 cells preloaded with fura-2 as described in Methods were exposed to KCl (40 mM) at the time indicated followed by the addition of (A) verapamil (20 μM) and (B) nifedipine (20 μM) at the indicated by arrows. (C) βTC3 cells preloaded with fura-2 were pretreated with nifedipine (20 μM) for 3-5 minutes prior to exposure to KCl (40 mM).


KCl
Verapamil
Fluorescence ratio (340/380 nm)
3
1.52
0.77
0.39
0.2
0.000
5.000
10.00
15.00
Minutes

A


KCl
Nifedipine
Fluorescence ratio (340/380 nm)
1.95
1.13
0.65
0.37
0.22
0.000
5.000
10.00
15.00
Minutes

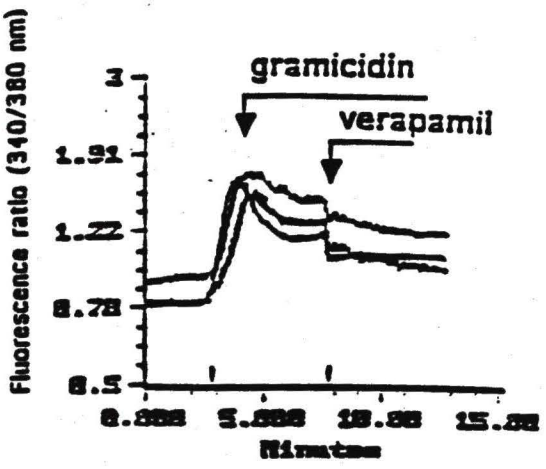
B


KCl
Fluorescence ratio (340/380 nm)
1.95
1.13
0.65
0.37
0.22
0.000
5.000
10.00
15.00
Minutes

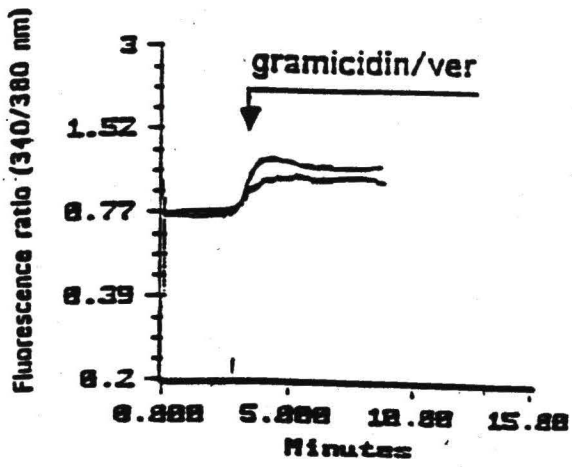
C

Fig. 8 Effects of verapamil and KN-62 on gramicidin D-induced increase in $[Ca^{2+}]_i$ in βTC3 cells.

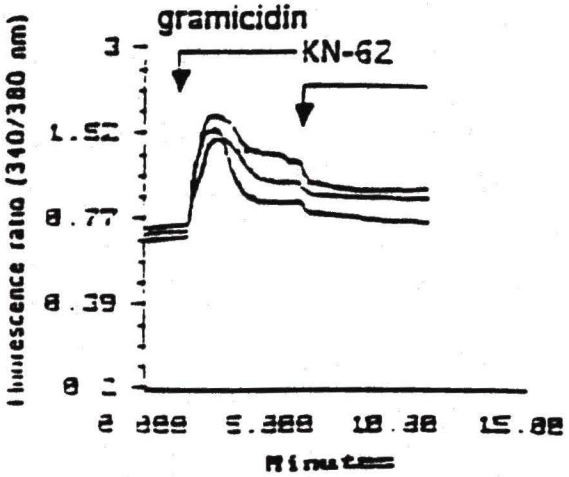
βTC3 cells were loaded with 0.5 μM fura-2 and exposed to gramicidin D (1 μM) at the time indicated. In (A) and (C), verapamil (ver, 20 μM) and KN-62 (10 μM) were added during the second phase of gramicidin D-induced increase in $[Ca^{2+}]_i$. In (B) and (D), verapamil (ver, 20 μM) and KN-62 (10 μM) were added simultaneously with gramicidin D.


gramicidin
verapamil
Fluorescence ratio (340/380 nm)
Minutes

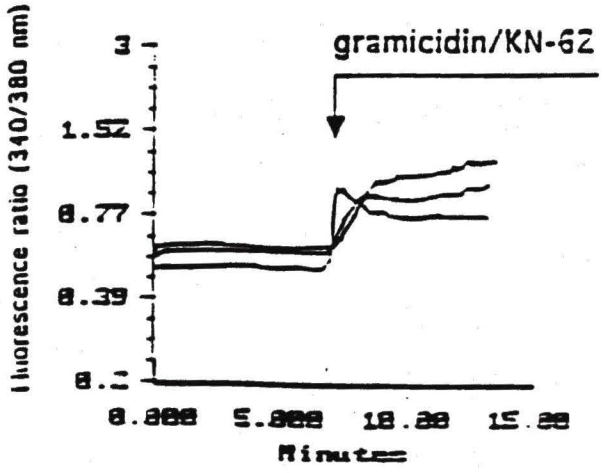
A


gramicidin/ver
Fluorescence ratio (340/380 nm)
Minutes

B


gramicidin
KN-62
Minutes

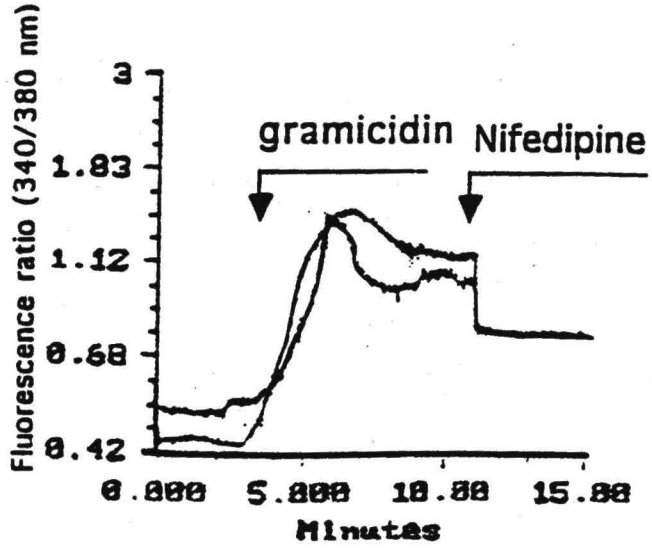
C


gramicidin/KN-62
Fluorescence ratio (340/380 nm)
Minutes

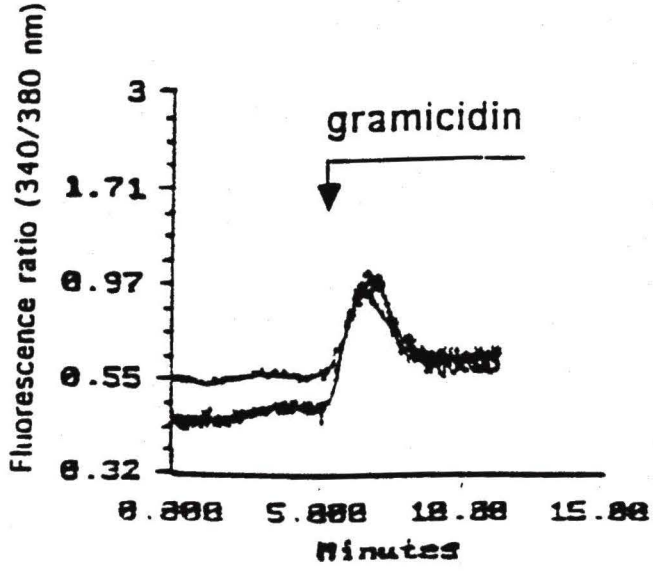
D

Fig.9 Effect of nifedipine on gramicidin D-induced increase in $[Ca^{2+}]_i$ in βTC3 cells.

βTC3 cells preloaded with fura-2 were exposed initially in (A) to gramicidin D (1 μM) followed by nifedipine (20 μM) addition at the time indicated by an arrow. In (B), βTC3 cells preloaded with fura-2 were pretreated with nifedipine (20 μM) for 3-5 minutes prior to exposure to gramicidin D (1 μM).


Fluorescence ratio (340/380 nm)
3
1.83
1.12
0.68
0.42
gramicidin
Nifedipine
0.000
5.000
10.00
15.00
Minutes

A


Fluorescence ratio (340/380 nm)
3
1.71
0.97
0.55
0.32
gramicidin
0.000
5.000
10.00
15.00
Minutes

B

Similar observations were found using KN-62 inhibitor. The addition of KN-62 during the second phase, inhibited gramicidin D-induced increase in $[Ca^{2+}]i$, whereas the simultaneous addition of KN-62 (10 μM) and gramicidin D produced a monophasic response (Figs. 8C and D, respectively (in 5 out of 6 cells tested)).

The same pattern of inhibition was observed using nifedipine (20 μM) in the second phase (Fig. 9A). Nevertheless, the pretreatment of cells with nifedipine (20 μM) prior to stimulation with gramicidin D partially inhibited the first phase of gramicidin D-induced increase in $[Ca^{2+}]_i$ while it suppressed the second phase greatly (Fig. 9B). The addition of gramicidin D (1 μM) to cells produced a first phase that was partially inhibited but, the $[Ca^{2+}]_i$ levels in the second phase were greatly reduced compared to normal levels seen after stimulation.

Taken together, these results suggest that VSCC are involved in mediating extracellular Ca^{2+} influx in both phases of Ca^{2+}-response induced by gramicidin D. In addition, gramicidin D and KCl appeared to share a similar pathway of Ca^{2+}-influx in the second phase of their responses. However, the ability of nifedipine to partially inhibit gramicidin D-induced increase in $[Ca^{2+}]_i$, suggested that an additional mechanism distinct from the VSCC contributed to this phase of response. This additional pathway of Ca^{2+}-influx was predicted to be a Na^+/Ca^{2+} exchanger mediating extracellular Ca^{2+}-influx. Therefore, experiments were performed to test its involvement in promoting the influx of extracellular Ca^{2+}.

<u>Effect of inhibitors of the Na^+/Ca^{2+} exchanger on gramicidin D-induced increase in $[Ca^{2+}]_i$</u>

As previously demonstrated, gramicidin D increased $[Na^+]_i$ in βTC3 cells (Results, section 1). Therefore, if βTC3 cells expressed functional Na^+/Ca^{2+} exchanger,

the influx of extracellular Na^+ was reasoned to activate the Na^+/Ca^{2+} exchanger and promote the influx of extracellular Ca^{2+} in exchange for intracellular Na^+. It was therefore hypothesized that the activation of such an exchanger may account for the additional Ca^{2+}-influx observed in cells exposed to gramicidin D.

Although the presence of a Na^+/Ca^{2+} exchanger in pancreatic β-cells has been suggested (179), it was necessary to test its presence in the insulinoma βTC3 cells. Under normal conditions, the Na^+/Ca^{2+} exchanger functions to promote the influx of extracellular Na^+ and the efflux of intracellular Ca^{2+} (forward mode). However, on the removal of extracellular Na^+, the exchanger will function in the reverse mode; that is to promote the influx of extracellular Ca^{2+} in exchange for Na^+. A similar approach was used in βTC3 cells. On switching normal KRB medium bathing βTC3 cells pre-loaded with fura-2 to a Na^+-free KRB medium (in which NaCl was substituted with N-methyl-D-glucamine at the same concentration), a sharp transient increase in $[Ca^{2+}]_i$ was observed (Fig. 10). This increase was characterized by a sharp peak that declined rapidly to basal levels within two minutes. Under such conditions, i.e., the removal of extracellular Na^+, cells became hyperpolarized and the activity of VSCC would be suppressed (135). Therefore, it was reasoned that the only mechanism that could promote the influx of extracellular Ca^{2+} under these conditions was Na^+/Ca^{2+} exchange. Such observations therefore, confirmed the presence of a Na^+/Ca^{2+} exchanger in βTC3 cells. The involvement of a Na^+/Ca^{2+} exchanger in promoting extracellular Ca^{2+} influx in cells exposed to gramicidin D was investigated by evaluating the effect of blockade of its activity. Amiloride, an inhibitor of Na^+/Ca^{2+} exchange activity, was added during the second phase of gramicidin D-induced increase in $[Ca^{2+}]_i$. Under these conditions, amiloride (100 μM) decreased $[Ca^{2+}]_i$ suggesting that a Na^+/Ca^{2+} exchange activity contributed to the second phase of Ca^{2+}-increase (Fig. 11A).

Fig. 10 Effect of sodium-free medium on $[Ca^{2+}]_i$ in βTC3 cells.

βTC3 cells were loaded with fura-2 in normal KRB medium (containing 115 mM NaCl) as described in Methods. At the time indicated by an arrow, normal KRB medium was replaced with Na^+-free KRB medium (in which NaCl was substituted with an equimolar amount of N-methyl D-glucamine) and levels of $[Ca^{2+}]_i$ were measured as previously described in Methods.

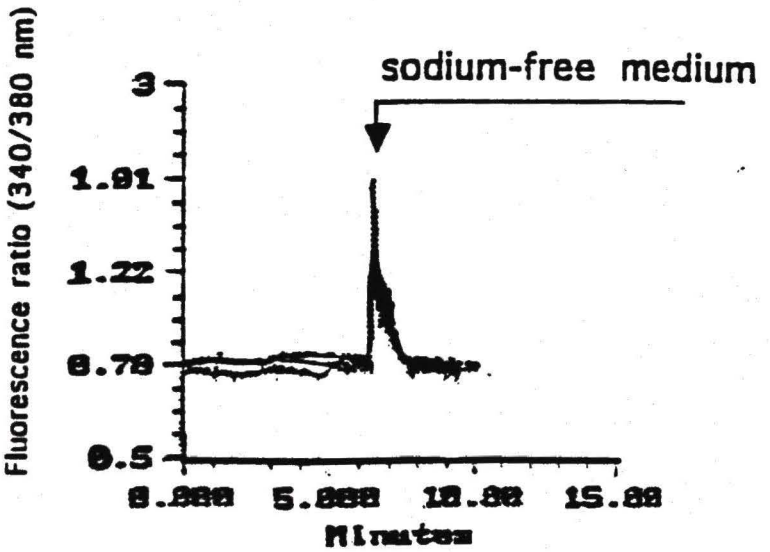
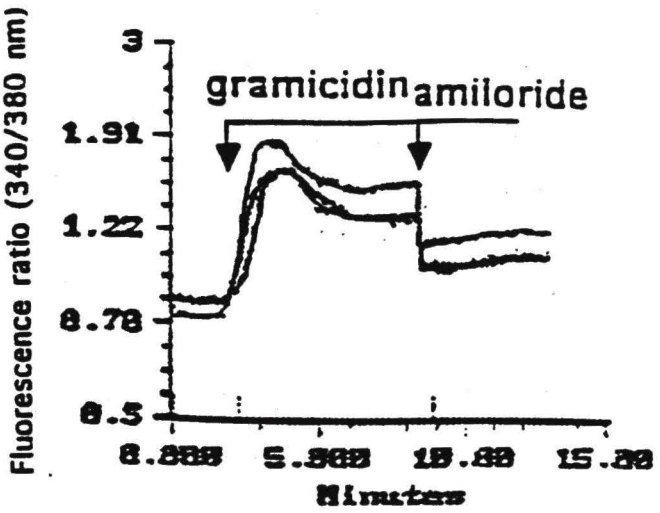
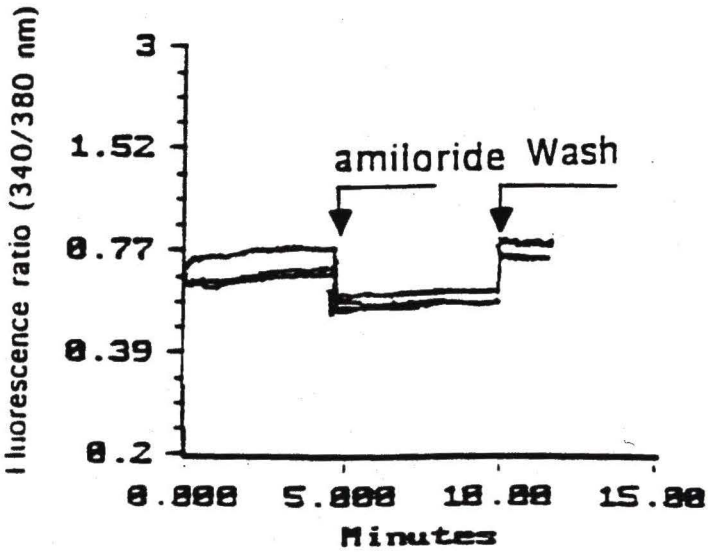

sodium-free medium
Fluorescence ratio (340/380 nm)
3
1.91
1.22
0.78
0.5
0.000
5.000
10.00
15.00
Minutes

Fig. 11 Effect of amiloride on gramicidin-induced increase in $[Ca^{2+}]_i$ in βTC3 cells.

(A) βTC3 cells were loaded with 0.5 μM fura-2 as described in Methods and exposed to gramicidin D (1 μM) followed by the addition of amiloride (50 μM) at the time indicated by an arrow. (B) Amiloride (50 μM) was added to βTC3 cells preloaded with fura-2 in the absence of gramicidin D. The addition of amiloride to fura-2 dye in the absence of cells resulted in a quenched fluorescence.


Fluorescence ratio (340/380 nm)
3
1.91
1.22
0.78
0.5
gramicidin
amiloride
0.000
5.000
10.00
15.00
Minutes

A


Fluorescence ratio (340/380 nm)
3
1.52
0.77
0.39
0.2
amiloride
Wash
0.000
5.000
10.00
15.00
Minutes

B

However, amiloride when added to loaded cells alone, induced a decrease in the 340/380 nm fluorescence ratio of a similar magnitude to that observed in the presence of gramicidin D (Fig. 11B). This observation suggested that the fluorescent nature of amiloride was responsible for the decrease in fluorescence ratio. Other potent inhibitors of Na^+/Ca^{2+} exchanger, including dimethylbenzamil (DMB) and 3,4-dichlorobenzamil (DCB) produced similar fluorescent effects in the absence of cells and therefore, could not be used. The lack of specific, non-fluorescent inhibitors of Na^+/Ca^{2+} exchanger therefore, hampered efforts to assess the involvement of such activity in the observed Ca^{2+}-response induced by gramicidin D.

In summary of this section of results, gramicidin D induced a profound increase in $[Ca^{2+}]_i$, a response that could not be mimicked qualitatively by any of the tested insulin secretagogues. The novel mechanism(s) of raising intracellular $[Ca^{2+}]_i$ seemed to involve at least two different pathways. The first involved VSCC whereas, the second likely involved Na^+/Ca^{2+} exchanger. The lack of specific, non-fluorescent inhibitors of Na^+/Ca^{2+} exchanger, however, hampered the direct assessment of the involvement of the Na^+/Ca^{2+} exchanger in the Ca^{2+}-influx in cells stimulated with gramicidin D.

Effects of gramicidin D and other insulin secretagogues on pH_i in βTC3 cell

Previous studies have reported that β-cells possess the Na^+/H^+ exchanger (166), and that such activity may play an important role in β-cell function. In addition, changes in ion flux in β-cells have been shown to be accompanied by changes in pH_i (150). Since gramicidin D increased both $[Na^+]_i$ (see section 1 of Results) and $[Ca^{2+}]_i$ (section two of Results) in βTC3 cells, it was necessary to test the effect of gramicidin D on pH_i in βTC3 cells.

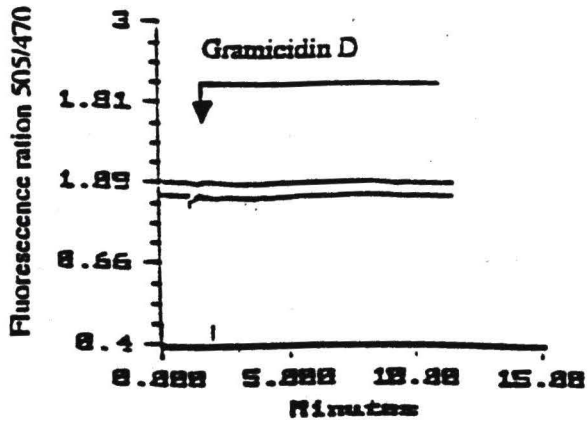
In order to assess the effect of gramicidin D on pH_i in βTC3 cells, cells were loaded with the pH-sensitive probe, BCECF-AM. An increase in the fluorescence ratio of 505/470 nm indicates alkalinization, whereas a decrease in the ratio indicates acidification (see Methods for detailed description of the use of this dye).

The exposure of βTC3 cells to gramicidin D (1 μM) had no effect on the BCECF fluorescence ratio indicating that gramicidin D treatment had no effect on pH_i (Fig. 12A). This observation was in contrast to effects of other insulin secretagogues tested. For example, glucose (20 mM) induced alkalinization as demonstrated in Fig. 12B. This effect was in agreement with a previously published report (166). Also, L-Leu (20 mM) mimicked the glucose effect to increase pH_i (Fig. 12C). The latter observation suggested that the observed increase in pH_i may result from the metabolism of glucose in the mitochondria. In addition, the effect of the phorbol ester, tetradeconylphorbol acetate (TPA, 100 nM), a potent activator of protein kinase C, on pH_i was evaluated. As shown in Fig. 12D, TPA increased pH_i. However, when PDD (4 α-phorbol 12,13-didecanoate, an inactive form of TPA that does not activate PKC) was tested, a similar increase in pH_i was observed (Fig. 12E) suggesting that the observed increase induced by TPA was likely due to the lipid-like effect of phorbol ester rather than the activation of PKC.

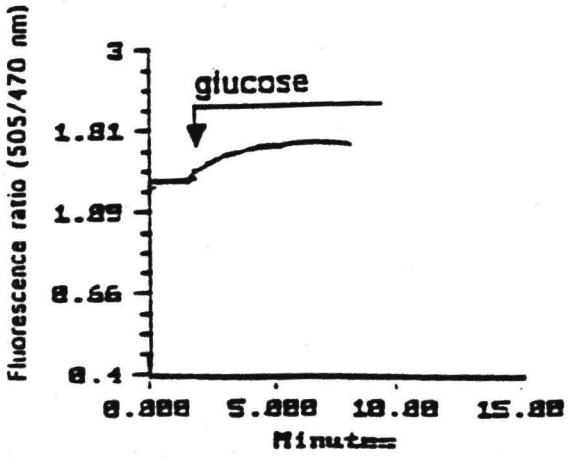
In summary, these data demonstrated that gramicidin D had no influence on pH_i in βTC3 cells, unlike other insulin secretagogues tested which increased pH_i. Furthermore, these data suggest that previously reported effects of TPA to induce cell alkalinization in support of the regulation of the Na^+/H^+ exchanger by PKC should be re-evaluated.

Fig. 12 Effect of gramicidin D on pH_i in βTC3 cells; comparison with other insulin secretagogues.

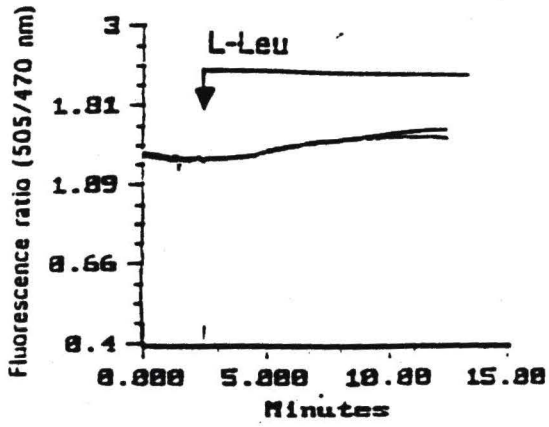
βTC3 cells were loaded with the pH-sensitive dye BCECF/AM (1 μM) as described in Methods and exposed to: (A) gramicidin D (1 μM), (B) glucose (20 mM), (C) L-Leu (L-Leucine, 20 mM), (D) TPA (tetradeconylphorbol acetate, 100 nM) and (E) PDD (4 α-phorbol-12,13-didecanoate, 100 nM) at the time indicated by arrows.


Fluorescence ration 505/470
3
1.81
1.89
0.66
0.4
Gramicidin D
0.000
5.000
10.00
15.00
Minutes

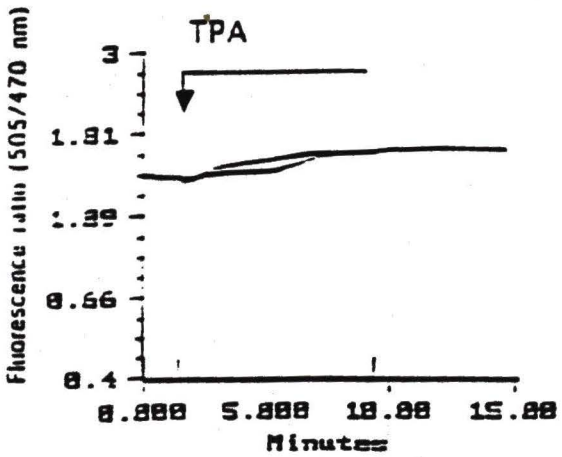
A


Fluorescence ratio (505/470 nm)
3
1.81
1.89
0.66
0.4
glucose
0.000
5.000
10.00
15.00
Minutes

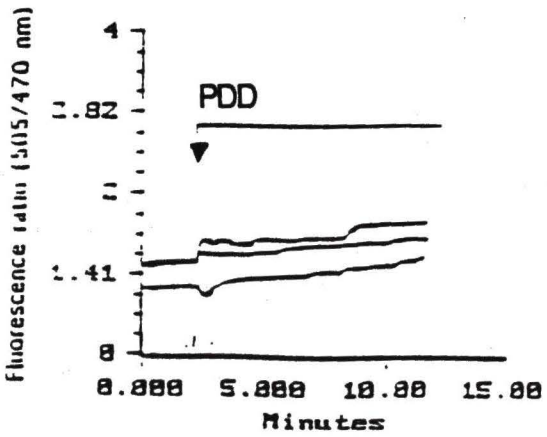
B


Fluorescence ratio (505/470 nm)
3
1.81
1.89
0.66
0.4
L-Leu
0.000
5.000
10.00
15.00
Minutes

C


Fluorescence ratio (505/470 nm)
3
1.81
0.66
0.4
TPA
0.000
5.000
10.00
15.00
Minutes

D


Fluorescence ratio (505/470 nm)
4
1.41
0
PDD
0.000
5.000
10.00
15.00
Minutes

E

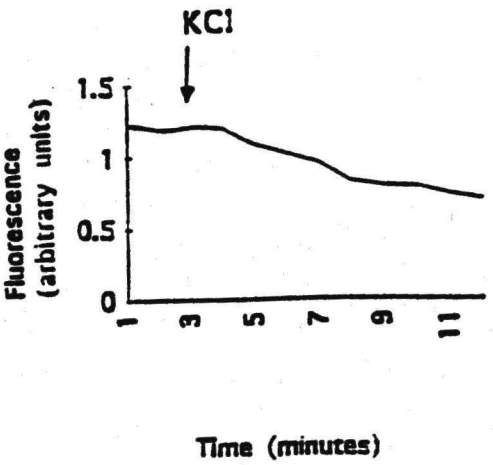
Effects of gramicidin D and other insulin secretagogues on membrane potential in βTC3 cells

As demonstrated in parts 1-3 of Results, gramicidin D increased $[Na^+]_i$ and $[Ca^{2+}]_i$, but had no effect on pH_i. Such a modulation of ion fluxes across plasma membranes would likely affect membrane potential in electrically sensitive cells such as β-cells. Therefore, the effect of gramicidin D on membrane potential was assessed using the fluorescent dye, Oxonol VI. Oxonol VI is an anionic dye commonly used for measuring membrane potential in numerous cells and has been used in the clonal β-cell line HIT cells (227-228). Depolarization of the plasma membrane results in the influx of dye into cells, resulting in a quenched fluorescence. Conversely, hyperpolarization of plasma membranes results in the efflux of dye from intracellular pools, resulting in an increased fluorescence. The fluorescence was followed in a spectrofluorophotometer at excitation and emission wavelengths of 590 nm and 660 nm, respectively.

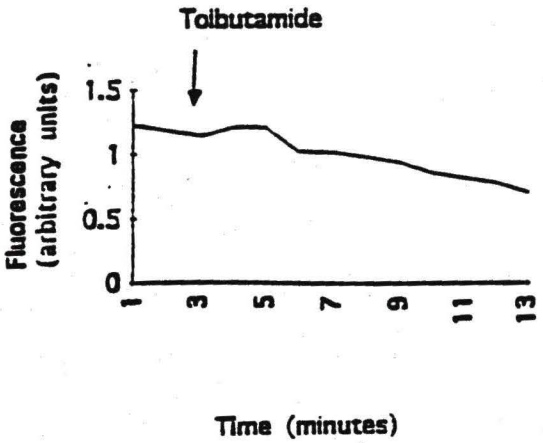
In these experiments, βTC3 cells were loaded with 1 μM Oxonol VI as described in Methods (section 6). Cells were then stimulated with secretagogues that are known to depolarize β-cells (KCl (40 mM) and tolbutamide). Both KCl (40 mM) and tolbutamide (100 μM) decreased fluorescence (Figs. 13A and 13B, respectively), indicating depolarization. These results were in agreement with the previously published reports (227-228). Next, the effect of gramicidin D on membrane potential in βTC3 cells was assessed. As shown in Fig. 13C, gramicidin D induced an initial increase in the fluorescence corresponding to hyperpolarization followed by a decrease in the fluorescence suggesting a depolarization effect on βTC3 cells. The net effect indicated membrane depolarization. In order to explain both opposing effects induced by gramicidin D, gramicidin D was added to Oxonol VI in the absence of cells and only an increase in fluorescence was observed (Fig. 13D).

Fig. 13 Effects of KCl, tolbutamide and gramicidin D on membrane potential in βTC3 cells.

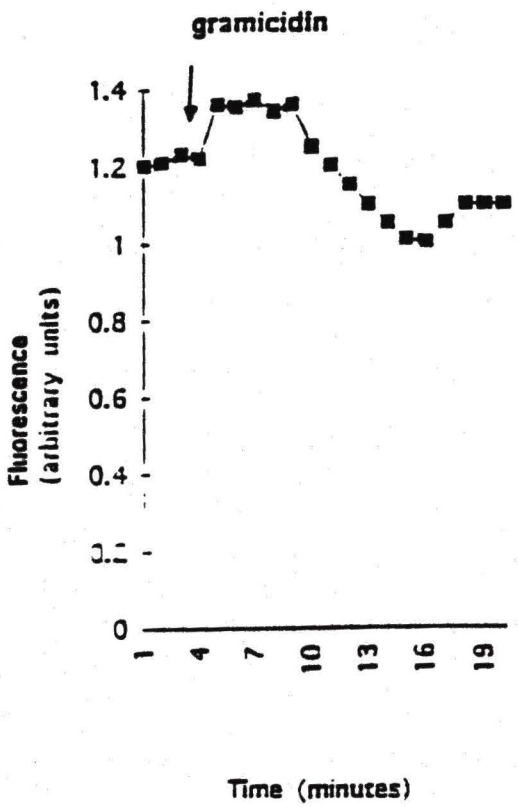
βTC3 cells were loaded with the membrane potential-sensitive dye Oxonol VI (1 μM) as described in Methods and exposed to: (A) KCl (40 mM), (B) tolbutamide (100 μM) and (C) gramicidin D (1 μM) at the indicated time. In (D), gramicidin D (1 μM) was added to Oxonol VI (1 μM) in the absence of βTC3 cells.


KCl
Fluorescence (arbitrary units)
1.5
1
0.5
0
1
3
5
7
9
11
Time (minutes)

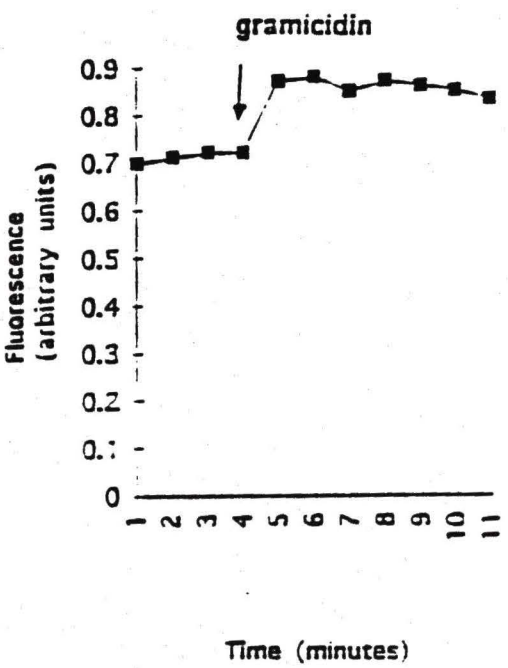
A


Tolbutamide
Fluorescence (arbitrary units)
1.5
1
0.5
0
1
3
5
7
9
11
13
Time (minutes)

B


gramicidin
Fluorescence (arbitrary units)
1.4
1.2
1
0.8
0.6
0.4
0
1
4
7
10
13
16
19
Time (minutes)

C


gramicidin
Fluorescence (arbitrary units)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
1
2
3
4
5
6
7
8
9
10
11
Time (minutes)

D

Therefore, the observed increase in fluorescence was due to interference of gramicidin D with Oxonol VI fluorescence. However, the decrease in fluorescence was not observed in the absence of cells. These data support the anticipated effect of gramicidin D to induce membrane depolarization.

Effect of Gramicidin D on insulin secretion from βTC3 cells.

An increase in $[Ca^{2+}]_i$ is critical for physiological insulin secretion from β-cells (23). It has been demonstrated that an increase in $[Ca^{2+}]_i$, even in the absence of glucose, is sufficient to trigger insulin secretion from β-cells (24). As demonstrated in the previous section of Results (section 2), a major response of gramicidin D was to increase $[Ca^{2+}]_i$ in βTC3. Therefore, the ability of gramicidin D to induce insulin secretion from βTC3 cells was evaluated. Two different models were utilized to assess the effect of gramicidin D on insulin secretion: (1) a "static model" and (2) a "perifusion model" (see Methods, section 7). The first model used for secretion studies was a static-model where the accumulation of the insulin secreted over a defined period of time was determined. This experimental model was used to assess the effect of gramicidin D on insulin secretion, the ability of other insulin secretagogues to modify its secretion response and to test the effect of different ion-channels inhibitors on gramicidin D-insulin release.

The second model of secretion studies used was a perifusion-model in which a constant flow of medium over immobilized cells was maintained (see Methods, section 7). The periodic collection of the perifusate then provided an analysis of the dynamics of gramicidin D-induced insulin secretion.

Static-model insulin secretion studies

Effect of gramicidin D on insulin secretion

As shown in Fig. 14A, gramicidin D (1 μM) stimulated insulin secretion from βTC3 cells and induced a 3.28-fold increase in insulin secretion relative to control cells incubated with vehicle alone (DMSO)($p < 0.001$, Student *t*-test, paired analysis). Basal insulin secretion rate was 716 ± 123 pmole/well/h, whereas the insulin secretion rate from cells stimulated by gramicidin D (1 μM) was 2349 ± 89 pmole/well/h. When higher concentrations of gramicidin D (5 and 10 μM) were tested, insulin secretion was not increased further (gramicidin D at 5 and 10 μM induced a 3.25-fold and a 3.06-fold increase in insulin secretion relative to control, respectively). Therefore, gramicidin D at 1 μM appeared to induce the maximal insulin secretion rate. A lower concentration of gramicidin D (250 nM) was tested and was found to stimulate insulin secretion (a 3.06-fold relative to control). These experiments demonstrated for the first time that gramicidin D was a potent insulin secretagogue.

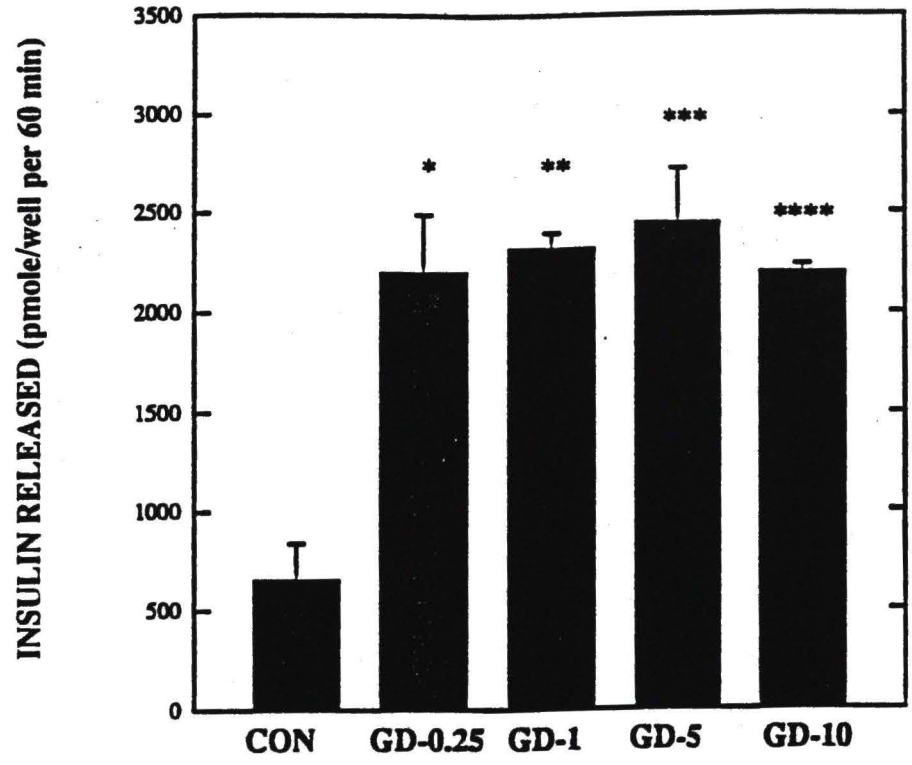
In order to correlate the effects of gramicidin D on $[Ca^{2+}]_i$ and insulin secretion, the effect of the chelation of extracellular Ca^{2+} with EGTA on gramicidin D-induced insulin secretion was studied. As demonstrated in Fig. 14B, the presence of 2 mM EGTA totally suppressed gramicidin D-induced insulin secretion. The identical effect of EGTA on $[Ca^{2+}]_i$ (Fig. 6) and insulin secretion therefore, suggested that gramicidin D-induced insulin release was mediated via the influx of extracellular Ca^{2+} rather than the elevation of Na^+.

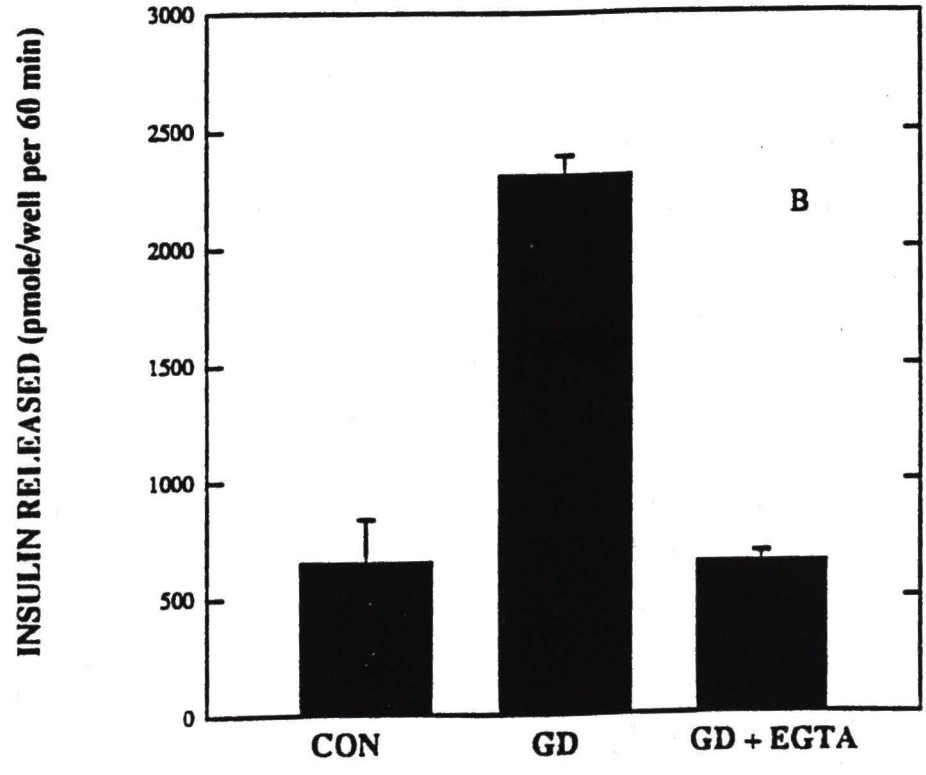
Effects of insulin secretagogues on gramicidin D-induced insulin secretion

Next, the ability of known insulin secretagogues to modify gramicidin D-induced insulin secretion was evaluated. Six (6) insulin secretagogues of different mechanisms of

Fig. 14 Effect of gramicidin D (GD) on insulin secretion rate from βTC3 cells.

Insulin secretion studies were performed as described in Methods. Results are shown as means ± SE of insulin released (pmole/well per 60 min) from at least three separate experiments. (A) Gramicidin D (0.25 μM, 1 μM, 5 μM and 10 μM) was added to βTC3 cells. (B) Gramicidin D (1 μM) was added in the presence of EGTA (2 mM)(C: control, *, ***$P < 0.05$ vs. control and **, ****$P < 0.001$ vs. control).


INSULIN RELEASED (pmole/well per 60 min)
3500
3000
2500
2000
1500
1000
500
0
*
**
***
****
CON
GD-0.25
GD-1
GD-5
GD-10


INSULIN RELEASED (pmole/well per 60 min)
3000
2500
2000
1500
1000
500
0
B
CON
GD
GD + EGTA

action were chosen (illustrated in Fig. 15). Glucose and ketoisocaproate (KIC) were studied because of their ability to increase intracellular Ca^{2+} via metabolism and the closure of K_{ATP} channels (115). L-Arg and KCl were studied because of their effects to increase $[Ca^{2+}]i$ via depolarizing the β-cells (113). By contrast, the mechanism of TPA-induced insulin release is not fully elucidated although it is known that TPA activates protein kinase C (52). Finally, carbachol was studied because of its ability to mobilize intracellular Ca^{2+}. Carbachol has been shown to potentiate glucose-induced insulin secretion (80).

In summary, the six insulin secretagogues to be tested have diverse mechanisms of action. These were tested in an attempt to dissect the mechanism of gramicidin D-induced insulin secretion by providing a better understanding of possible activities that may be either involved or missing in the gramicidin D-induced insulin secretion pathway.

Glucose (20 mM) stimulated insulin secretion by 1.74 ± 0.11 fold relative to control cells incubated in the absence of the carbohydrate (Fig. 16). This response was significantly less than that induced by gramicidin D (1 μM, 3.28-fold). The combination of glucose and gramicidin D (1 μM) however, induced insulin secretion by 4.82 ± 0.61-fold relative to control (vehicle alone). These data demonstrated the ability of glucose to potentiate gramicidin D-induced insulin secretion. This observation was significant because glucose failed to influence gramicidin D-induced increase in $[Ca^{2+}]_i$ (see Results, Fig. 5B). Therefore, it seemed while an increase in $[Ca^{2+}]_i$ was required for insulin secretion, other signals could modulate the secretion event. In the case of glucose, these additional signals are likely generated from glucose metabolism. Next, it was of interest to identify the source of such intermediates that were likely potentiating the gramicidin D secretion event; i.e., to localize their place of production in β-cells and identify if they were of glycolytic or mitochondrial origins.

Fig. 15 Mechanisms of stimulating insulin release by insulin secretagogues.

Six insulin secretagogues with their proposed mechanisms of action are depicted in this figure. (1) Glucose and (2) KIC (ketoisocaproate) are believed to increase $[ATP]_i$ resulting in a closure of K_{ATP} channels. (3) L-Arg (L-Arginine) and (4) KCl induce membrane depolarization and the activation of Ca^{2+} influx via VSCC. (5) TPA (tertadeconylphorbol acetate)activates PKC an enzyme that stimulates insulin release by unknown mechanisms although it has been shown that PKC inhibites the K_{ATP} channel leading to depolarization of plasma membrane. (6) Carbachol mobilizes intracellular Ca^{2+}.

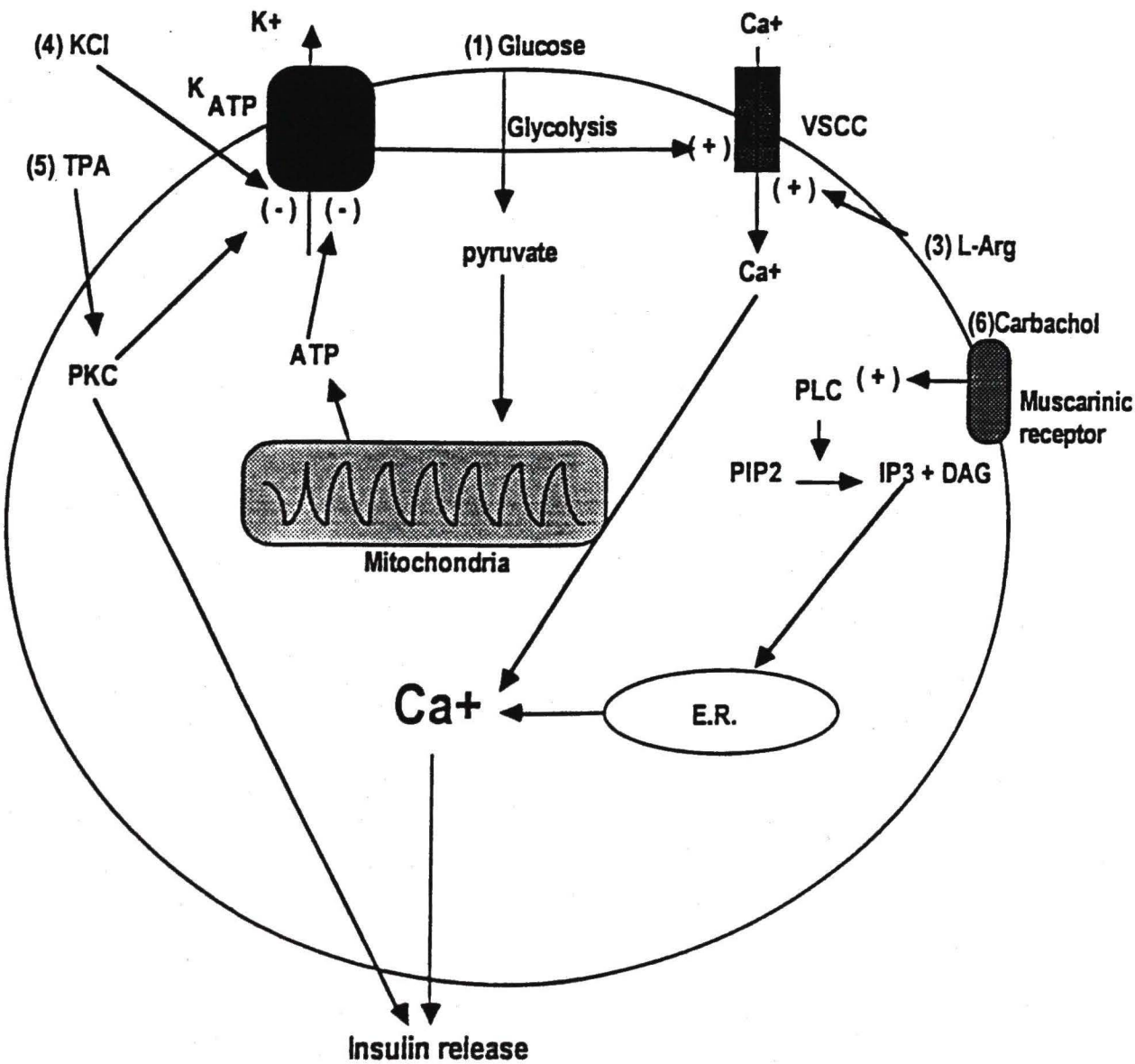

(4) KCl
K+
(1) Glucose
Ca+
K ATP
Glycolysis
VSCC
(5) TPA
(+)
(-)
(-)
(+)
(3) L-Arg
pyruvate
Ca+
(6)Carbachol
ATP
PKC
PLC
(+)
Muscarinic receptor
PIP2
IP3 + DAG
Mitochondria
Ca+
E.R.
Insulin release

Fig. 16 Effect of glucose (20 mM) on gramicidin D-induced insulin secretion from βTC3 cells.

Insulin secretion studies were performed in βTC3 cells as described in Methods. Results are shown as mean ± SE of insulin released of at least 3 separate experiments (pmole/well per 60 minutes). GD: gramicidin D, C: control (*P < 0.03 vs. control and **P < 0.08 vs. gramicidin D).

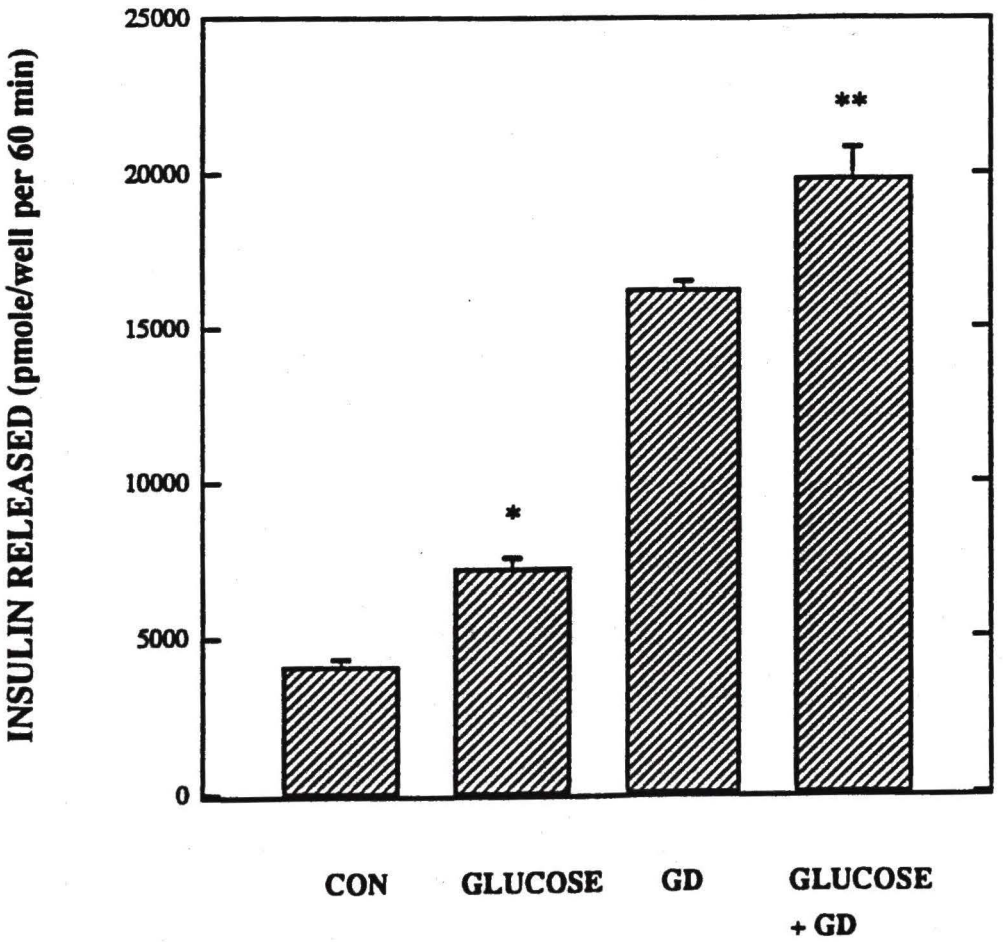

INSULIN RELEASED (pmole/well per 60 min)
25000
20000
15000
10000
5000
0
*
**
CON
GLUCOSE
GD
GLUCOSE
+ GD

Fig. 17 Effects of ketoisocaproate (KIC) on gramicidin D (GD) and glucose-induced insulin secretion from βTC3 cells.

The effects of KIC (20 mM), a mitochondrial fuel, on gramicidin D and glucose-induced insulin secretion was evaluated by adding a combination of KIC/gramicidin D or KIC/glucose to βTC3 cells. Insulin release was assayed as described in Methods (*$P < 0.01$ vs. gramicidin D and **$P < 0.001$ vs. glucose).

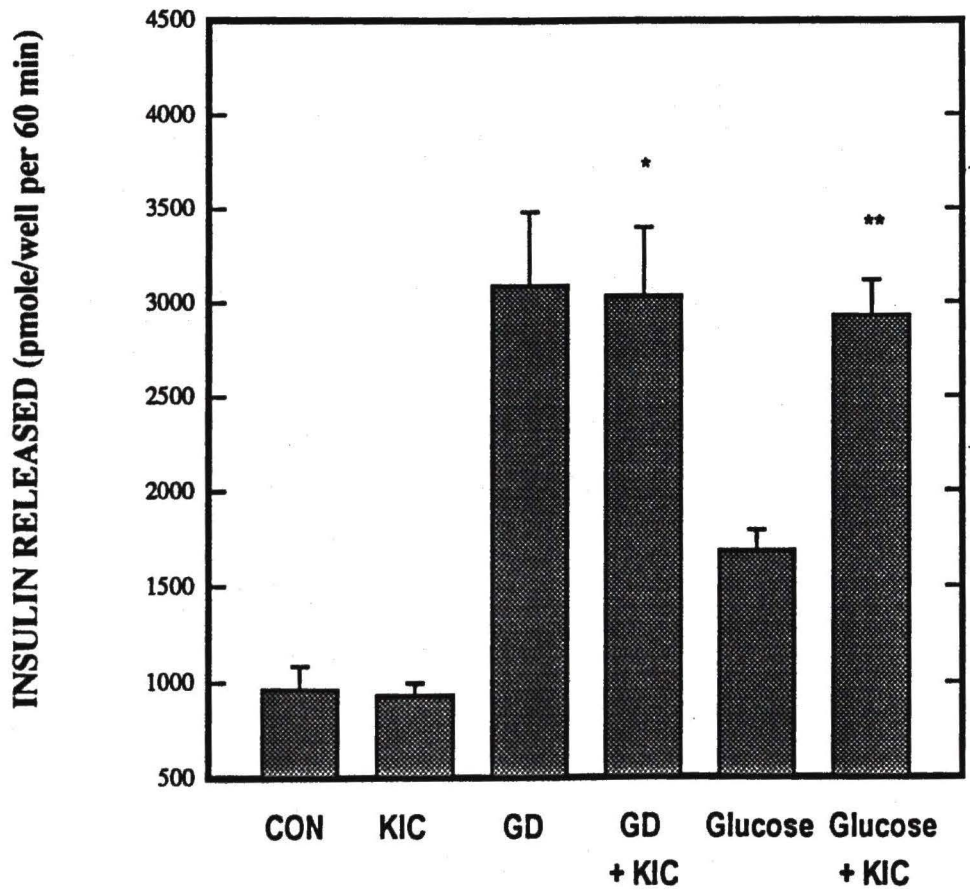
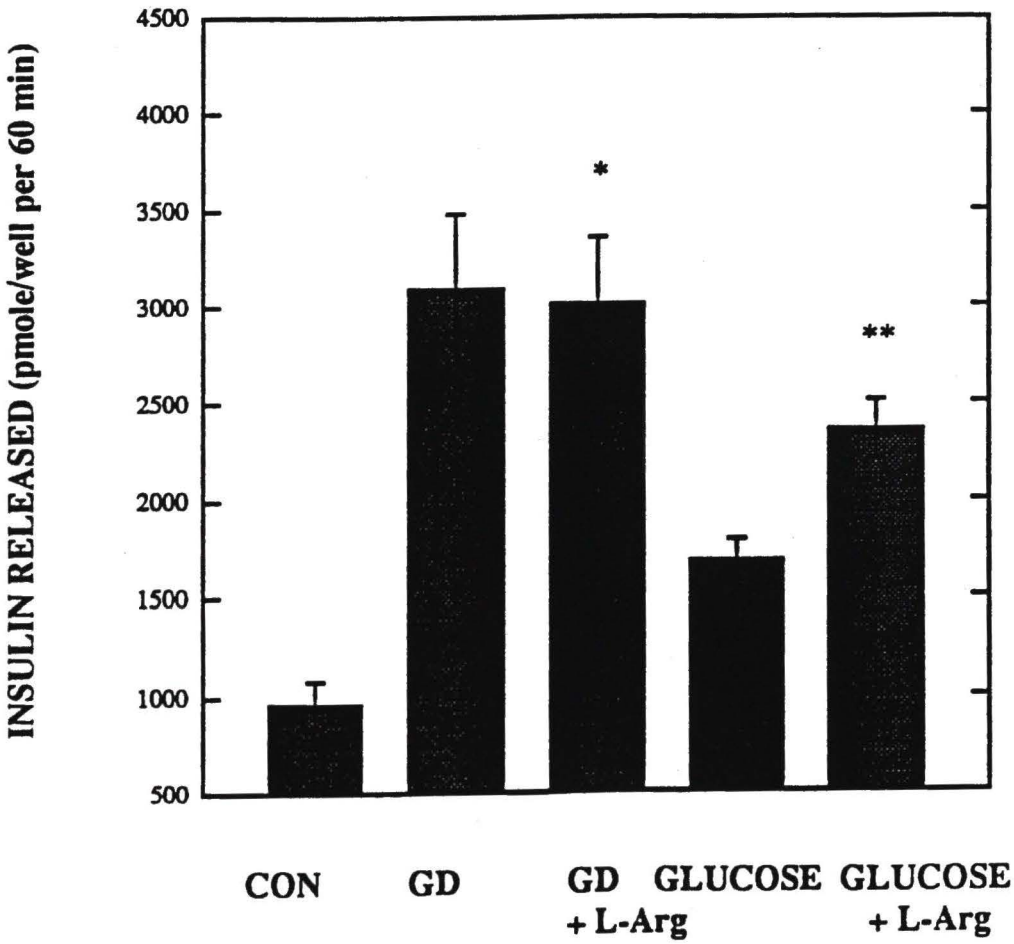

INSULIN RELEASED (pmole/well per 60 min)
4500
4000
3500
3000
2500
2000
1500
1000
500
*
**
CON
KIC
GD
GD + KIC
Glucose
Glucose + KIC

Fig. 18 Effects of L-arginine (L-Arg) on glucose and gramicidin D-induced insulin secretion from βTC3 cells.

L-Arg (20 mM), a basic amino acid, was added to glucose- (20 mM) or gramicidin D (1 μM)-stimulated cells and levels of insulin were assayed and compared with insulin released from stimulated-cells with gramicidin D or glucose in the absence of L-Arg (*, **$P < 0.01$ vs. gramicidin D and glucose).


INSULIN RELEASED (pmole/well per 60 min)
4500
4000
3500
3000
2500
2000
1500
1000
500
*
**
CON
GD
GD + L-Arg
GLUCOSE
GLUCOSE + L-Arg

Therefore, the effect of KIC (20 mM), a mitochondrial fuel, on glucose and gramicidin D-induced insulin secretion was evaluated. As shown in Fig. 17, while KIC augmented glucose-induced insulin secretion (a combination of glucose and KIC stimulated insulin secretion by 3.04 $\pm$ 0.2 fold), KIC had no effect on gramicidin D-induced insulin secretion. This observation suggested that glycolytic intermediates were responsible for potentiation of gramicidin D-induced secretion by glucose.

The third secretagogue to be tested for its ability to modulate gramicidin D-induced insulin secretion was L-Arg. While L-Arg (20 mM) potentiated glucose-induced insulin secretion (a combination produced a 2.45 $\pm$ 0.15 fold of insulin release), it had no effect on gramicidin D-induced insulin secretion (Fig. 18). This observation was consistent with the anticipated effect of L-Arg to induce membrane depolarization, since gramicidin D was shown in this study (section 4 of Results) to exert a depolarizing effect on plasma membranes, it was unlikely that L-Arg would exert an additional depolarizing effect. In fact, these data support the current hypothesis explaining the mechanism by which basic amino acids stimulate insulin secretion (101).

The ability of depolarizing concentrations of KCl (40 mM) to modulate gramicidin D-induced insulin release was tested. As shown in Fig. 19, KCl (40 mM) induced insulin secretion by 6.20 $\pm$ 0.78 fold relative to control. A combination of KCl and gramicidin D induced a 4.6 $\pm$ 0.51-fold increase in insulin secretion relative to control. The addition of gramicidin D to KCl inhibited KCl-induced insulin secretion by 26 %. This result was unexpected since the combination of both stimuli had additive effects on the increase in $[Ca^{2+}]_i$ (see results, Fig. 5C). However, the high levels of cytosolic Na^+ may exert inhibitory effects on insulin secretion that are yet to be discovered.

Fig. 19 Effect of KCl on gramicidin D-induced insulin secretion from βTC3 cells.

The effect of KCl (40 mM) on gramicidin D-induced insulin secretion was tested by the adding a combination of both stimuli to βTC3 cells and insulin secretion was measured as described in Methods (*$P < 0.05$ vs. gramicidin D (GD)).

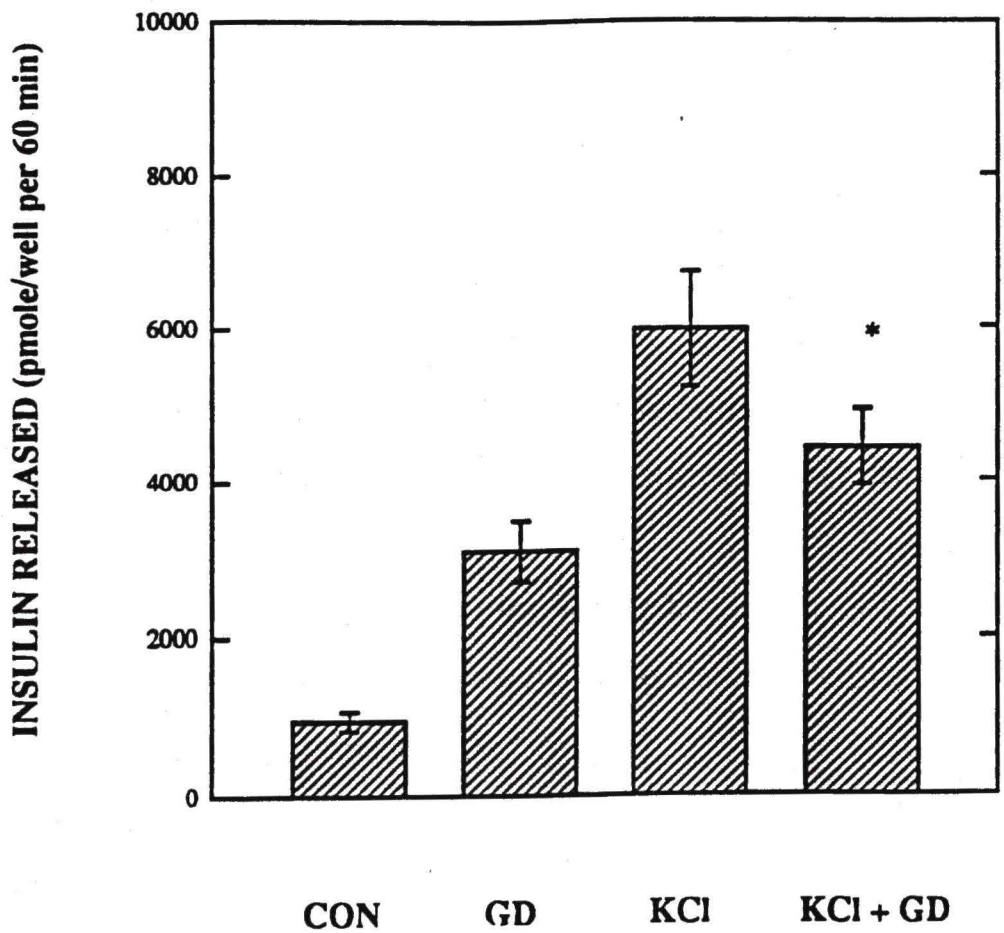
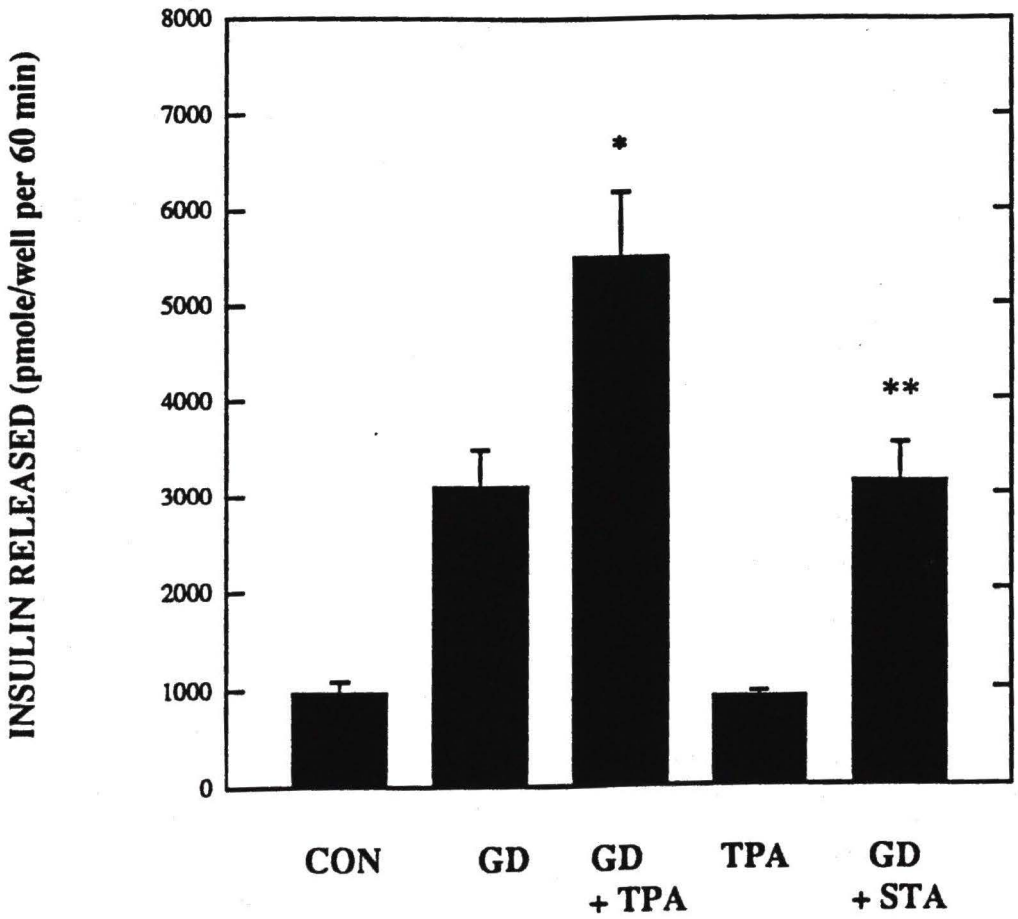

INSULIN RELEASED (pmole/well per 60 min)
10000
8000
6000
4000
2000
0
*
CON
GD
KCl
KCl + GD

Fig. 20 Effects of TPA (100 nM) and staurosporine (20 nM) on gramicidin D-induced insulin secretion from βTC3 cells.

Insulin secretion studies were performed as described in Methods. Results are shown as means ± SE of insulin released (pmole/well per 60 minutes)(*, **P < 0.01 vs. gramicidin D (GD), Stau: staurosporine (20 nM)).


INSULIN RELEASED (pmole/well per 60 min)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
**
CON
GD
GD + TPA
TPA
GD + STA

TPA is a potent activator of PKC, that has been shown to potentiate glucose-induced insulin secretion from pancreatic islets (53). Therefore, its effect on gramicidin D-induced insulin secretion was tested. While TPA (100 nM) had no effect on basal insulin secretion, the combination of gramicidin D and TPA stimulated insulin secretion by 4.80 $\pm$ 0.72 fold (Fig. 20). This result suggested that the TPA-induced activation of PKC potentiated gramicidin D-induced insulin secretion. In other experiments, staurosporine, a potent inhibitor of PKC (257), had no effect on gramicidin D-induced insulin secretion (Fig. 20). Finally, the effect of carbachol on gramicidin D-induced insulin release was investigated. As shown in Fig. 21, carbachol (500 μM) had no effect on gramicidin D-induced insulin secretion although carbachol produced a transient increase in $[Ca^{2+}]_i$ in cells pretreated with gramicidin D (see Fig. 5D). However, such transient increase appeared to be insufficient to further potentiate gramicidin D-induced insulin secretion.

In summary, gramicidin D is a new insulin secretagogue that is absolutely Ca^{2+}-dependent. Of all insulin secretagogues tested for their effects on its insulin release, three insulin secretagogues (glucose, KCl and TPA) were found to have additive effects, whereas, the remaining three secretagogues failed to affect gramicidin D-induced insulin secretion (KIC, L-Arg and carbachol). The data obtained in this series of experiments are summarized in Table 1.

Effects of ion-channel inhibitors on gramicidin D-induced insulin secretion

This section of the static-model secretion studies was designed to probe the involvement of VSCC and the Na^+/Ca^{2+} exchanger in gramicidin D-induced insulin secretion. These experiments utilized the same inhibitors as tested on the Ca^{2+}-response induced by gramicidin D (see Results, section 2, part two).

Fig. 21 Effect of carbachol on gramicidin D-induced insulin secretion from βTC3 cells.

The effect of carbachol (500 μM), a muscarinic agonist, on gramicidin D-induced insulin secretion was tested. Insulin release was measured as described in Methods (*P < 0.04 vs. control and **P < 0.01 vs. gramicidin D).

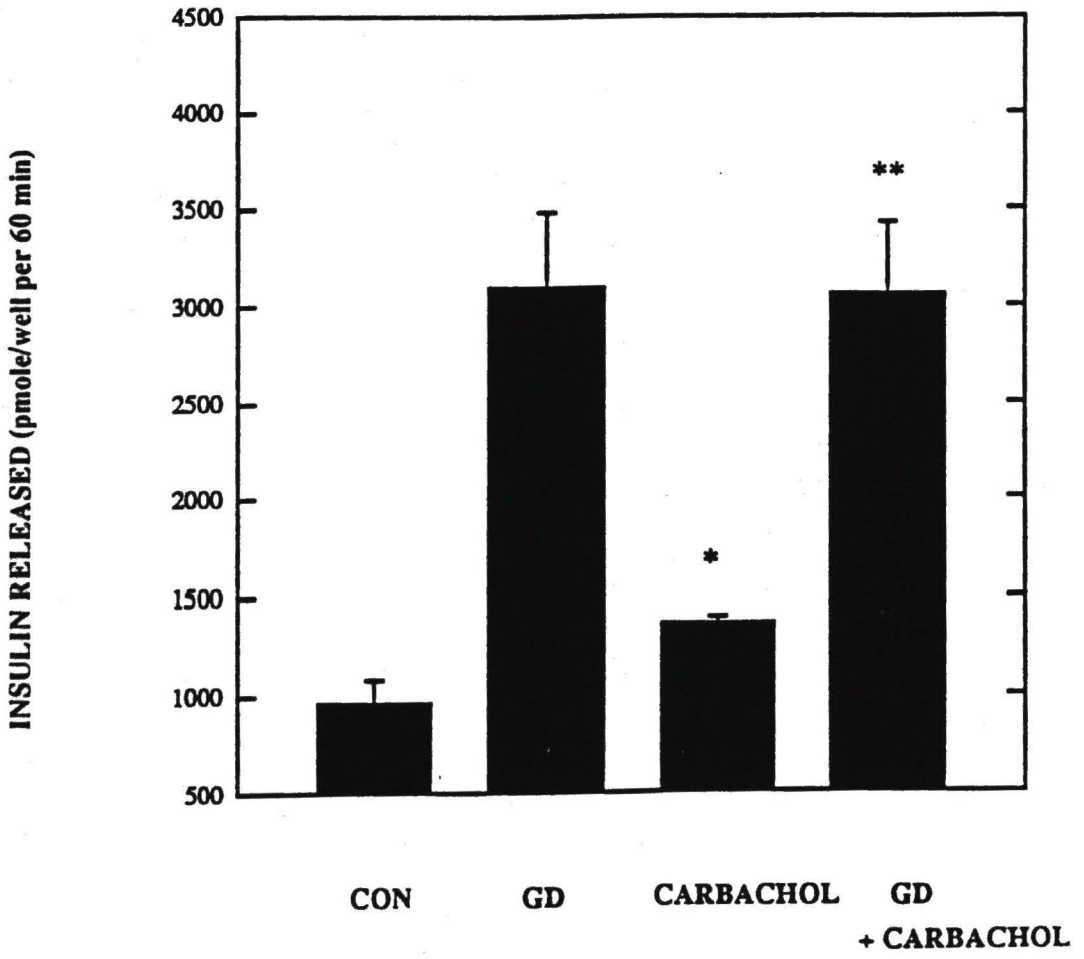

INSULIN RELEASED (pmole/well per 60 min)
4500
4000
3500
3000
2500
2000
1500
1000
500
*
**
CON
GD
CARBACHOL
GD
+ CARBACHOL

Table 1: Effects of insulin secretagogues on gramicidin D-induced insulin secretion from βTC3 cells.

Insulin secretagogues	Effect on gramicidin D induced insulin secretion
Glucose (20 mM)	additive (4.82 fold relative to control)
TPA (100 nM)	additive (4.8 fold relative to control)
KCl (40 mM)	additive (4.6 fold relative to control)
KIC (20 mM)	no effect
L-Arg (20 mM)	no effect
Carbachol (500 μM)	no effect

If the elevation of $[Ca^{2+}]_i$ represented the key signal for initiation of insulin secretion, then these inhibitors would suppress insulin release to an extent similar to that observed on $[Ca^{2+}]_i$.

Effects of inhibitors of VSCC on gramicidin D and KCl-induced insulin secretion

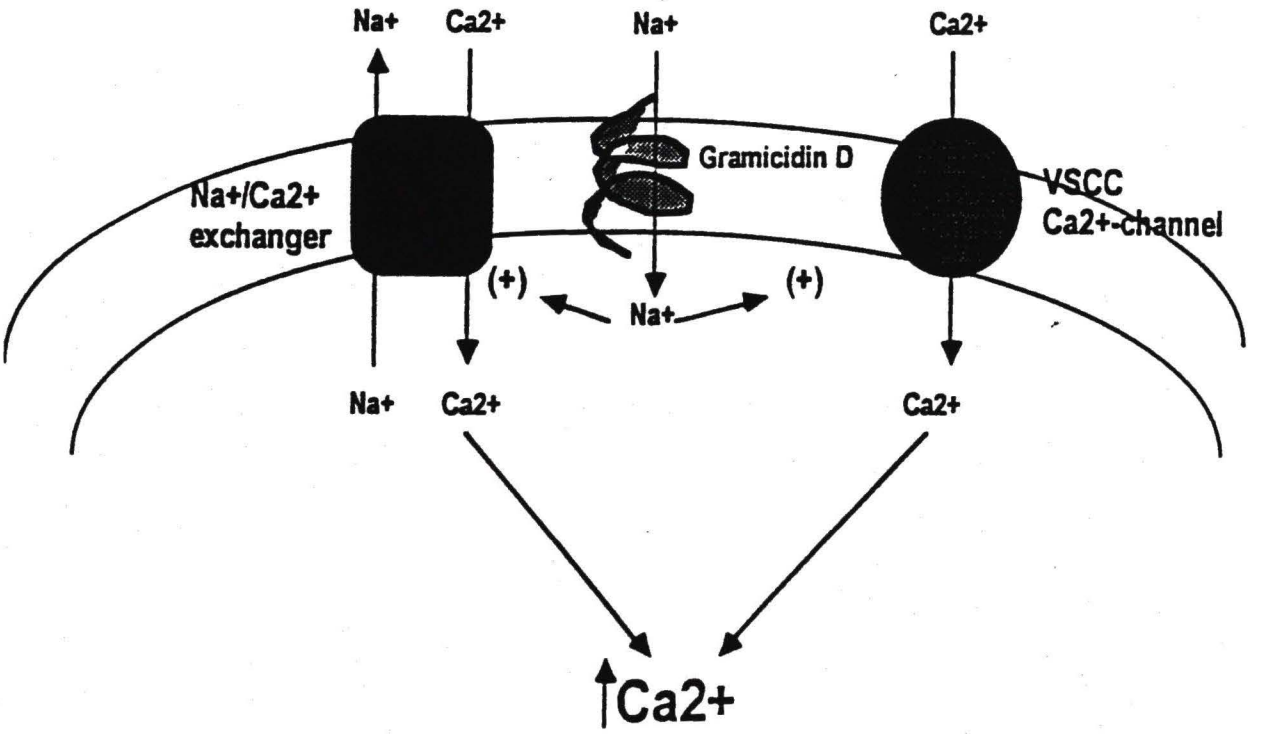
The observed depolarizing effect in cells exposed to gramicidin D was expected to activate L-type VSCC (see Fig. 22). The L-type Ca^{2+} channels are considered the key Ca^{2+}-channels mediating extracellular Ca^{2+}-influx in response to depolarization induced by glucose and KCl (40 mM)(see Introduction). Potent inhibitors against L-type Ca^{2+} channels were used to assess the involvement of L-type Ca^{2+}-channels in the gramicidin D-induced insulin secretion.

As demonstrated in Fig. 23A verapamil, an inhibitor of L-Type Ca^{2+} channel activity, dose-dependently inhibited gramicidin D-induced insulin secretion. At a maximally effective concentration (20 μM), verapamil inhibited insulin secretion by 42.6%; verapamil at a similar concentration had no effect on basal secretion in the presence of vehicle alone. The half maximal effect of verapamil (IC_{50}) was calculated to be approximately 0.94 μM, a concentration that is consistent with reported values for the effect of verapamil on VSCC in β-cells (0.5 μM, 225).

Also, verapamil inhibited KCl-induced insulin secretion in a dose-dependent manner with an identical IC_{50} value (0.94 μM)(Fig. 23B). These observations were consistent with the target of verapamil being identical in both of gramicidin D- and KCl-induced insulin release and was likely the L-type Ca^{2+} channel. However, verapamil inhibited KCl-induced insulin secretion by 80.8 % at the highest concentration used (20 μM).

Fig. 22 Proposed pathways of Ca^{2+}-influx from the extracellular medium induced by gramicidin D.

There are two possible mechanisms involved in mediating extracellular Ca^{2+}-influx: (1) VSCC and (2) Na^{+}/Ca^{2+} exchanger.


Na+
Ca2+
Na+
Ca2+
Gramicidin D
Na+/Ca2+
exchanger
VSCC
Ca2+-channel
(+)
Na+
(+)
Na+
Ca2+
Ca2+
↑Ca2+

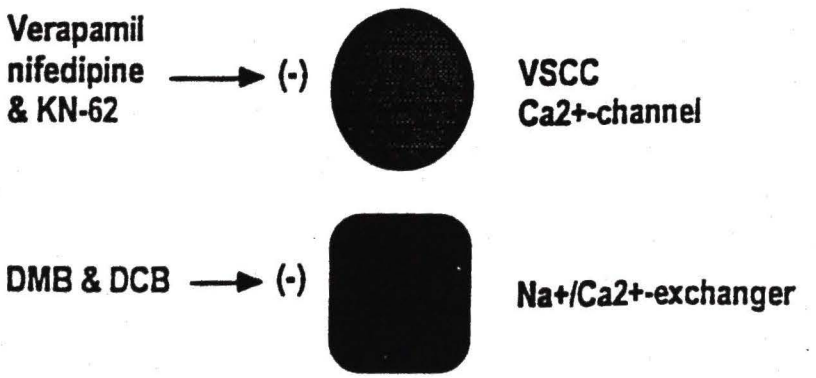
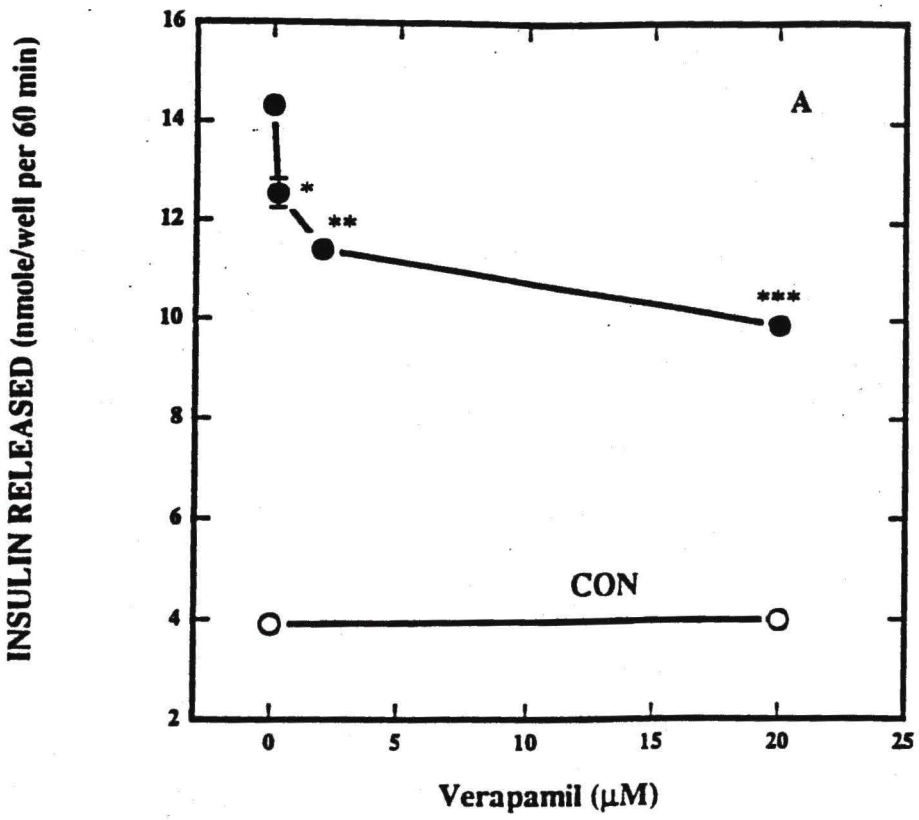
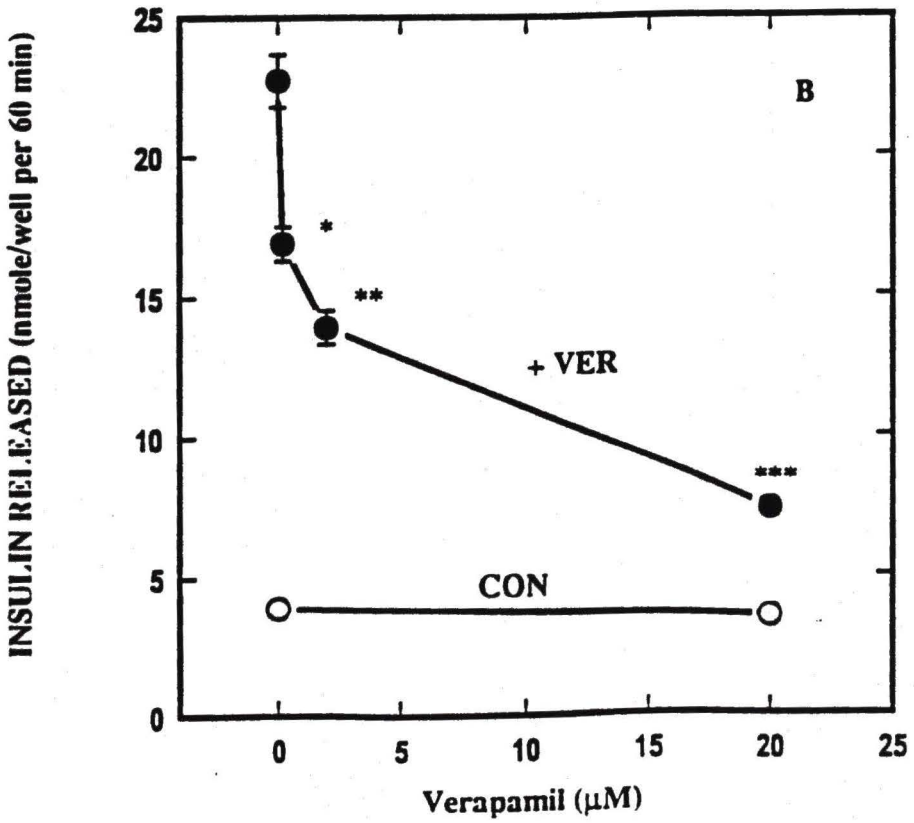

Verapamil
nifedipine
& KN-62
(-)
VSCC
Ca2+-channel
DMB & DCB
(-)
Na+/Ca2+-exchanger

Fig. 23 Effects of verapamil on gramicidin D- and KCl-induced insulin secretion from βTC3 cells.

(A) βTC3 cells were incubated with gramicidin D (1 μM) or vehicle alone in the absence or presence of increasing concentrations of verapamil (0.2-20 μM) and insulin secretion rates were determined as described in Methods (*, **, ***P < 0.002 vs. gramicidin D). (B) βTC3 cells were incubated with KCl (40 mM) in the presence or absence of increasing concentrations of verapamil and insulin secretion rates were measured as described previously (*, **, ***P < 0.001 vs. KCl).


INSULIN RELEASED (nmole/well per 60 min)
16
14
12
10
8
6
4
2
A
*
**
***
CON
0
5
10
15
20
25
Verapamil (μM)

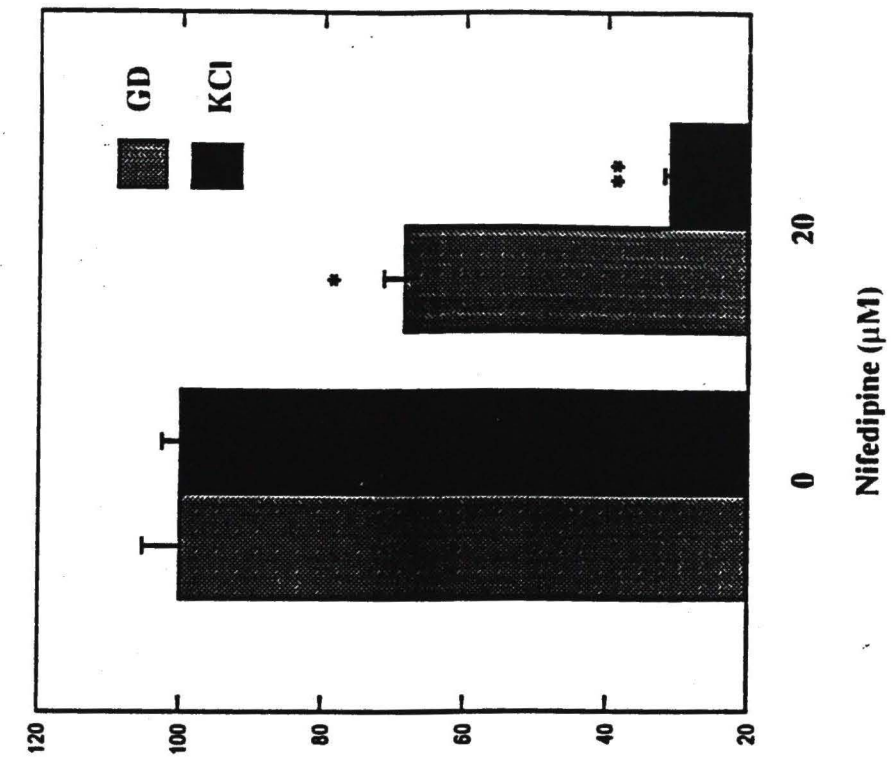

INSULIN RELEASED (nmole/well per 60 min)
25
20
15
10
5
0
B
*
**
+ VER
***
CON
0
5
10
15
20
25
Verapamil (μM)

Thus, while this data is consistent with the involvement of VSCC in gramicidin D-induced insulin secretion, the partial inhibition of gramicidin D-induced insulin secretion in contrast to greater inhibition of secretion induced by KCl suggested the involvement of an additional mechanism(s) in gramicidin D-induced secretion. Although verapamil is reported as a selective inhibitor of VSCC, there is some evidence for additional non-specific effects on other targets including the Na^+/Ca^{2+}-exchanger (239). Therefore, the effects of another inhibitor of VSCC, nifedipine, on gramicidin D and KCl-induced insulin secretion were studied. As demonstrated in Figs. 24A-B, increasing concentrations of nifedipine inhibited both gramicidin D- and KCl-induced insulin secretion but did so to different extents. Gramicidin D and KCl induced a 3.28-fold and 6.21-fold stimulation of insulin secretion, respectively.

At the highest concentration used, nifedipine (20 μM) inhibited gramicidin D- and KCl-induced insulin secretion by 31.3% and 80 %, respectively. The differential effects of nifedipine were therefore similar to data obtained with verapamil and suggested the involvement of VSCC in both gramicidin D- and KCl-indued insulin secretion but to different extents. Also, KN-62 which has been shown to suppress Ca^{2+}-influx via VSCC in HIT cells (256)(see Figs. 6C-D), also inhibited both KCl- and gramicidin D-induced insulin secretion by 38 % and 19 %, respectively (Fig. 25). A similar phenomenon was reproduced with all of the tested VSCC inhibitors; each antagonist had stronger inhibitory effects on KCl-induced insulin secretion when compared with gramicidin D-induced insulin secretion. This observation provided a strong evidence that gramicidin D-induced insulin secretion was mediated in part, by the activation of VSCC but that an additional mechanism(s) also contributed to this response.

Fig. 24 Effects of nifedipine on gramicidin D- and KCl-induced insulin secretion from βTC3 cells.

βTC3 cells were incubated with gramicidin D (1 μM) or KCl (40 mM) in the presence or absence of increasing concentrations of nifedipine (0.2-20 μM) and insulin secretion rates were measured as described in Methods. (A) Absolute values of insulin secretion are plotted as a function of nifedipine concentration. (B) Values are represented as a percentage of maximal (100 %) secretion in the presence of gramicidin D and KCl (*$P < 0.002$ vs. gramicidin D and KCl).


GD
KCl
120
100
80
60
40
20
INSULIN RELEASED (% OF CONTROL)
*
**
0
20
Nifedipine (μM)

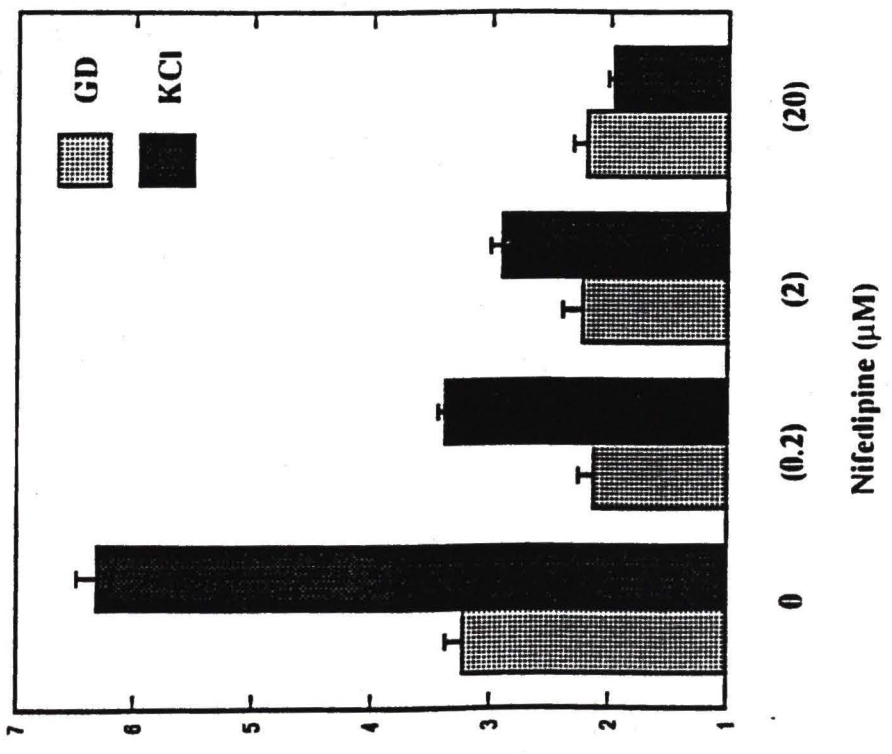
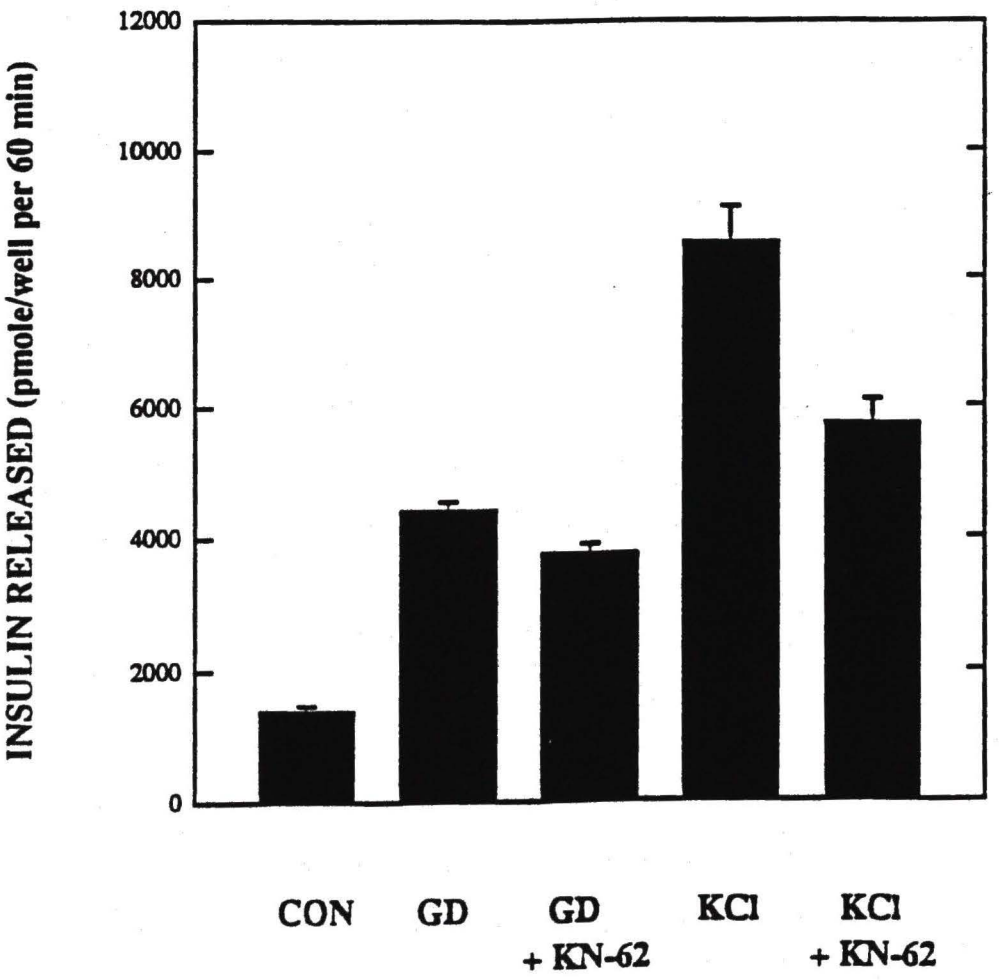

GD
KCl
7
6
5
4
3
2
1
INSULIN RELEASED (nmole/well per 60 min)
0
(0.2)
(2)
(20)
Nifedipine (μM)

Fig. 25 Effects of KN-62 on KCl and gramicidin D-induced insulin secretion from βTC3 cells.

βTC3 cells were incubated with basal KRB containing vehicle (0.1 % DMSO)(CON), gramicidin D (1 μM) with or without KN-62 (10 μM) and modified KRB containing KCl (40 mM) with or without KN-62 (10 μM). Insulin was assayed as described in Methods.


INSULIN RELEASED (pmole/well per 60 min)
12000
10000
8000
6000
4000
2000
0
CON
GD
GD
+ KN-62
KCl
KCl
+ KN-62

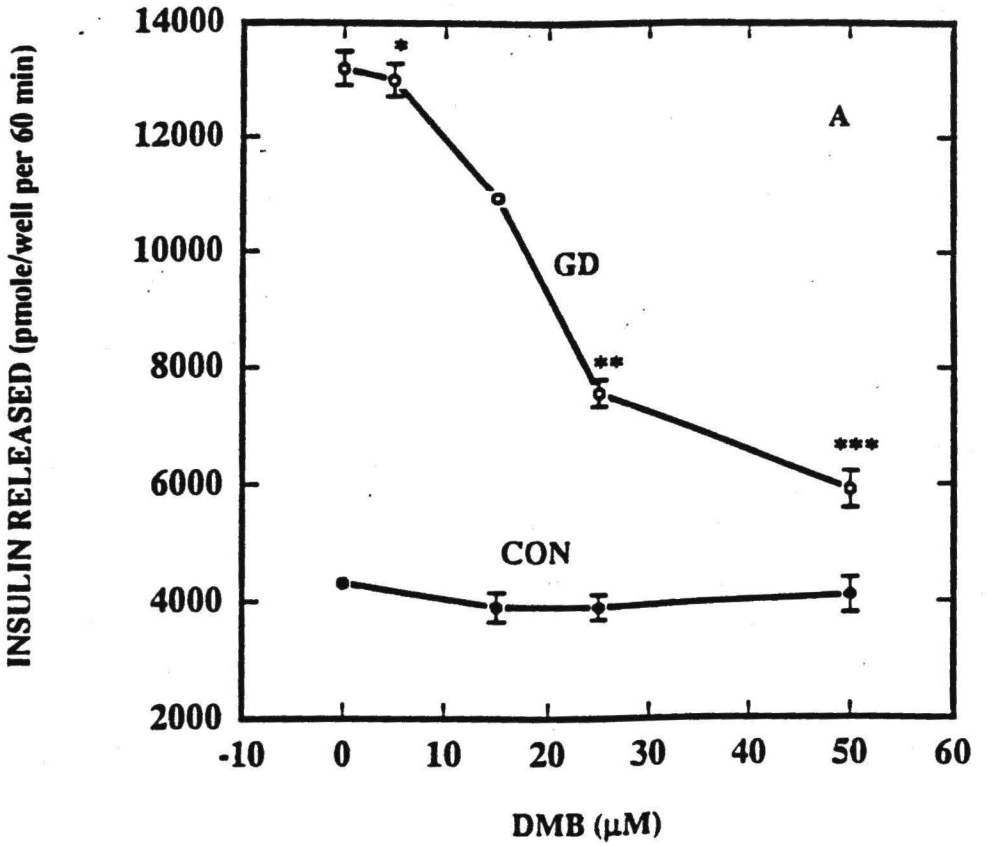
Effects of inhibitors of Na^{+}/Ca^{2+} exchanger activity on gramicidin D-induced insulin secretion

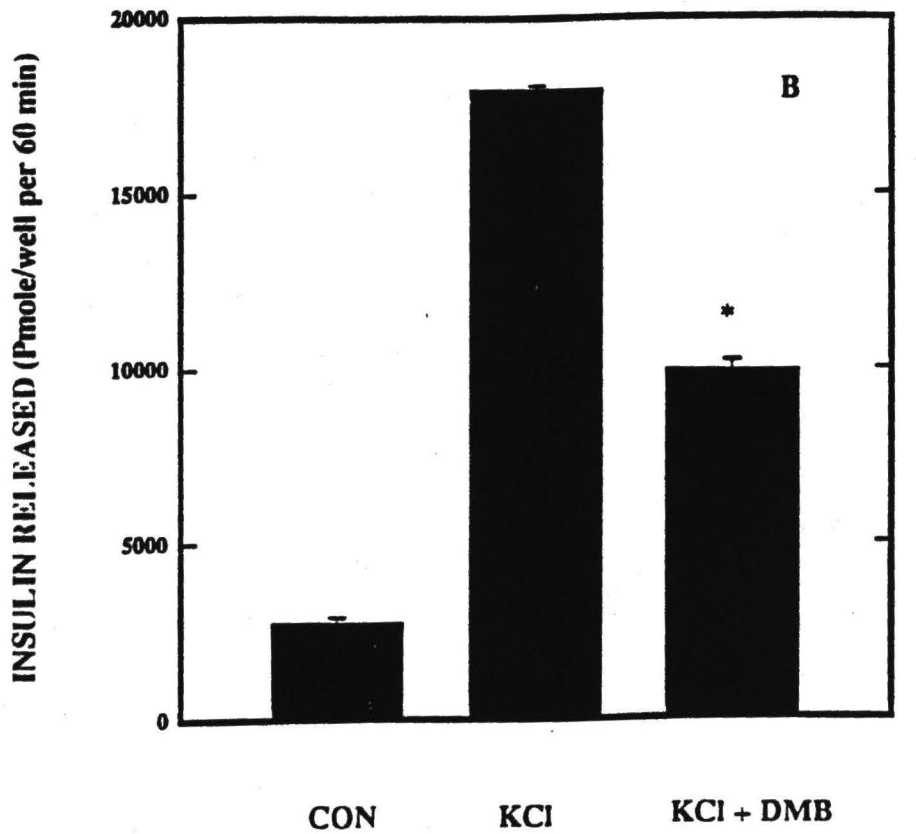
The presence a Na^{+}/Ca^{2+} exchange activity was confirmed previously in βTC3 cells (see Results, Fig. 10). The following experiments were performed to assess the actual involvement of a Na^{+}/Ca^{2+} exchanger in the gramicidin D-induced insulin secretion. Dimethylbenzamil (DMB, 25 μM) a potent inhibitor of Na^{+}/Ca^{2+} exchanger (229, 243), dose-dependently inhibited gramicidin D-induced insulin secretion (Fig. 26A). At a maximally effective concentration (50 μM), DMB inhibited gramicidin D-induced insulin secretion by approximately 80 %. DMB at a similar concentration had no effect on basal secretion in the presence of the vehicle alone. The half maximal effect of DMB (IC_{50}) was calculated to be approximately 18 μM (Fig. 26A), a concentration that is consistent with reported value for the effect of DMB on Na^{+}/Ca^{2+} exchanger activity *in vitro* (11 μM, 243).

It was important however, to test the effect of DMB effect on KCl-induced insulin secretion to assess its specificity. DMB (50 μM) also inhibited KCl-induced insulin secretion by 48 % (Fig. 26B). This observation was unexpected since KCl effects on β-cell secretion were thought to be mediated by VSCC exclusively. Since KCl was unlikely to activate Na^{+}/Ca^{2+} exchanger, this observation suggested that DMB also targeted the L-type Ca^{2+} channels. The extent to which DMB inhibited KCl-induced (48 %) was significantly less than the observed inhibition of gramicidin D-induced insulin release. This result suggested there were at least two components of Ca^{2+}-influx activated by gramicidin D. Since these data suggested that DMB targeted both the VSCC and the Na^{+}/Ca^{2+} exchanger, a more potent effect of DMB on gramicidin D-induced secretion would be anticipated as the result of inhibition of both activities.

Fig. 26 Effects DMB on gramicidin D- (A) and KCl- (B) induced insulin secretion from βTC3 cells.

(A) The effect of DMB (5-50 μM), a potent inhibitor of the Na^+/Ca^{2+} exchanger, on gramicidin D-induced insulin secretion was evaluated. Insulin release was assayed as described in Methods (*$P < 0.05$ vs. control and **, ***$P < 0.004$ vs. gramicidin D). (B) The specificity of DMB for the Na^+/Ca^{2+} exchanger and VSCC was assessed indirectly by testing its effect on KCl-induced insulin secretion (*$P < 0.002$ vs. KCl).


INSULIN RELEASED (pmole/well per 60 min)
14000
12000
10000
8000
6000
4000
2000
-10
0
10
20
30
40
50
60
DMB (μM)
A
GD
CON
*
**
***


INSULIN RELEASED (Pmole/well per 60 min)
20000
15000
10000
5000
0
B
*
CON
KCl
KCl + DMB

Another inhibitor of the Na^+/Ca^{2+} exchanger, dichlorobenzamil (50 μM, DCB (180)) was also tested on gramicidin D-induced insulin secretion. As shown in Fig. 27, DCB also inhibited gramicidin D-induced insulin secretion by 50 %. In an attempt to verify its specificity for the Na^+/Ca^{2+} exchanger and not VSCC, its effect on KCl-induced insulin secretion was also evaluated. As shown in Fig. 27, DCB (50 μM) inhibited KCl-induced insulin secretion by 48 % suggesting that DCB also targeted VSCC. Although these data may suggest the involvement of the βTC3 cell Na^+/Ca^{2+} exchanger in mediating extracellular influx in response to gramicidin D, the effects of the Na^+/Ca^{2+} exchanger inhibitors were not specific, and appeared to inhibit VSCC as well.

In summary of the results of this section, gramicidin D-induced insulin secretion was suggested to involve the activation of both VSCC (L-type) and Na^+/Ca^{2+} exchanger in mediating extracellular Ca^{2+}-influx and stimulation of insulin release from β-cells. However, the lack of specific inhibitor against the Na^+/Ca^{2+} exchanger makes it difficult to accurately assess the involvement of the Na^+/Ca^{2+} exchanger in gramicidin D mechanism of insulin secretion at this time.

Effect of inhibitor of the $Na^{\pm}/H^{\pm}$ exchanger on gramicidin D-induced insulin secretion

Although the influx of Na^+ by gramicidin D failed to affect pH_i in βTC3 cell, it was necessary to eliminate the involvement of the Na^+/H^+ exchanger in gramicidin D-induced insulin secretion. Therefore, the effect of ethylisopropyl amiloride (EIPA)(100 nM), a potent inhibitor of the Na^+/H^+ exchanger, on gramicidin D-induced insulin secretion was tested (230). EIPA had no significant effect on gramicidin D-induced insulin secretion (Fig. 28). This data suggested the Na^+/H^+ exchanger played no role in the mechanism of gramicidin D-induced insulin secretion.

Fig. 27 Effects of DCB on KCl- and gramicidin D-induced insulin secretion from βTC3 cells.

The effect of DCB (50 μM), a potent inhibitor of the Na^+/Ca^{2+} exchanger, on KCl- and gramicidin D-induced insulin secretion was assessed. DCB was also tested on control and no effect on basal insulin secretion levels was observed.

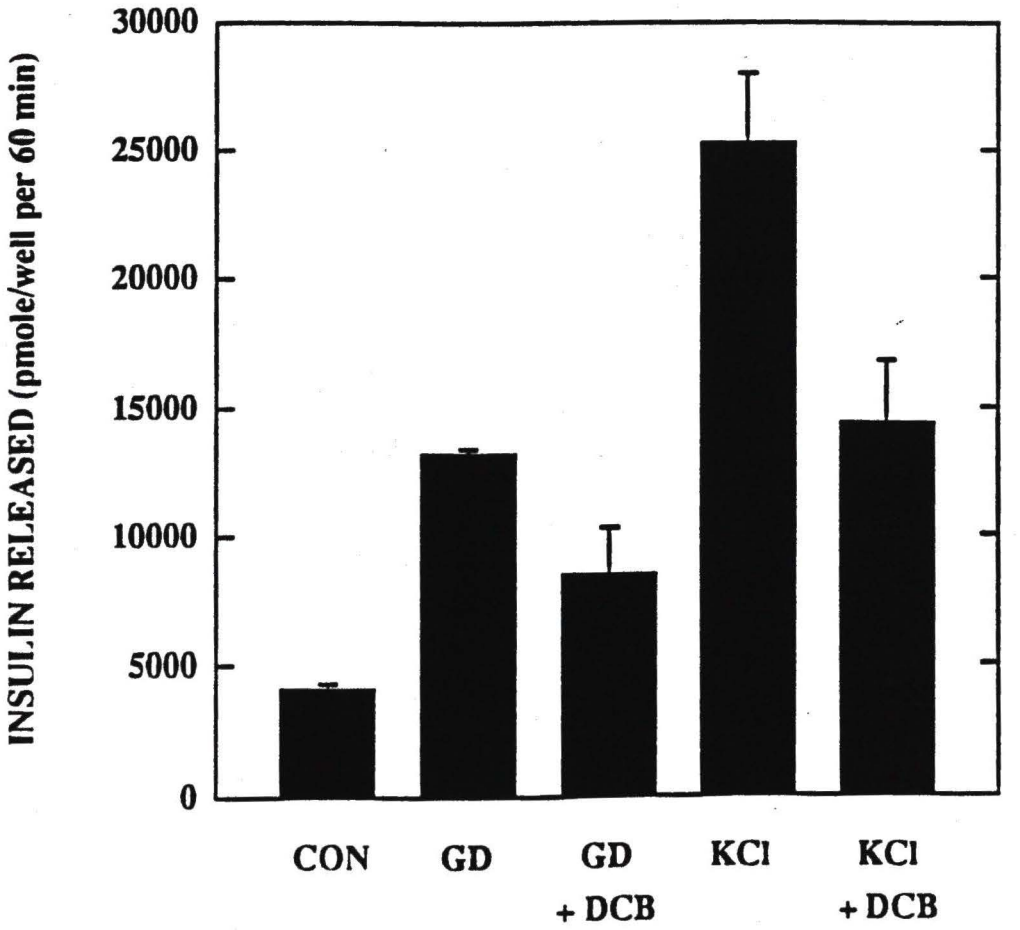
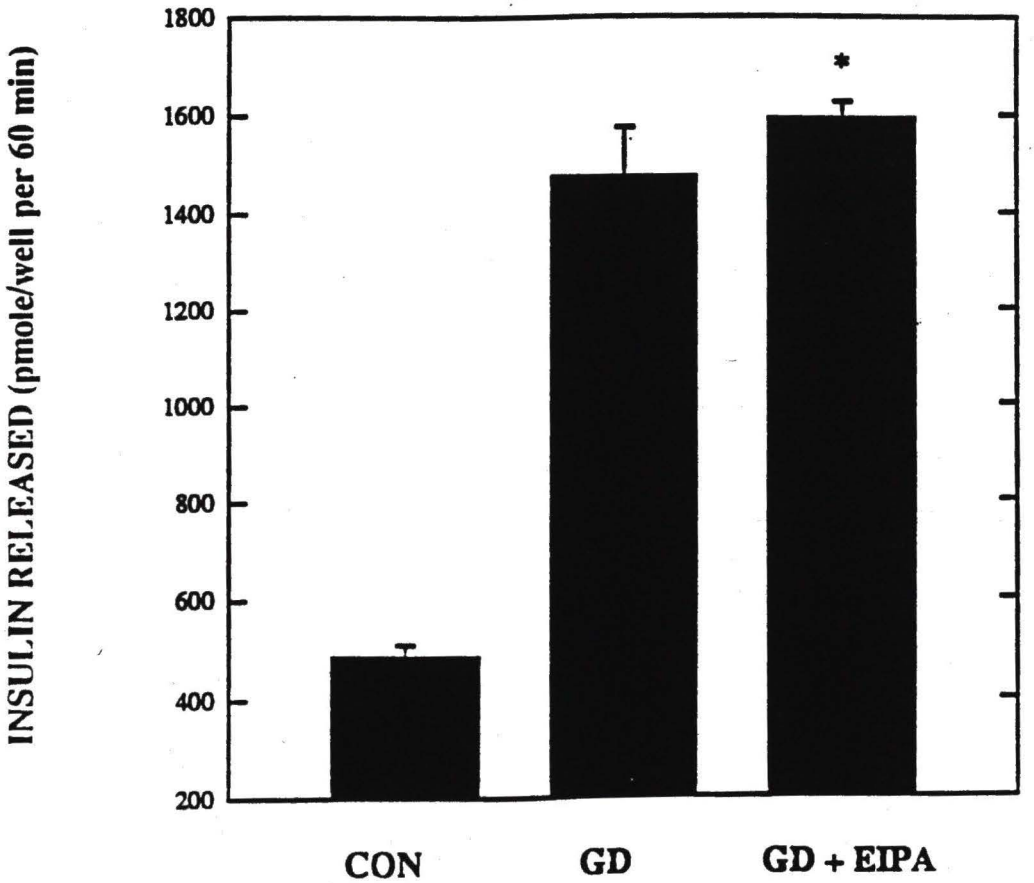

INSULIN RELEASED (pmole/well per 60 min)
30000
25000
20000
15000
10000
5000
0
CON
GD
GD + DCB
KCl
KCl + DCB

Fig. 28 Effect of EIPA on gramicidin D-induced insulin secretion from βTC3 cells.

In order to eliminate the involvement of the Na^+/H^+ exchanger in gramicidin D-induced insulin secretion, the effect of EIPA (100 nM), a potent inhibitor of such activity, was tested (*P > 0.3 vs. gramicidin D). EIPA had no effect on basal insulin secretion levels.


INSULIN RELEASED (pmole/well per 60 min)
1800
1600
1400
1200
1000
800
600
400
200
*
CON
GD
GD + EIPA

A comparison of the effects of inhibitors of VSCC and Na^+/Ca^{2+} exchanger on KCl- and gramicidin D-induced insulin release is shown in Table 2.

Perifusion of βTC3 cells with gramicidin D

The static-model of insulin secretion helped characterize the potential mechanisms involved in the insulin exocytotic pathway activated by gramicidin D and suggested the involvement both of the VSCC and Na^+/Ca^{2+} exchange activities. The secretion data confirmed that a difference existed between gramicidin D and KCl effects on β-cells revealed in the fura-2 studies. To study these mechanisms further, it was necessary to analyze the dynamics of insulin secretion induced by both gramicidin D andKCl using a perifusion model (see Methods for detailed description). It was reasoned that this model also would help dissect the insulin response with ion-channel inhibitors. As shown in Fig. 29A, gramicidin D (1 μM) induced a biphasic insulin secretion response. The response was characterized by an initial peak at 7-12 minutes after stimulation followed by a second phase of insulin secretion which lasted 30 minutes. Also, KCl (40 mM) induced a biphasic insulin secretion response (Fig. 29B). This result was consistent with a previously published report (218).

Next, the effects of two different inhibitors of ion-channels on gramicidin D-induced insulin secretion using the perifusion model were studied. Nifedipine, a selective inhibitor of VSCC, which does not target the Na^+/Ca^{2+} exchanger was tested on gramicidin D-induced insulin secretion (135). Also, DMB, a putative inhibitor of the Na^+/Ca^{2+} exchanger, but was demonstrated in this study to target the VSCC as well, was tested for its ability to modulate gramicidin D-induced insulin release. As shown in Fig. 30A, nifedipine (5 μM) modestly inhibited the first phase of insulin secretion-induced by gramicidin D, whereas it suppressed the second phase of secretion.

Table 2: Effects of ion-channel inhibitors on gramicidin D (GD)- and KCl-induced insulin secretion from βTC3 cells.

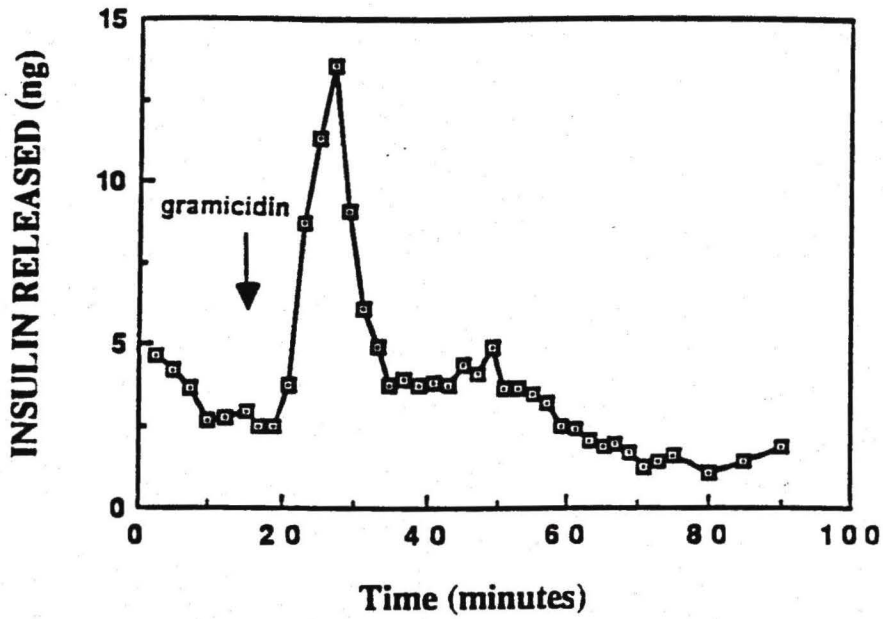
A comparison of the effects of VSCC and Na^+/Ca^{2+} exchanger inhibitors on KCl- and gramicidin D-induced insulin release is shown.

Inhibitor	% of inhibition of GD-induced insulin secretion	% of inhibition of KCl-induced insulin secretion
A. VSCC Inhibitors		
1. Verapamil (20 μM)	42.6	80.8
2. Nifedipine (20 μM)	31.3	80
3. KN-62 (10 μM)	19	38
B. VSCC and Na^+/Ca^{2+} exchanger Inhibitors		
1. DMB (50 μM)	80	48
2. DCB (50 μM)	50	48
C. Na^+/H^+ exchanger Inhibitor		
1. EIPA (100 nM)	no effect	not tested

Fig. 29 Effects of Perifusion of βTC3 cells with gramicidin D (1 μM) or KCl (40 mM) on insulin release.

βTC3 cells (5-10 x10^6) prepared as described in Methods were perifused with: (A) gramicidin D (n= 3) or (B) KCl (40 mM, n=1) at a flow rate 0.5 ml/minute and insulin content of samples was assayed as described in Methods.

A


INSULIN RELEASED (ng)
15
10
5
0
gramicidin
0
20
40
60
80
100
Time (minutes)

B

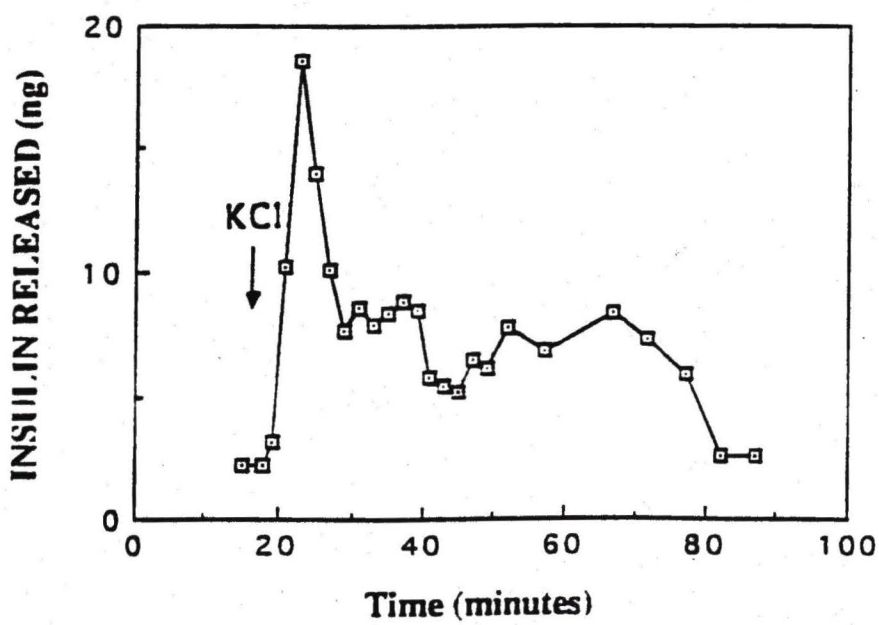
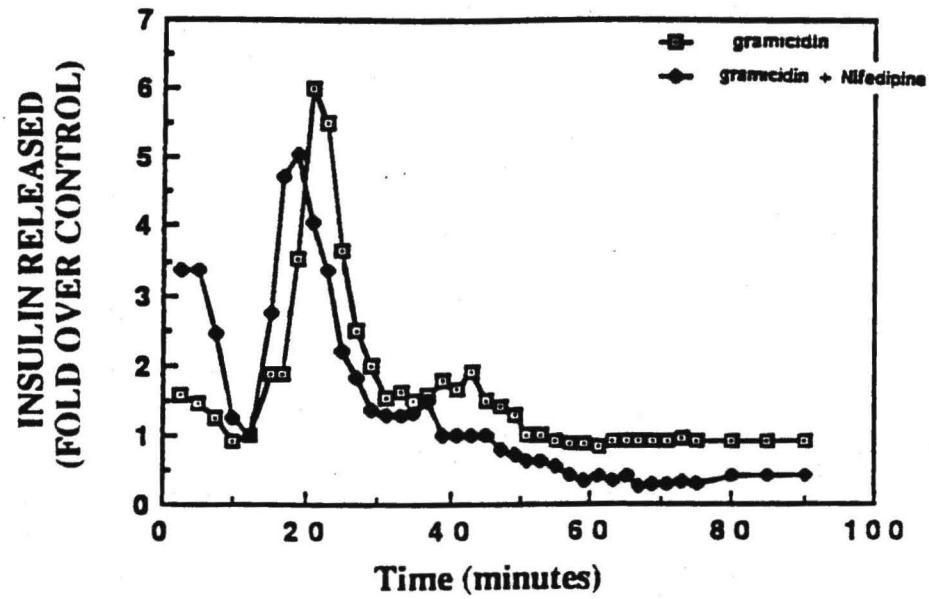

INSULIN RELEASED (ng)
20
10
0
KCl
0
20
40
60
80
100
Time (minutes)

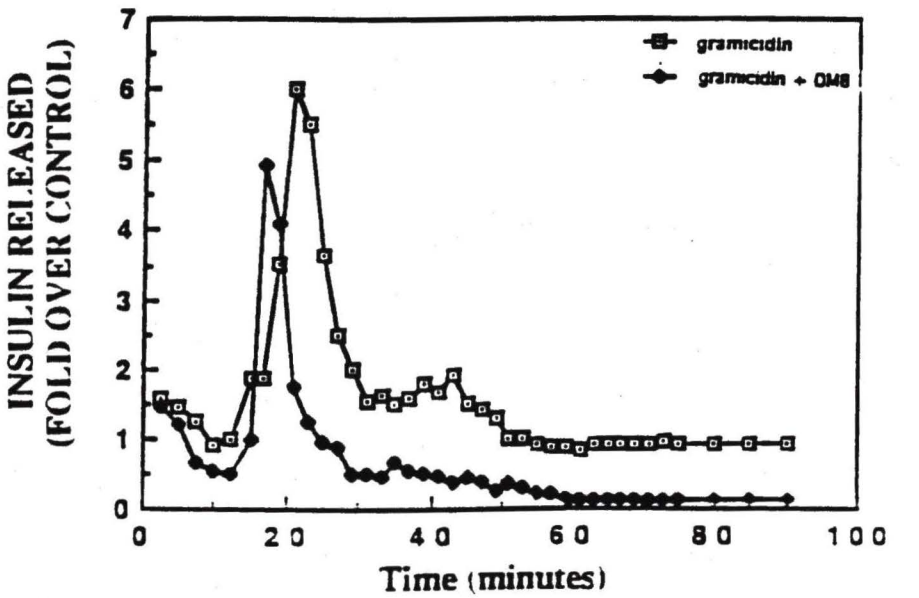
Fig. 30 Effects of nifedipine and DMB on insulin release from βTC3 perifused with gramicidin D (1 μM).

βTC3 cells (5-10 x10^6) were perifused for 5 minutes with: (A) nifedipine (5 μM) or (B) DMB (25 μM) prior to perifusion with gramicidin D (1 μM)(n = 3). Samples were collected and their insulin content was determined by radioimmunoassay.

A


INSULIN RELEASED
(FOLD OVER CONTROL)
gramicidin
gramicidin + Nifedipine
0
1
2
3
4
5
6
7
0
20
40
60
80
100
Time (minutes)

B


INSULIN RELEASED
(FOLD OVER CONTROL)
gramicidin
gramicidin + OM8
0
1
2
3
4
5
6
7
0
20
40
60
80
100
Time (minutes)

This result suggested that the first phase of secretion was likely involving an activity that was different from VSCC, whereas the second phase of secretion was involving, at least in part, the VSCC since insulin secretion was suppressed. When DMB (25 μM) was tested for its ability to influence the insulin response, it was demonstrated that DMB inhibited the first phase of secretion while it abolished the second phase of insulin secretion (Fig. 30B). These observations may suggest the activation of Na^+/Ca^{2+} exchanger during the first phase of secretion as well as the second phase of insulin secretion induced by gramicidin D. However, the ability of DMB to inhibit the VSCC may also suggest that the observed inhibition was due to inhibiting VSCC by DMB. On the other hand, nifedipine, an inhibitor of VSCC but not the Na^+/Ca^{2+} exchanger, only partially inhibited the first phase of insulin secretion suggesting the involvement of an additional mechanism in the first phase. The additional mechanism is postulated to involve the Na^+/Ca^{2+} exchanger.

In summary of the perifusion results, gramicidin D-induced a biphasic insulin response. The VSCC is suggested to be involved in both phases of insulin secretion. However, the Na^+/Ca^{2+} exchanger is likely involved in the first phase but its role in the second phase can not be confirmed due to the lack of specific inhibitors.

Effect of gramicidin D on Cam-kinase II and myosin light chain kinase activities

As demonstrated in the previous sections, gramicidin D induced an influx of extracellular Na^+ and Ca^{2+} in βTC3 cells. Insulin secretion was totally dependent on Ca^{2+} since EGTA abolished gramicidin D-induced insulin secretion (Fig. 14B). This result suggested that raising $[Ca^{2+}]_i$ was the key event that triggered insulin secretion by gramicidin D. While the mechanism by which an increase in $[Ca^{2+}]_i$ stimulates insulin secretion is still poorly understood, this process is thought to be mediated by the

activation of Ca^{2+}-dependent protein kinases. These kinases have been suggested to function at distal steps in insulin exocytosis (233). Emphasis has been placed at the potential involvement of: (i) the multifunctional Ca^{2+}/calmodulin-dependent protein kinase (Cam kinase II); and (ii) the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK)(34-39)(57, 257). It was of interest to test the ability of gramicidin D to induce the activation of such key kinases in βTC3 cells.

Activation of Cam kinase II

Cam kinase II activity has been detected in primary β-cells (40). In initial experiments employing an assay established in the laboratory utilizing autocamtide-2 as exogenous substrate, βTC3 cells were found to possess Cam kinase II-like activity. Furthermore, immunoblot analyses performed by Dr. Easom using a chicken anti-Cam kinase II antibody (raised against a synthetic peptide of a primary sequence that is universal to all known Cam kinase isoenzymes), have demonstrated the presence of at least two isoenzyme forms of Cam kinase II in βTC3 cells. The principal objective of the following experiments was to determine whether the gramicidin D-induced increase in $[Ca^{2+}]_i$ activated Cam kinase II in βTC3 cells or not. The assay was based on a novel characteristic of Cam kinase II. After an increase in $[Ca^{2+}]_i$, the enzyme is autophosphorylated (THR^{286} in the α-subunit of Cam kinase II). Such autophosphorylation traps calmodulin and converts the enzyme into a partially Ca^{2+}/calmodulin-independent form (autonomous form). This autonomous activity can be determined by the enzymatic phosphorylation of Cam kinase II-specific peptide substrate "autocamtide-2" in the absence of Ca^{2+}(205).

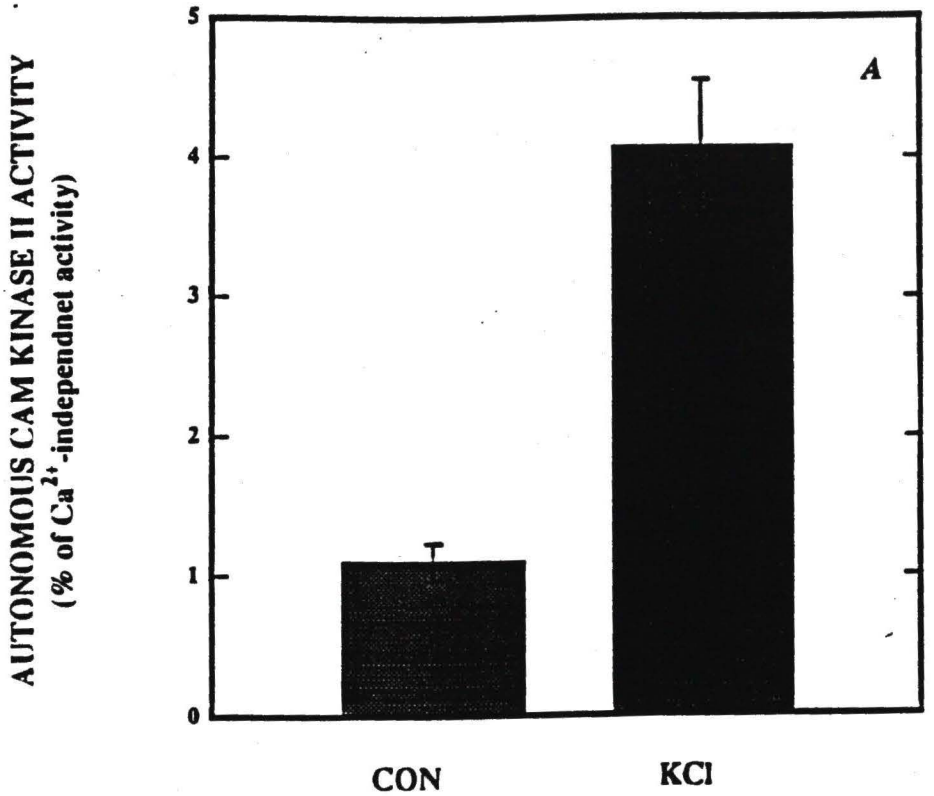
Using conditions established for assay of Cam kinase, the ability of gramicidin D to promote generation of autonomous Cam kinase II activity was assessed. First, It was

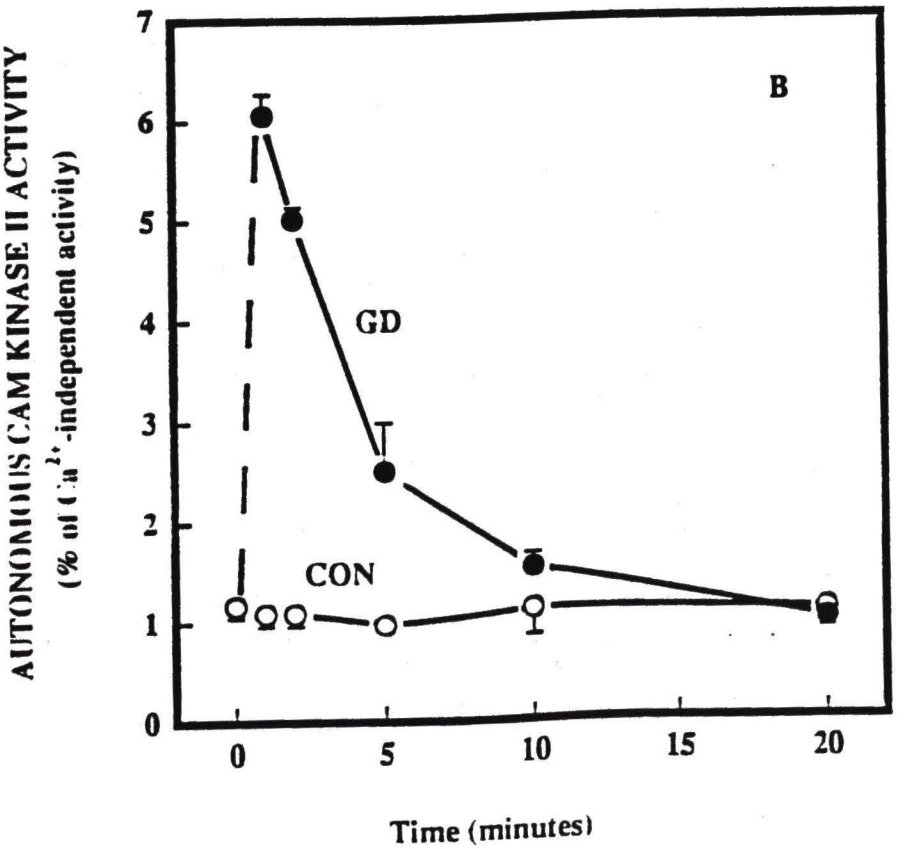
necessary to validate the assay using a stimulus (KCl) that has been shown to activate Cam kinase II. Cam kinase II was assayed at 2 minutes after stimulation by KCl (40 mM). As illustrated in Fig. 31A, KCl induced a 3.71 $\pm$ 0.6 fold increase in the autonomous activity relative to control ($p < 0.001$ vs. control). This result validated the assay. Next, the activity of Cam kinase II in cells treated with gramicidin D (1 μM) was followed over a period of time (0-20 minutes). In a resting cell, the proportion of Cam kinase II in the autonomous activity state (Ca^{2+}-independent form) was 1.00 $\pm$ 0.23 (Fig. 31B) and did not change throughout the course of the experiment (0-20 minutes). However, in cells exposed to gramicidin D (1 μM), activation of Cam kinase II peaked at 1-2 minutes as detected by increasing the autonomous activity ratio (5.56 $\pm$ 0.24 and 4.58 $\pm$ 0.2 fold relative to control at 1 and 2 minutes, respectively)($p < 0.01$ vs. control, Fig. 31B). However, the autonomous activity ratio declined to basal levels at 20 minutes. These data suggested that gramicidin D-induced increase in $[Ca^{2+}]_i$ activated Cam kinase II and the degree of activation was similar to that induced by KCl.

Since inhibitors of ion-channels suppressed gramicidin D-induced increase in $[Ca^{2+}]_i$ (see Results, section 2) and gramicidin D-induced insulin secretion (section 5 of Results), the effects of ion-channel inhibitors on gramicidin D-induced activation of Cam kinase II were studied. These experiments were performed in an attempt to correlate the two parameters (inhibition of both insulin release and Cam kinase II activation). As shown in Fig. 32, DMB (25 μM) inhibited gramicidin D-induced activation of Cam kinase II by 61 $\pm$ 15 %. However, DMB (25 μM) inhibited gramicidin D-induced insulin secretion by 38 % (Fig. 26A). Such observation suggested that the inhibition of insulin secretion did not correlate with the inhibition of Cam kinase.

Fig. 31 Effects of KCl (A) and gramicidin D (B) on Cam kinase activation in βTC3 cells.

(A) βTC3 cells were stimulated with KCl (40 mM) and Cam kinase II was assayed as described in Methods by measuring its autonomous activity (Ca^{2+}-independent activity). (B) βTC3 cells were treated with gramicidin D (1 μM) over a period of time (0-20 minutes) and the activity of Cam kinase was assayed as previously described in Methods.


AUTONOMOUS CAM KINASE II ACTIVITY
(% of Ca2+-independnet activity)
A
5
4
3
2
1
0
CON
KCl


AUTONOMOUS CAM KINASE II ACTIVITY
(% of Ca2+-independent activity)
B
GD
CON
7
6
5
4
3
2
1
0
0
5
10
15
20
Time (minutes)

In contrast to DMB, verapamil (20 μM) abolished gramicidin D-induced activation of Cam kinase II. Although under such conditions verapamil did not abolish the influx of extracellular Ca^{2+}-induced by gramicidin D (Fig. 8B) nor insulin secretion (only 42.6 % inhibition, Fig. 24A). These results are in support of a hypothesis suggesting the lack of correlation between insulin secretion and activation of Cam kinase II (256).

In summary of this section of results, gramicidin D-induced the activation of Cam kinase II. DMB and verapamil inhibited gramicidin D-induced insulin secretion and Cam kinase II activation to different extents. These data are summarized in Table 3.

Assay of myosin light chain phosphorylation

MLCK is a Ca^{2+}/calmodulin-dependent protein kinase which by phosphorylating of a specific substrate, MLC_{20} (MLC_{20}), regulates microfilament polymerization (234, 263). This enzyme has been suggested to regulate insulin secretion. Therefore, it was of interest to test the involvement of this enzyme in gramicidin D-induced insulin secretion. The experimental strategy in this section was divided into two parts. The first set of experiments was designed to test the effect of wortmannin, a potent myosin light chain kinase inhibitor (232), on gramicidin D-induced insulin secretion. In the second set of experiments the ability of gramicidin D to affect the phosphorylation levels of myosin light chain was evaluated.

As shown in Fig. 33A, wortmannin (10 μM) inhibited both KCl- and gramicidin D-induced insulin secretion by 87 % and 63 %, respectively. Wortmannin had no effect on basal insulin secretion rate. These results suggested that myosin light chain kinase played a role in insulin secretion induced by gramicidin D and KCl.

Fig. 32 Effects of verapamil and DMB on gramicidin D-induced activation of Cam kinase II in βTC3 cells.

The abilities of DMB (25 μM) and verapamil (ver, 20 μM) to inhibit gramicidin D-induced activation of Cam kinase II were tested (*P < 0.005 vs. control and ***P < 0.5 vs. gramicidin D). The autonomous Cam kinase II activity was assayed as described in Methods (section 8). Verapamil (20 μM) and DMB (25 μM) had no effect on basal levels of Cam kinase II autonomous activity.

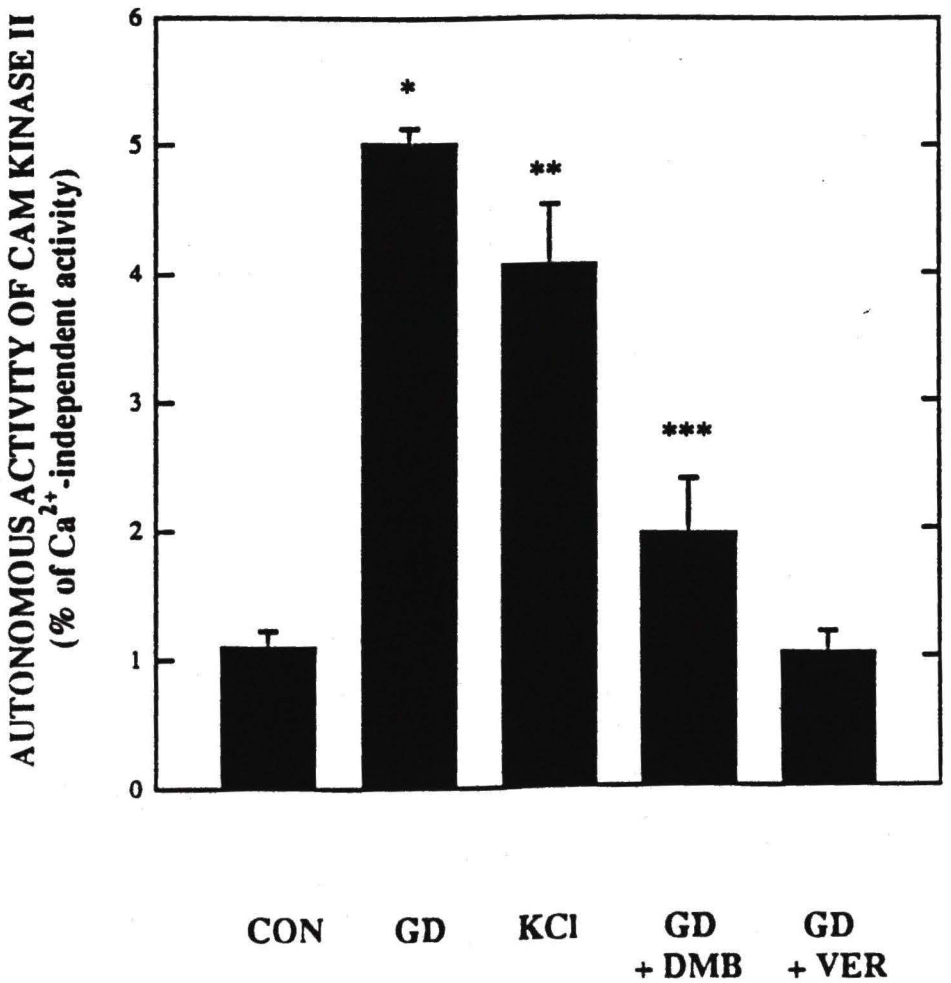

AUTONOMOUS ACTIVITY OF CAM KINASE II
(% of Ca2+-independent activity)
6
5
4
3
2
1
0
*
**
***
CON
GD
KCl
GD
+ DMB
GD
+ VER

Table 3: Effects of verapamil and DMB on gramicidin D-induced insulin secretion and activation of Cam kinase II.

The percentage of inhibition of gramicidin D-induced insulin secretion and Cam kinase II activation by DMB and verapamil are shown.

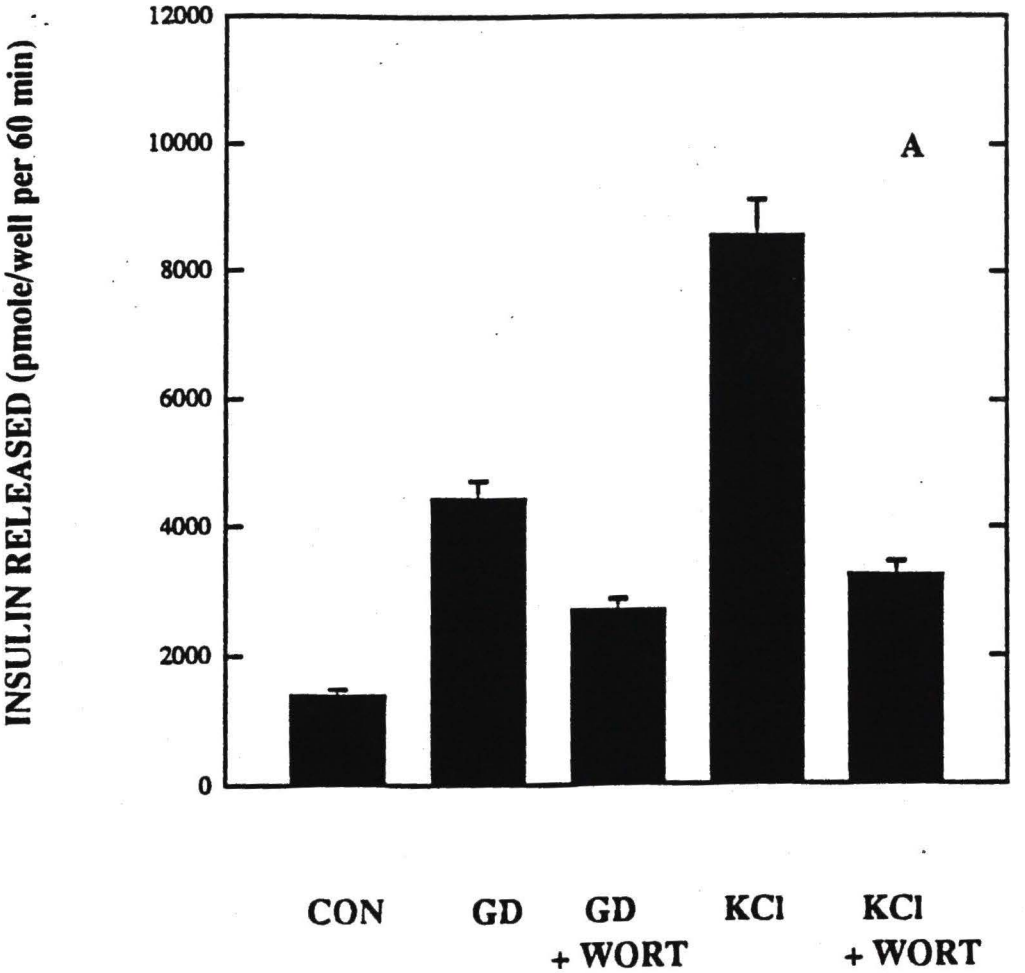
Inhibitor	% of inhibiton of insulin release	% of inhibition of Cam kinase II
Verapamil (20 μM)	42.6	100
DMB (25 μM)	38	61

In the second set of experiments, myosin light chain kinase activation was followed in intact βTC3 cells by an assay of the phosphorylation level of its specific substrate MLC_{20}. Phosphorylation of MLC_{20} can be detected by the charge-induced separation of phosphorylated and the non-phosphorylated light chain forms in an electrically-driven field generated in native urea-glycerol PAGE; phosphorylated forms of MLC_{20} migrate at a faster rate than non-phosphorylated forms. Both forms can be detected by immunoblotting using antibodies with an equal affinity towards non-phosphorylated and phosphorylated forms. By densitometry, the proportion of phosphorylated MLC_{20} can be calculated as a percentage of total MLC_{20}. The level of phosphorylation will reflect the activation of myosin light chain kinase. This method has been widely used for the assay of MLCK in numerous muscle systems (235).

As shown in Fig. 33B, immunoblot analyses detected 4 bands in the extracts obtained from non-stimulated cells (treated with vehicle alone). This observation suggested the presence of both non-phosphorylated and phosphorylated forms of MLC_{20} (mono- and di-phosphorylated corresponding to the 2 and 3 bands, respectively) in resting βTC3 cells. The 4th band may represent the non-essential MLC_{17} that is not phosphorylated by MLCK (232). These results were similar to that reported in non-muscle cell cultures (232). Analysis of extracts of stimulated cells with gramicidin D showed a similar pattern. By densitometry, no significant differences between the ratios of phosphorylated and nonphosphorylated forms of MLC_{20}, were detected (Table 4). Similar results were obtained using KCl as a stimulus. Therefore, no change in myosin light chain phosphorylation in response to either KCl or gramicidin D could be detected using this method. Since KCl failed to elicit increased phosphorylation levels of MLC_{20}, this method for assay of MLC_{20} in non-muscle systems is questionable.

Fig. 33 Involvement of MLCK in gramicidin D-induced insulin secretion from βTC3 cells.

(A) The effects of wortmannin (wort, 10 μM), a potent inhibitor of the myosin light chain kinase on KCl- and gramicidin D-induced insulin secretion were tested. Insulin release was assayed as described in Methods. (B) βTC3 cells were stimulated with gramicidin D (1-5 μM) and MLC_{20} was extracted and subjected to imunoblotting as described in Methods (section 9). Immunoblotting utilized rabbit anti-bovine tracheal myosin light chain antibodies (ST: purified smooth muscle myosin light chain extracts were used as a marker, lane 1: control (DMSO treatment), lanes 2, 3: gramicidin D (1 μM), lane 4: gramicidin D (5 μM) and lane 5: KCl (40 mM)).


INSULIN RELEASED (pmole/well per 60 min)
12000
10000
8000
6000
4000
2000
0
A
CON
GD
GD + WORT
KCl
KCl + WORT


5 4 3 2 1 ST
B
Nonophosphorylated
Monophosphorylated
Diphosphorylated
MLC17

Table 4: Effect of gramicidin D on phosphorylation state of MLC_{20} in βTC3 cells.

MLC_{20} extracts from βTC3 cells were separated in urea-gel page electrophoresis as described in Methods (section 9). Proportions of non-phosphorylated MLC_{20}, mono-phosphorylated MLC_{20} (MLC_{20}-P) and di-phosphorylated MLC_{20} (MLC_{20}-P-P) are expressed as percentage of total MLC_{20}.

MLC_{20}-form	Control	KCl (40 mM)-stimulation	GD (1 μM)-stimulation	GD (5 μM)-stimulation
% MLC_{20}	47.4	47.3	48.1	49.5
% MLC_{20}-P	24.2	26.5	26.1	22.5
% MLC_{20}-P-P	28.2	26.2	25.8	28

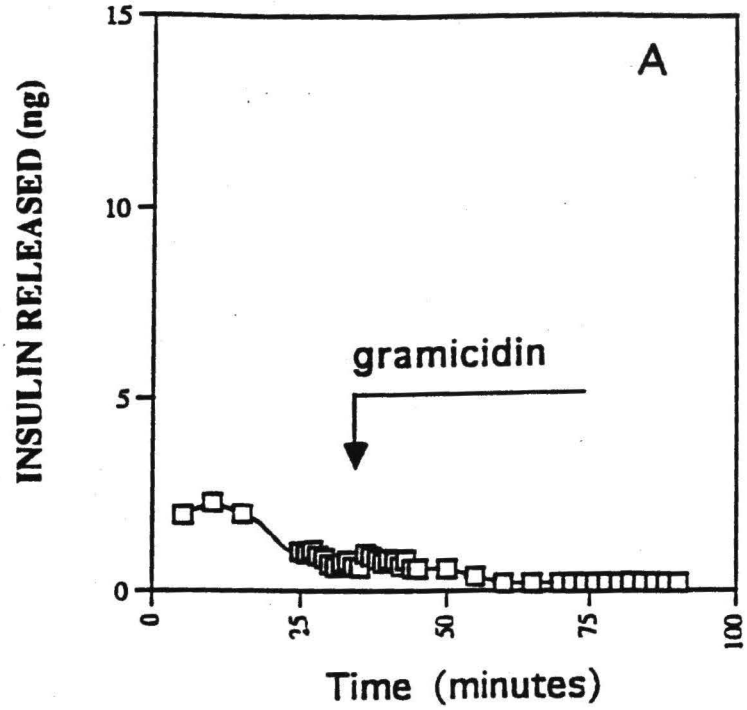
Effect of gramicidin D on pancreatic islets

All the experiments described in this dissertation to this point were performed using a transformed β-cell line (βTC3 cells). In order to apply physiological significance to these observations, it was necessary to test the ability of gramicidin D to stimulate insulin secretion from the primary β-cell. For this purpose, islets of Langerhans were isolated from rat pancreati. As demonstrated in Fig. 34B, glucose (17 mM) induced a 5.8-fold increase in insulin release relative to control (incubated in the presence of 3 mM glucose). By contrast, gramicidin D in the presence of basal levels of glucose (3 mM), failed to stimulate insulin secretion as assessed using either the perifusion or static model (Figs. 34 A-B). These observations suggested that either gramicidin D failed to increase $[Na^+]_i$ in freshly isolated primary β-cells or the increase in $[Na^+]_i$ was insufficient to induce an increase in $[Ca^{2+}]_i$ and insulin secretion. Irregardless of the explanation, these results clearly demonstrated that primary β-cells and βTC3 cells respond distinctly to gramicidin D.

In order to resolve this difference, the effect of ouabain; a drug that inhibits the Na^+/K^+ ATPase and therefore, expected to increase $[Na^+]_i$ and mimicking gramicidin D effect, on insulin release from pancreatic islets and βTC3 cells was tested. As shown in Fig. 35B, ouabain (0.5 mM) induced a 2-fold increase in insulin release from pancreatic islets, relative to control (in the presence of 3 mM glucose). This result was similar to previously reported effects of ouabain in islets (175). Gramicidin D (1 μM) failed to potentiate ouabain-induced insulin release.

Fig. 34 Effect of gramicidin D (1 μM) on insulin secretion from pancreatic islets.

(A) The perifusion model and (B) static model of insulin secretion were used for characterization the effect of gramicidin D on insulin release from islets. Pancreatic islets isolated as described in Methods (section 3) were treated with gramicidin D (1 μM) and insulin release was assayed as described in Methods. Glucose (17 mM) was used as a positive control in (B).


A
INSULIN RELEASED (ng)
15
10
5
0
gramicidin
0
25
50
75
100
Time (minutes)

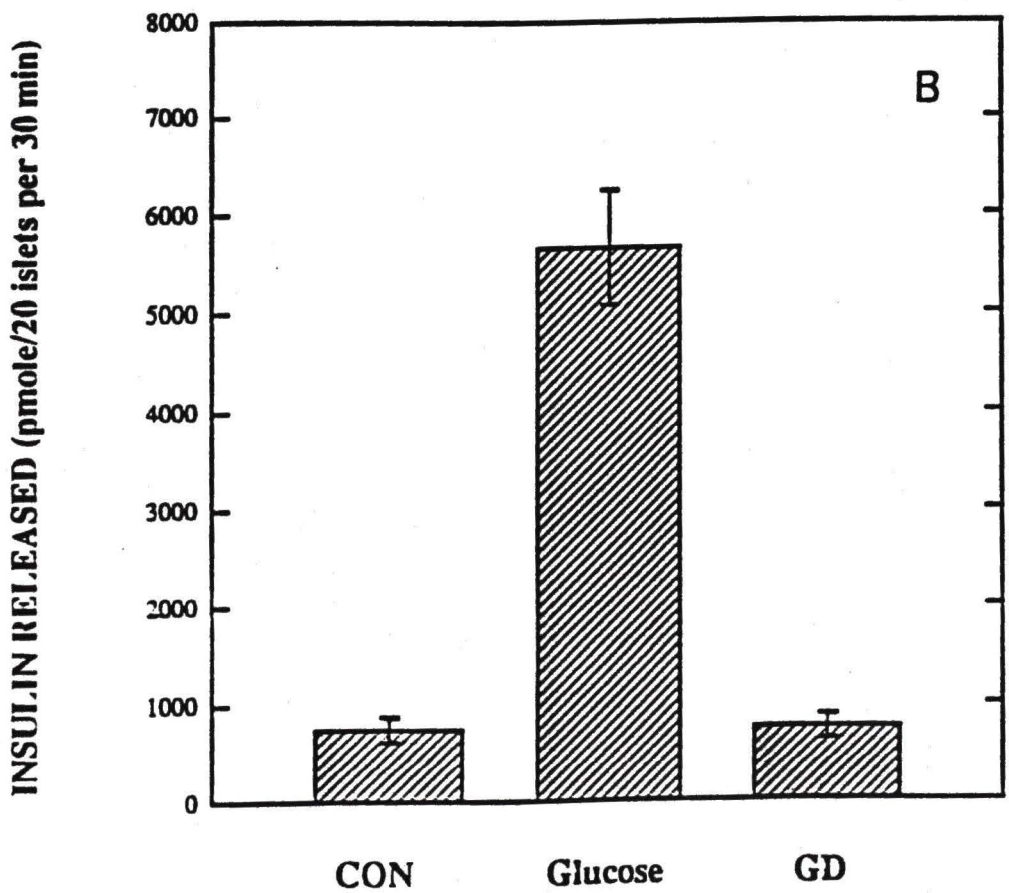
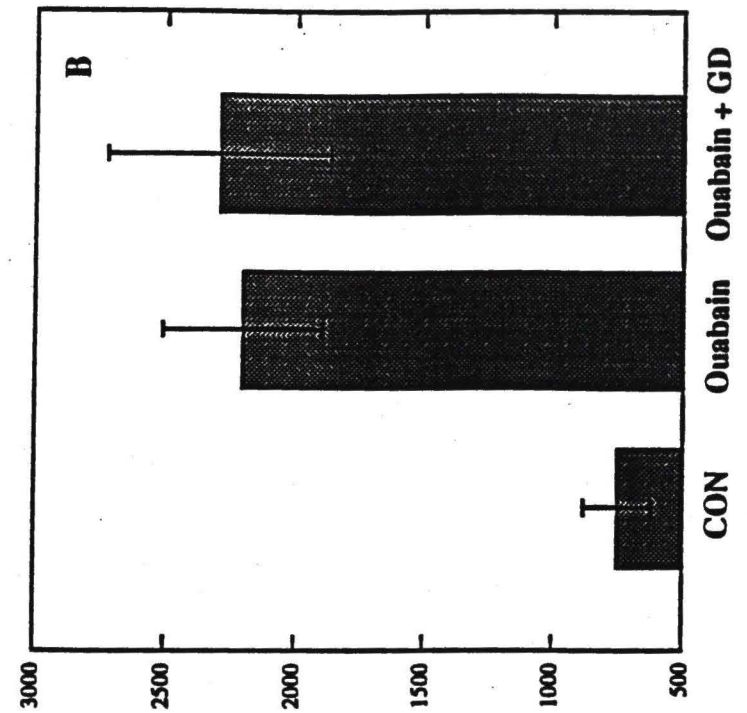
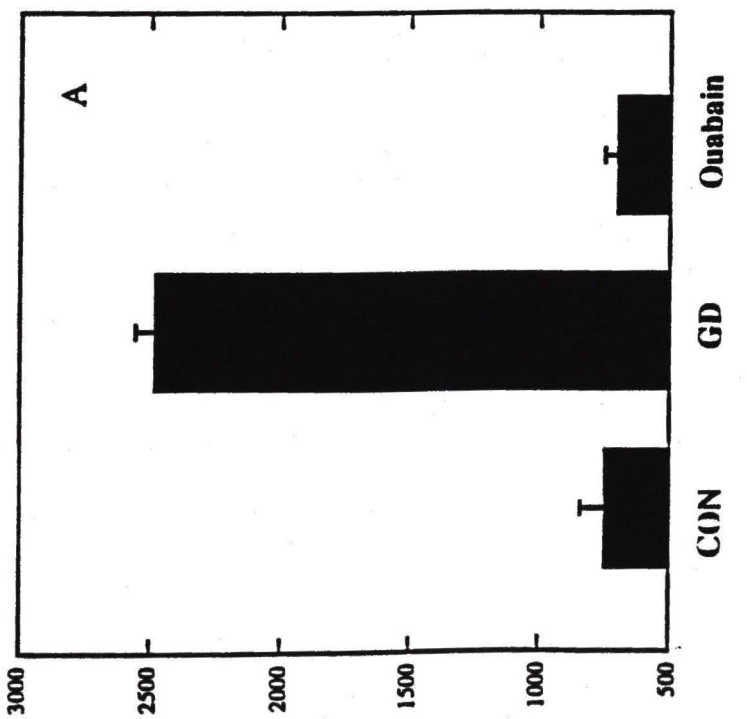

B
INSULIN RELEASED (pmole/20 islets per 30 min)
8000
7000
6000
5000
4000
3000
2000
1000
0
CON
Glucose
GD

Fig. 35 Effects of ouabain on insulin release rates from (A) βTC3 cells and (B) pancreatic islets.

(A) βTC3 cells and (B) pancreatic islets were treated with ouabain (0.5 mM) and insulin release was determined as described in Methods.


B
INSULIN RELEASED (pmole/20 islets per 30 min)
3000
2500
2000
1500
1000
500
CON
Ouabain
Ouabain + GD


A
INSULIN RELEASED (pmole/well per 60 min)
3000
2500
2000
1500
1000
500
CON
GD
Ouabain

By contrast, ouabain failed to stimulate insulin release from βTC3 cells under conditions where gramicidin D induced a 3.28-fold increase in insulin release (Fig. 35B). These data identified a distinct response of islets and βTC3 cells to ouabain and suggested that the Na^+/K^+ ATPase in βTC3 cells was either insensitive to inhibition by ouabain or functionally impaired.

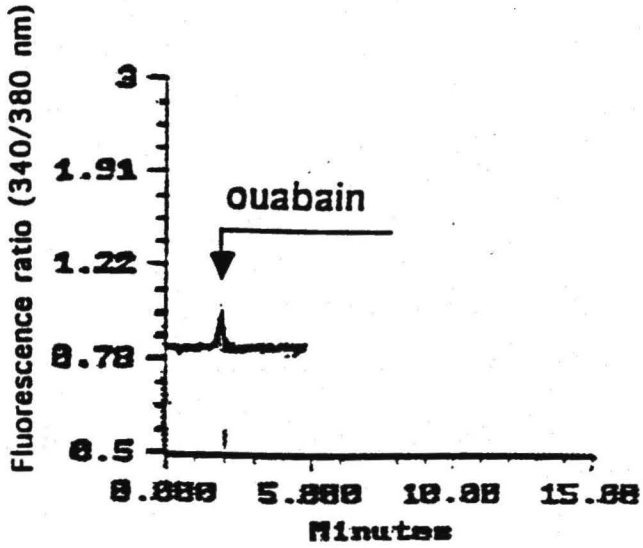
Characterization of the $Na^{\pm}/K^{\pm}$ ATPase activity in βTC3 cells

Experiments described above have suggested that a difference in the function of the Na^+/K^+ ATPase in islets versus βTC3 cells may account for the distinct responses for ouabain and gramicidin D. It was of interest therefore, to characterize the Na^+/K^+ ATPase activity in both βTC3 cells and pancreatic islets. The strategy of this set of experiments was divided into two parts. The first part tested the effect of ouabain, a potent inhibitor of the Na^+/K^+ ATPase, on $[Ca^{2+}]_i$ and $[Na^+]_i$ in βTC3 cells. Ouabain by inhibiting the Na^+/K^+ ATPase was expected to increase $[Na^+]_i$ and $[Ca^{2+}]_i$. In the second part, a direct assay of the Na^+/K^+ ATPase activity in βTC3 cells and pancreatic islets was performed to evaluate the levels of Na^+/K^+ ATPase activity in βTC3 cells and isolated islets.

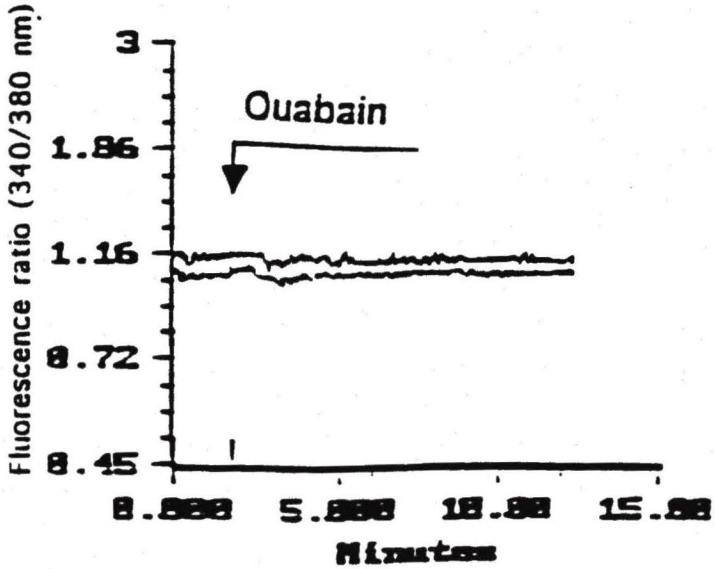
As shown in Fig. 36A, the addition of ouabain (1 mM) to βTC3 cells caused a transient increase in $[Ca^{2+}]_i$ which declined rapidly to basal levels. However, no effect for ouabain was observed on $[Na^+]_i$ (Fig. 36B) suggesting a possible defect in the βTC3 cells Na^+/K^+ ATPase. It was possible that ouabain increased $[Na^+]_i$ but to levels that could not be detected. Although the previous observations suggested the presence of a deficient Na^+/K^+ ATPase in βTC3 cells, a direct assay of the pump activity indicated that the Na^+/K^+ ATPase was active.

Fig 36 Effects of ouabain (1 mM) on $[Ca^{2+}]_i$ (A) and $[Na^+]_i$ (B) in βTC3 cells.

(A) βTC3 cells preloaded with fura-2 were treated with ouabain (1 mM) at the indicated time and $[Ca^{2+}]_i$ was measured by recording the fluorescence ratio of fura-2 at 340:380 nm. (B) βTC3 cells were loaded with the fluorescent Na^+-sensitive dye SBFI/AM (5 μM) as described in Methods and exposed to 1 mM ouabain at the indicated time.


Fluorescence ratio (340/380 nm)
3
1.91
1.22
0.78
0.5
ouabain
0.000
5.000
10.00
15.00
Minutes

A


Fluorescence ratio (340/380 nm)
3
1.86
1.16
0.72
0.45
Ouabain
0.000
5.000
10.00
15.00
Minutes

B

The specific activity was 7 μMole P_i/mg protein/hour (Fig. 37). However, the specific activity of the pancreatic Na^+/K^+ ATPase was almost twice that found in βTC3, and was 13.3 μMole P_i/mg protein/ hour.

Finally, the effect of ouabain on $[Ca^{2+}]i$ was evaluated in primary β-cells. As shown in Fig. 38A-B, ouabain (0.5 mM) induced a gradual increase in $[Ca^{2+}]_i$ and contrasted with the transient increase in $[Ca^{2+}]_i$ on ouabain addition to βTC3 cells.

However, although gramicidin D failed to stimulate insulin secretion from pancreatic islets, it increased $[Ca^{2+}]_i$ in primary β-cells (Fig. 38C). There is no current explanation for inability of gramicidin D to stimulate insulin secretion from pancreatic islets although it increased $[Ca^{2+}]_i$.

The present study has identified a novel difference between the βTC3 cell line, which is commonly used by researchers, and pancreatic islets. These results suggested the presence of a functionally impaired or ouabain-insensitive Na^+/K^+ ATPase in βTC3 cells. The data are summarized in Table 5.

Fig. 37 Determination of the specific activity of the Na^+/K^+ ATPase in pancreatic islets and βTC3 cells.

The activity of Na^+/K^+ ATPase was measured by quantitating the amount of released P_i from ATP in plasma membrane homogenates isolated of pancreatic islets and βTC3 cells as described in Methods. The specific activity is expressed as μMole P_i/mg/hour.

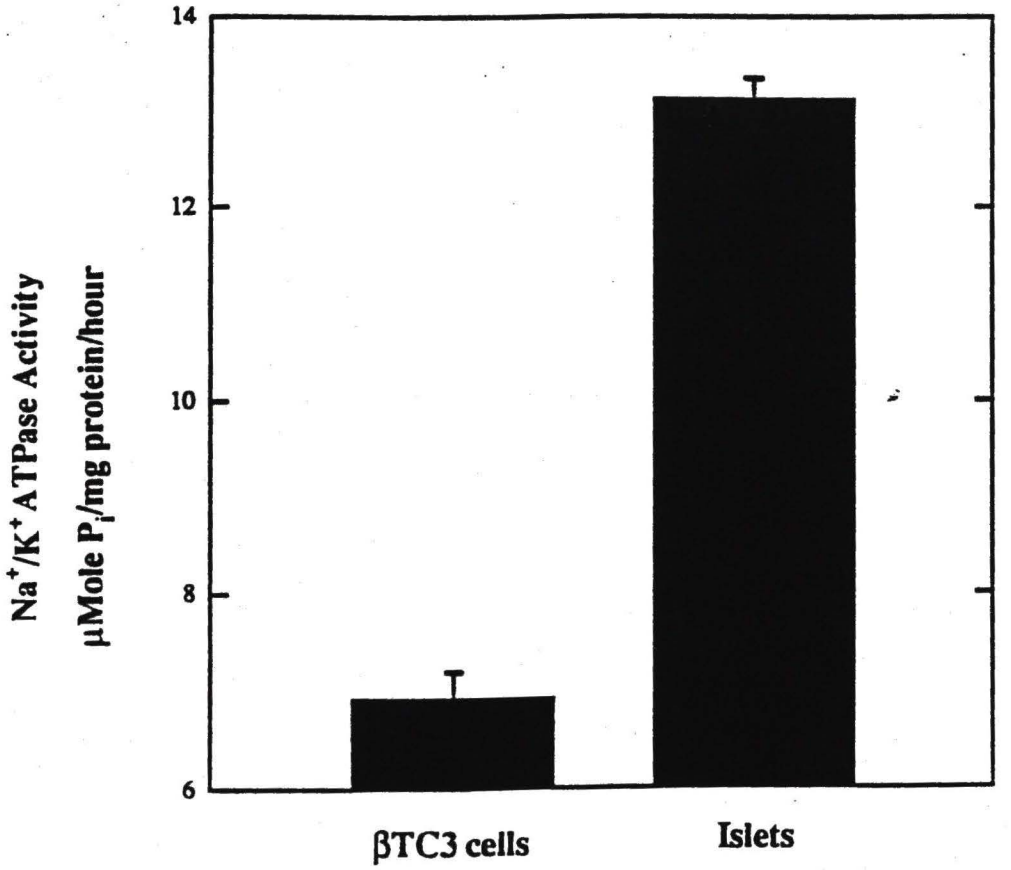
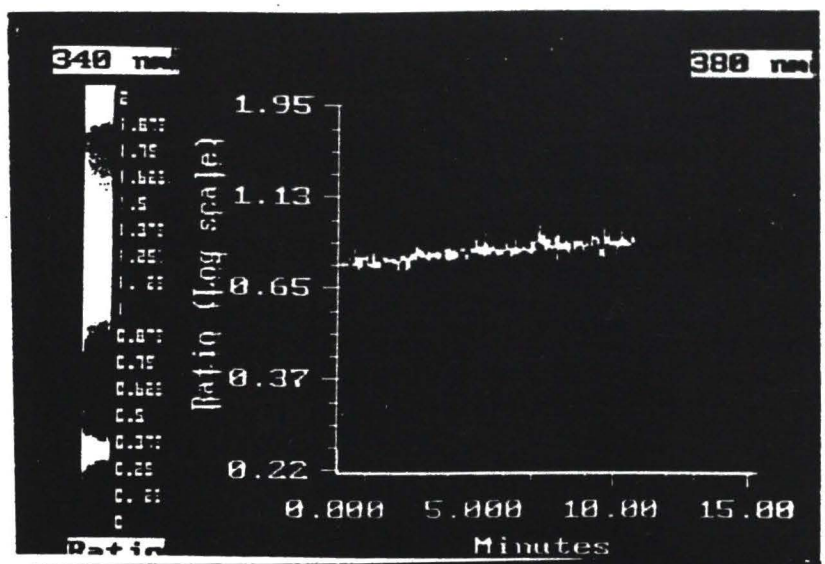

14
12
10
8
6
Na+/K+ ATPase Activity
µMole Pi/mg protein/hour
βTC3 cells
Islets

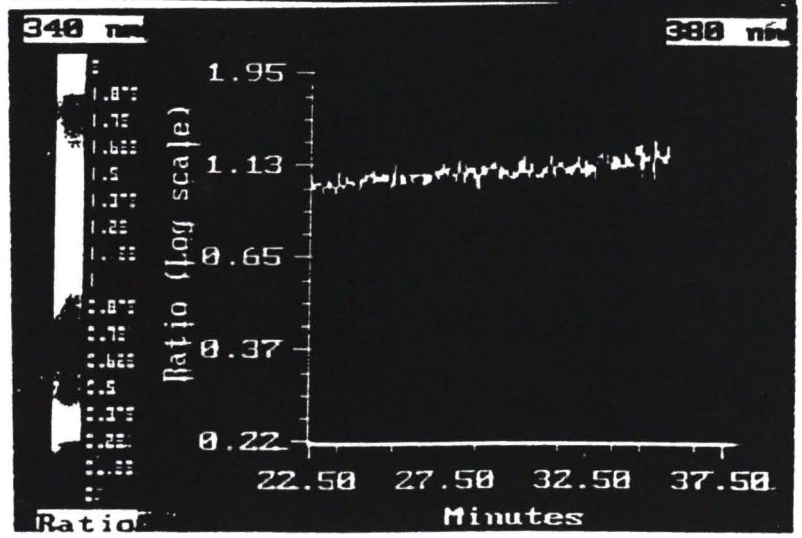
Fig. 38 Effects of gramicidin D and ouabain on $[Ca^{2+}]_i$ in single pancreatic β-cells.

Single pancreatic β-cells isolated by dispase digestion of islets, were loaded with 0.5 μM fura-2 as described in Methods and exposed to: (A) ouabain (0.5 mM) or (C) gramicidin D (1 μM) at the indicated time. (B) is a continuation of (A).

A


340 nm
380 nm
1.95
1.13
0.65
0.37
0.22
Ratio (Log scale)
0.000
5.000
10.00
15.00
Minutes

B


340 nm
380 nm
1.95
1.13
0.65
0.37
0.22
Ratio (Log scale)
22.50
27.50
32.50
37.50
Minutes

C

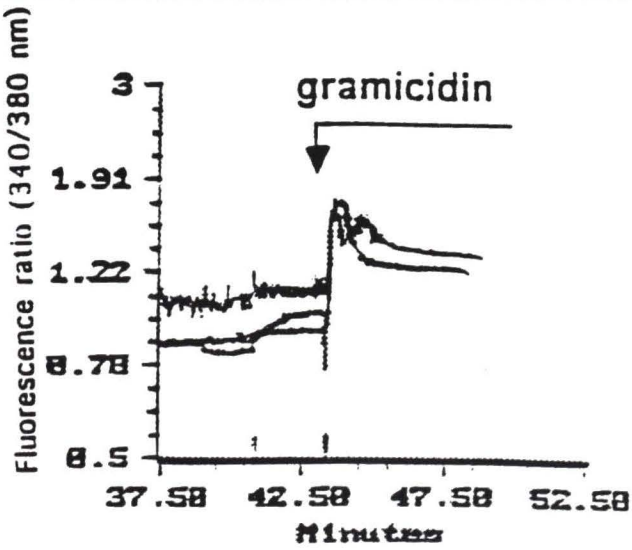

gramicidin
3
1.91
1.22
0.78
0.5
Fluorescence ratio (340/380 nm)
37.50
42.50
47.50
52.50
Minutes

Table 5: A comparison between βTC3 cells and primary β-cells.

Four different responses were identified between βTC3 cells and primary β-cells.

Response	βTC3 cells	Primary β-cells
Effect of gramicidin D on insulin release	stimulating	no effect
Effect of ouabain on insulin release	no effect	stimulating
Effect of ouabain on $[Ca^{2+}]_i$	transient increase	gradual increase
Specific activity of Na^+/K^+ ATPase	7 μMole P_i/mg protein/hour	13.3 μMole Pi/mg protein/hour

DISCUSSION

The purpose of this study was to use the modulation of ion fluxes by gramicidin D to study their influence on insulin secretion from the pancreatic β-cell. In this study, gramicidin D was identified as a potent, Ca^{2+}-dependent insulin secretagogue in transformed βTC3 cells but not in the primary β-cell. Gramicidin D has been demonstrated to be an effective Na^+-ionophore in a variety of cells including mouse pancreatic β-cells, fibroblasts and lymphocytes. A similar property of gramicidin D towards βTC3 cells was demonstrated by the ability of gramicidin D to promote the elevation of Na^+ in these cells. The response was essentially immediate suggesting that the intercalation of the pore-forming antibiotic was rapid. The increase was monophasic and sustained, a response that was similar to that observed in other cells (210). Using SBFI as a selective probe for Na^+, no other secretagogue tested (glucose, KCl, KIC and L-Arg) was able to mimic the effect of gramicidin D to increase $[Na^+]_i$. This observation contradicted previous reports that have demonstrated that glucose-induced a decrease in $[Na^+]_i$ in pancreatic islets (176-177). This observation may reflect a different mechanism of Na^+ homeostasis in the βTC3 cell. Although the presence of an impaired Na^+/K^+ ATPase in βTC3 cells (as suggested in section 8 of Results) may provide a possible explanation for the difference response observed using these cells, glucose-induced decrease in $[Na^+]_i$ was reported to be mediated via a mechanism independent of the Na^+/K^+ ATPase (176). Therefore, the presence of a possible deficient Na^+/K^+ ATPase is unlikely to account for the lack of effect of glucose on $[Na^+]_i$ in βTC3 cells.

This difference may therefore, reflect a different ability of insulinoma βTC3 cells

and primary β-cells to respond to glucose. Numerous reports have shown that a given insulin secretagogue can produce different responses on tumor β-cells and primary β-cells. In this study, the elevation of $[Ca^{2+}]_i$ induced by glucose in βTC3 cells was significantly less relative to primary β-cells. This may be the result of the reduced ability of insulinoma cells to metabolize glucose due to the reduced ratio of glucokinase to hexokinase (264).

The elevation of Na^+ in electrically excitable cells such as β-cells would be predicted to induce membrane depolarization. Indeed, gramicidin D induced membrane depolarization in cell types; e.g., HL-60 cells and GH_4C_1 pituitary cells (237-239). Using the membrane potential-sensitive dye Oxonol VI, gramicidin D was also demonstrated to induce membrane depolarization in βTC3 cells which was qualitatively similar to depolarization induced by agents known to depolarize β-cells (i.e., KCl and tolbutamide). The distinct temporal response of gramicidin D (i.e., increase in fluorescence observed (hyperpolarization)) was likely an artifact due to binding of gramicidin D to the fluorescent dye since a similar increase was observed in the absence of cells. A more accurate assessment of gramicidin D-induced membrane depolarization would require the use of patch clamp microphysiological techniques. Unfortunately, these were not available at the time of the study.

The potential involvement of Na^+ in β-cell electrical activity is not clear and an area of intense study. A physiological significance is suggested from observations that glucose-induced insulin secretion is suppressed in medium containing low concentrations of Na^+ (247) and enhanced under conditions favoring an increase in intracellular Na^+ (248-249). The presence of extracellular Na^+ has been shown to be necessary for carbohydrate-induced membrane depolarization in insulinoma cells, RINm5F (250). In addition, a direct involvement of Na^+ in electrical activity has been provided by the

characterization of Na^+-dependent cation potentials in canine (251-252) and human β-cells (253). The elevation of Na^+ in βTC3 cells supports a role for Na^+ in regulating membrane potential in the mouse tumor βTC3 β-cell line. A similar suggestion has been reported in normal mouse β-cells (254). Indeed, the present study provides an evidence for the ability of Na^+ to influence membrane potential in β-cells.

The elevation of Na^+ and membrane depolarization induced by gramicidin D was accompanied by a profound increase in $[Ca^{2+}]_i$ in βTC3. These events were correlated with respect to time since both events occurred simultaneously as far as could be evaluated. The action of gramicidin D as a Na^+-ionophore suggests that the principal response of βTC3 cells to an elevation in $[Na^+]_i$ is to increase $[Ca^{2+}]_i$. The correlation of the two events is supported by the stable elevation in the concentration of both Na^+ and Ca^{2+} after gramicidin D stimulation. However, while the gramicidin D-induced increase in $[Na^+]_i$ was monophasic, gramicidin D-induced increase in $[Ca^{2+}]_i$ was biphasic. The significance of these observations is not fully understood but may reflect the intricate mechanisms of Ca^{2+} homeostasis including the plasma membrane and endoplasmic reticulum Ca^{2+}-ATPase activities which is a characteristic of the β-cell whose function is critically regulated by Ca^{2+}-fluxes and/or the involvement of multiple mechanisms in increasing $[Ca^{2+}]_i$.

The elevation of Ca^{2+} induced by gramicidin D was shown to be exclusively from the extracellular medium since its chelation by EGTA completely abolished this response (Fig. 6). The same treatment also abolished gramicidin D-induced insulin secretion (Fig. 14B). These results not only identify gramicidin D as an insulin secretagogue but also suggest that its secretion response is totally dependent on the influx of extracellular Ca^{2+}. A support for this conclusion is provided by the ability of carbachol to elicit an identical transient increase in $[Ca^{2+}]_i$ when added alone or in the

presence of gramicidin D. Thus, the intracellular pools of Ca^{2+} were intact , whether or not the cell had been stimulated with gramicidin D. The additive effect of carbachol on gramicidin D-induced increase in $[Ca^{2+}]_i$ failed to potentiate a further secretion by gramicidin D suggesting that such modest increase in $[Ca^{2+}]_i$ was insufficient to contribute further to secretion.

The biphasic Ca^{2+}-response induced by gramicidin D was characterized by a first phase that peaked at 1-2 minutes and a second phase in which $[Ca^{2+}]_i$ was maintained at levels higher than basal concentrations. Gramicidin D induced a 7.73-fold increase in $[Ca^{2+}]_i$ relative to basal levels in the first peak (basal $[Ca^{2+}]_i$ was 67 nM, and the peak was 518 nM). However, in the second phase, $[Ca^{2+}]_i$ was 168 nM, a 2.5-fold response relative to basal levels. A number of insulin secretagogues were tested and they did not exert a similar pattern in raising $[Ca^{2+}]_i$ with the exception of KCl. Glucose induced a modest increase in $[Ca^{2+}]_i$. Glucose-induced increase in $[Ca^{2+}]_i$ was similar to that previously reported in βTC3 cells (195).

The elevation in $[Ca^{2+}]_i$ induced by depolarizing concentrations of KCl was similar to gramicidin D in βTC3 cells. Careful examination revealed a difference between gramicidin D and KCl in the first phase of Ca^{2+}-increase. In contrast to the gradual increase/decrease in the first peak of increased $[Ca^{2+}]_i$ induced by gramicidin D, KCl-induced increase in $[Ca^{2+}]_i$ was characterized by a fast incline/decline in $[Ca^{2+}]_i$ in the first peak. This observation suggests that the mechanisms of KCl- and gramicidin D-induced increase in $[Ca^{2+}]_i$ were distinct. This conclusion was supported by the observed additive effects of KCl and gramicidin D to increase $[Ca^{2+}]_i$ (Fig. 5C). The profile of the combined response was characterized by a sharp increase in $[Ca^{2+}]_i$ which persisted and no decline to lower levels was observed as normally seen with either stimulus added individually (see Figs. 4B and 5A).

As mentioned in the Introduction, glucose-induced insulin secretion is thought to involve the following sequence of events; (i) the metabolism of glucose leads to the closure of K_{ATP} channels and membrane depolarization, (ii) cell depolarization then activates VSCC leading to Ca^{2+} influx and (3) Ca^{2+} then activates insulin secretory granules exocytosis by yet unidentified mechanisms. The observed effects of gramicidin D to induce membrane depolarization, an increase in $[Ca^{2+}]_i$ and insulin release suggested that these steps were linked in a way that mimicked glucose. Next, the focus of this study was to identify the mechanisms involved in gramicidin D-induced increase in $[Ca^{2+}]_i$ rather than the understanding of mechanisms by which increased $[Ca^{2+}]_i$ stimulates insulin release. Such understanding was anticipated to help establish new methods of treatment of Type II diabetes mellitus. Current treatment of diabetic patients utilize drugs that increase $[Ca^{2+}]_i$. It was of interest therefore, to understand the mechanisms by which gramicidin D increased $[Ca^{2+}]_i$ in order to develop new potential drugs that can mimic gramicidin D effects.

The mechanisms involved in gramicidin D-induced increased in $[Ca^{2+}]_i$ involves VSCC. This hypothesis was supported by the observed ability of VSCC inhibitors to suppress gramicidin D-induced increase in $[Ca^{2+}]_i$. Verapamil and nifedipine inhibited both phases of gramicidin D-induced increase in $[Ca^{2+}]_i$. This was the first piece of evidence in support of the involvement of VSCC in mediating extracellular Ca^{2+} influx induced by the ionophore. Also, KN-62 which has been shown to inhibit VSCC in insulinoma cells (255), mimicked the inhibitory effects of verapamil. All VSCC inhibitors exerted similar inhibitory effects on KCl-induced increase in $[Ca^{2+}]_i$. The effects of VSCC inhibitors to partially inhibit the second phase of increased $[Ca^{2+}]_i$ may reflect the different abilities of drugs to interfere with VSCC in the activation versus resting state of VSCC channel (135).

In addition, the conditions to totally suppress Ca^{2+} influx through VSCC were established by pretreatment of βTC3 cells with nifedipine for 3-5 minutes prior to stimulation with KCl (see Fig. 7C). However, a similar effect of verapamil was not observed because of the ability of verapamil to detach cells from the immobilized coverslip, an effect may due to the different mechanisms of action of each inhibitor. While nifedipine binds to the α-subunit (pore-forming subunit) of VSCC, verapamil binds to a regulatory subunit of VSCC. In addition, some reports suggested an inhibitory effect for verapamil on Na^{+}/Ca^{2+} exchanger, another mechanism that mediate extracellular Ca^{2+} influx (239). Under the same conditions needed to totally abolish Ca^{2+}-influx via VSCC, gramicidin D still induced an increase in $[Ca^{2+}]_i$ (see Fig. 9B). The pretreatment with nifedipine (3-5 minutes prior to stimulation with gramicidin D) suppressed the second phase almost completely, whereas the first peak was partially inhibited. This observation suggested that the second phase of Ca^{2+}-response induced by gramicidin D was attributed to VSCC channels whereas the first phase involved a mixed activation of VSCC channels and another mechanism.

The profound effect of gramicidin D on $[Ca^{2+}]_i$ was responsible for insulin release. This was supported by suppressing gramicidin D-induced insulin secretion by VSCC inhibitors which have been shown to inhibit gramicidin D-induced increase in $[Ca^{2+}]_i$. Careful analyses of the effects of VSCC inhibitors of on gramicidin D- and KCl-induced insulin secretion revealed significant differences. In the case of each of the VSCC inhibitors tested (nifedipine, verapamil and KN-62), KCl-induced insulin secretion was inhibited to a greater extent than gramicidin D-induced insulin secretion. Verapamil dose-dependently inhibited both gramicidin D-induced insulin secretion and KCl-induced insulin secretion; the half maximal effect of verapamil (IC_{50}) was calculated to be approximately 0.94 μM in each case. The close correspondence of these values together

with the reported IC_{50} (0.5 μM, 225) supports a common site of action involving VSCC. However, verapamil at the maximally effective concentration (20 μM) inhibited KCl- and gramicidin D-induced insulin secretion by 80.8 % and 42.6 %, respectively. Similar findings were observed in the case of nifedipine and to a lesser degree, KN-62. At the highest concentration used, nifedipine (20 μM) inhibited KCl- and gramicidin D-induced insulin secretion by 80 % and 31.3 %, respectively. Also, KN-62 (10 μM) inhibited KCl and gramicidin D-induced insulin secretion by 33 % and 19 %, respectively.

There are therefore, four pieces of data that suggest the presence of mechanism(s) in addition to the activation of VSCC in the gramicidin D-induced insulin secretion: (1) the different Ca^{2+}-profile in the first phase of increased $[Ca^{2+}]_i$ induced by gramicidin D when compared to KCl (KCl-induced increase in $[Ca^{2+}]_i$ is thought to be mediated solely via VSCC), (2) the additive effect of gramicidin D and KCl in increasing $[Ca^{2+}]_i$, (3) the ability of nifedipine to abolish KCl- but not gramicidin D-induced increase in $[Ca^{2+}]_i$ and (4) the more profound inhibitory effects of VSCC inhibitors (when tested at the same concentration) on KCl-induced insulin secretion relative to the response induced by gramicidin D.

It has been shown in some cells that an increase or a decrease in intracellular pH induced an increase in $[Ca^{2+}]_i$ (245, 246, increase and decrease, respectively). Since an increase in $[Na^+]_i$ would be anticipated to activate the Na^+/H^+ exchanger and cause acidification of cells by promoting the efflux of Na^+ and influx of H^+, it was reasoned that gramicidin D-induced activation of this process may contribute to the increase in $[Ca^{2+}]_i$ and insulin release. Furthermore, since the Na^+/H^+ exchanger has been implicated in insulin secretion, the direct stimulation of such activity could modulate gramicidin D-induced insulin secretion. However, two pieces of evidence obtained in this study support the conclusion that the Na^+/H^+ exchanger plays no role in the

mechanism of gramicidin D-induced increase in $[Ca^{2+}]_i$ and insulin release. The first was the lack of effect of gramicidin D on pH_i (Fig. 12A). The lack of effect of gramicidin D on pH_i could be explained by the proven inhibitory effect of high intracellular Na^+ concentrations on the Na^+/H^+ exchanger activity. It has been shown that Na^+ competes with H^+ for the same transporting site and, at high levels of Na^+, the exchanger loses its ability to transport H^+ (258-260). Gramicidin D was likely exerting similar effects since it flooded the cells with Na^+. The second evidence was the inability of EIPA, a potent inhibitor of such activity, to inhibit gramicidin D-induced insulin secretion (Fig. 28).

The phorbol ester TPA, has been used shown to promote an increase in pH_i (166) and this observation has been used as evidence in support of the involvement of PKC in the regulation of Na^+/H^+ exchange activity. A similar response of TPA was observed in these studies when used as a positive control for the study of the effects of gramicidin D on pH_i. Significantly, 4 α-PDD, a pharmacologically inactive analogue of TPA, mimicked TPA effects on cell pH_i (Fig. 12E). This observation suggests that the phorbol ester effects on cell pH_i are likely to be lipid-mediated and not the result of the activation of PKC. While this observation does not eliminate a role for PKC in regulating the Na^+/H^+ exchanger activity (based on the fact that the amino acid sequences of the Na^+/H^+ exchanger possessed potential phosphorylation sites for PKC), it does require that such studies be re-evaluated.

In mammalian cells, there are only two known pathways that can mediate the influx of extracellular Ca^{2+} under physiological conditions. The first pathway involves the VSCC whereas, the second pathway involves Na^+/Ca^{2+} exchanger. Under normal conditions, the Na^+/Ca^{2+} exchanger promotes Na^+ influx and Ca^{2+} efflux (forward mode). However, the exchanger can reverse its function and promote Ca^{2+} influx for

Na^+ efflux under conditions where the Na^+-gradient across the plasma membrane is disrupted (reverse mode). Since gramicidin D dissipated the Na^+-gradient in βTC3 cells, it was reasoned that the ionophore may activate the βTC3 cell Na^+/Ca^{2+} exchanger and that this mechanism may account for the observed effects on Ca^{2+} and insulin release.

That the Na^+/Ca^{2+} exchanger exists in βTC3 cells was supported by the effect of the removal of extracellular Na^+ to induce a transient increase in $[Ca^{2+}]_i$ (Fig. 10). This response was similar to that reported in primary β-cells (181) and was considered to be the result of the disruption of the Na^+ gradient across the plasma membrane and likely the result of the activation of the reverse mode of the Na^+/Ca^{2+} exchanger. In an attempt to re-establish Na^+ gradient, the βTC3 cell Na^+/Ca^{2+} exchanger promotes the efflux of Na^+ and influx of Ca^{2+}. When the inhibitors of Na^+/Ca^{2+} exchanger (DMB and DCB) were tested on gramicidin D-induced insulin secretion, both exerted inhibitory effects. DMB dose-dependently inhibited gramicidin D-induced insulin secretion. The half maximal effect of DMB (IC_{50}) was calculated to be approximately 18 μM (Fig. 24A), a concentration that was similar to reported IC_{50} of DMB on the Na^+/Ca^{2+} exchanger (11 μM)(243). Also, DCB (50 μM) inhibited gramicidin D-induced insulin release. These data suggested initially, that the Na^+/Ca^{2+} exchanger was involved in gramicidin D-induced insulin secretion. However, at a maximally effective concentration (50 μM), DMB and DCB also inhibited KCl-induced insulin secretion. Since KCl-induced insulin secretion is thought to be mediated via VSCC exclusively and such conditions would not be expected to activate Na^+/Ca^{2+} exchange, these data suggested that both antagonists also targeted and inhibited the VSCC. This result was unexpected and questioned the use of DMB and DCB as selective inhibitors of Na^+/Ca^{2+} exchanger.

On the other hand, the more profound inhibitory effect of DMB (50 μM) on gramicidin D-induced insulin secretion relative to KCl-induced insulin secretion supports

an involvement of at least two different components of Ca^{2+}-influx in the gramicidin D-stimulated cells. The effect of DMB to inhibit both the VSCC and Na^{+}/Ca^{2+} exchange activities, would explain the more profound inhibitory effects of DMB on gramicidin D-induced insulin secretion when compared to KCl-induced insulin secretion. This effect was not reproduced by DCB.

Analysis of the dynamics of gramicidin D-induced insulin secretion, using the perifusion model, revealed a biphasic insulin release response (Fig. 29). In an attempt to probe the involvement of VSCC and Na^{+}/Ca^{2+} exchanger in both phases of insulin secretion, nifedipine (VSCC inhibitor) and DMB (Na^{+}/Ca^{2+} exchanger and possibly VSCC inhibitor) were tested on gramicidin D-induced insulin secretion. The data obtained suggested that the VSCC was involved in both phases of insulin secretion since nifedipine suppressed insulin secretion in both phases with the first phase of secretion being less affected. However, the inability of nifedipine, a VSCC inhibitor, to totally suppress insulin secretion in the first phase suggested the involvement of an additional mechanism in such phase. This additional mechanism is suggested to involve the Na^{+}/Ca^{2+} exchanger. DMB inhibited the first phase of secretion which may suggest the involvement of Na^{+}/Ca^{2+} exchanger in the first phase, a result that was in support of the previous hypothesis suggesting the involvement of an additional mechanism in the first phase of insulin secretion. The fura-2 studies confirmed that an additional mechanism to VSCC promoting extracellular Ca^{2+}-influx exists in the first phase of gramicidin D Ca^{2+} response (see Fig. 9B). Also, DMB inhibited the second phase of insulin secretion. However, the involvement of Na^{+}/Ca^{2+} exchanger in the second phase of secretion based on DMB inhibition of such phase is possible but can not be definitively assessed due to the potential inhibitory effect on VSCC. Therefore, using the perifusion model, the data suggests that VSCC are involved in both phases of insulin secretion, whereas the

Na^+/Ca^{2+} exchanger is likely involved in the first phase of insulin secretion. The involvement of the exchanger in the second phase of secretion can not be ruled out as well.

The best method to evaluate the role of the Na^+/Ca^{2+} exchange in gramicidin D-induced insulin release and β-cell function in general, would be to study the ability of antisense message of the Na^+/Ca^{2+} exchanger to diminish the expression of active protein in βTC3 cells. Antisense message could be introduced to βTC3 cells either by microinjection or transfection with a plasmid expression vector. One group performed microinjection of the antisense message of the Na^+/Ca^{2+} exchanger and was able to show that cells lost the Na^+/Ca^{2+} exchange activity (242). In order to perform these experiments, it would be necessary to identify the cDNA sequence of the isoform of the β-cell Na^+/Ca^{2+} exchanger.

It is clear that the involvement of Na^+/Ca^{2+} exchanger in gramicidin D-induced insulin release cannot be verified due to technical difficulties. Not only did the fluorescent nature of all three inhibitors of the exchanger activity (amiloride, dichlorobenzamil (DCB) and dimethylbenzamil (DMB) prevent their use in the fura-2 studies, but also their non-specific effects hampered the direct assessment of the Na^+/Ca^{2+} exchange activity in gramicidin D-induced insulin secretion. In conclusion, gramicidin D-induced insulin secretion is suggested to involve both the VSCC and the Na^+/Ca^{2+} exchanger. However, the inhibitors of the Na^+/Ca^{2+} exchanger activity were not specific and appeared to target VSCC and therefore, the actual involvement of the Na^+/Ca^{2+} exchanger is not fully characterized at this time of study due to the lack of selective inhibitors.

Although gramicidin D-induced insulin secretion was absolutely dependent on increased Ca^{2+} influx, other cellular intermediates could still potentiate its secretion

response without affecting $[Ca^{2+}]_i$. Glucose potentiated gramicidin D-induced insulin secretion although it had no effect on gramicidin D-induced increase in $[Ca^{2+}]_i$. These signals or intermediates seemed to be generated from the metabolism of glucose in glycolysis and not the mitochondria since KIC, a mitochondrial fuel, had no potentiating effect on gramicidin D-induced insulin secretion. On the other hand, insulin secretagogues that affect membrane potential (L-Arg and KCl) exerted different effects on gramicidin D-induced insulin secretion. L-Arg had no effect on gramicidin D-induced insulin secretion and is consistent with the proposed effect of L-Arg to induce membrane depolarization (101).

KCl and gramicidin D induced a 6.21-fold and a 3.28-fold increase in insulin release. However, a combination of both stimuli induced a 4.6-fold increase in insulin release. This data therefore, reflected a net inhibition of KCl-induced insulin secretion by gramicidin D and was unexpected since KCl and gramicidin D had additive effects in increasing $[Ca^{2+}]_i$ (Fig. 5C). It was anticipated that a combination of both secretagogues would induce a rate of insulin secretion greater than that induced by either alone as a result of greater levels Ca^{2+} achieved by both stimuli. A possible explanation for such result is the increase in $[Na^+]_i$ to the level induced by gramicidin D may exert inhibitory effects on Ca^{2+}-induced insulin release. While KCl and gramicidin D induced quantitatively similar increases in $[Ca^{2+}]_i$, KCl stimulated insulin secretion by almost twice the fold as gramicidin D. In other words, it seems that the insulin secretion process in β-cells is preferably occurring in an environment of an increased $[Ca^{2+}]_i$ that is not accompanied by an increased $[Na^+]i$. It has been shown that an increase in $[Na^+]_i$ inhibited PKA in rat thyroid and brain (240-241). PKA is known to regulate insulin secretion (62-66). Therefore, gramicidin D may inhibit KCl-induced insulin secretion via the suppression of the activity of PKA. Experiments to further test the possible inhibition

of PKA by gramicidin D would involve an assay of PKA in β-cell homogenates after gramicidin D-stimulation. Also, forskolin, an agent that increases cAMP via the activation of adenylate cyclase, could be tested for its ability to affect gramicidin D-induced insulin secretion. Forskolin would not be expected to affect gramicidin D-induced insulin secretion while potentiating glucose- or KCl-induced insulin secretion.

Gramicidin D-induced increase in $[Ca^{2+}]_i$ resulted in the activation of Cam kinase II. Gramicidin D induced a marked activation of Cam kinase II as demonstrated by ionophore-induced autophosphorylation and conversion to a partially Ca^{2+}-independent active kinase. This activation was rapid, reaching a maximum (5.56-fold relative to control) after approximately 1 minute and therefore, correlated with the initial increase in $[Ca^{2+}]_i$. This response was very similar to the previously reported effects of glucose and KCl-induced activation of Cam kinase II in isolated islets (40) and KCl in βTC3 cells (Easom, unpublished observations).

The activation state of Cam kinase II declined to basal levels by 20 minutes. This monophasic activation of Cam kinase contrasted the biphasic increase in $[Ca^{2+}]_i$. Therefore, if Cam kinase is directly involved in regulating insulin secretion, its role is likely occurring during the initial minutes of insulin secretion, i.e., in the first phase of insulin secretion. Alternatively, the phosphorylation event in the early phase is sufficient for secretion to proceed for a longer period of time. Our results confirm the hypothesis of Rasmussen *et al* . (38), who suggested that a Ca^{2+}/calmodulin-dependent protein kinase was involved in the first phase of insulin secretion in pancreatic islets. On the other hand, the decline in the activity of Cam kinase II may reflect the activation of a phosphatase and the enzymatic removal of the phosphate group at the autophosphorylation site of Cam kinase II. The identity of regulation of this phophatase in β-cell is not known.

The role of Cam kinase II in regulating insulin secretion is not fully elucidated. While experiments performed in the laboratory have correlated glucose-induced the activation of Cam kinase II with insulin secretion (40), Wolheim *et al* (256) have shown in a permeabilized insulinoma cell that, KN-62, a Cam kinase II inhibitor, has no effect on insulin release. The current study provides an evidence that Cam kinase II activation may not be essential for insulin secretion. This was demonstrated by ability of verapamil to inhibit gramicidin D-induced insulin secretion by 42.6 % (Fig. 23A) while abolishing gramicidin D-induced activation of Cam kinase II (Fig. 32). However, since the effect of verapamil was not studied at all time points, the possibility that a transient, modest activation of Cam kinase II cannot not be ruled out.

A novel mechanism controlling the activation state of Cam kinase II in βTC3 cells exists. Although the detailed mechanism(s) is not fully elucidated, initial experiments performed in the laboratory have demonstrated that a certain Ca^{2+}-threshold was needed to activate Cam kinase II. The Ca^{2+}-threshold was estimated to be approximately 500 nM (Easom, R.A. and Bahtt, H.B., unpublished observations). Interestingly, $[Ca^{2+}]_i$ in gramicidin D-stimulated cells reached this level only in the initial peak and then declined to 168 nM in the second phase. This may provide a reasonable explanation for detection of an active of Cam kinase II in the initial minutes but the absence of an active Cam kinase II during the second phase of increased $[Ca^{2+}]_i$ in gramicidin D-stimulated cells. In order to verify such hypothesis, experiments are currently being performed to assay the enzyme in cells stimulated with a combination of KCl and gramicidin D. The simultaneous addition of both stimuli produced a monophasic increase in $[Ca^{2+}]_i$ in which $[Ca^{2+}]_i$ was maintained at the Ca^{2+}-threshold needed to activate Cam kinase II (Fig. 5C). It may be predicted therefore, that a stable activation of Cam kinase II occur in cells.

The involvement of protein kinase C in gramicidin D-induced insulin release is unlikely since staurosporine, a potent inhibitor of PKC, failed to inhibit gramicidin D-induced insulin secretion. In the light of this data, the more definitive experiment to assay the activation of PKC (by demonstration the translocation of PKC from a cytosolic form to a membrane-associated form) was not necessary. Staurosporine has been shown to inhibit glucose-induced insulin secretion in pancreatic islets (257). On the other hand, TPA, a potent activator of PKC, potentiated gramicidin D-induced insulin secretion suggesting that the activation of PKC is capable of modulating gramicidin D-induced insulin secretion. This result is likely to reflect the reported ability of PKC to sensitize insulin secretion to Ca^{2+}, probably as the result of the modification of the cytoskeleton in β-cell.

The effect of gramicidin D to increase $[Ca^{2+}]_i$ was expected to result in the activation of MLCK specially since its affinity for Ca^{2+}/calmodulin is considerably less than that for Cam kinase II (265). The ability of wortmannin, a potent inhibitor of MLCK, to inhibit gramicidin D-induced insulin secretion by 63 % (Fig. 33A) suggested that MLCK may be involved in gramicidin D-induced insulin secretion. This result was similar to the reported effect of wortmannin to suppress IgE-stimulated histamine release in mast cells (261) which was used to support a role for MLCK in its secretion process. However, wortmannin may have other non-specific effects that may complicate the interpretation of this data. For example, it has been shown that wortmannin is a stong inhibitor of phosphatidyl inositol 3-kinase (244). While there is no evidence to suggest the involvement of this enzyme in insulin release, the potential involvement of this enzyme in insulin release has not been fully elucidated yet. Unfortunately, there are no available specific inhibitors of MLCK that are currently known.

In an attempt to support a role for MLCK in gramicidin D-induced insulin release, a direct assay of the ability of gramicidin D to induce the activation of MLCK was performed. Using an assay developed in smooth muscle (see Methods, section 9), gramicidin D failed to increase the phosphorylation levels of MLC_{20}, a specific substrate of MLCK, suggesting that the enzyme was not activated. However, KCl, used as a positive control, also failed to influence the levels of MLC_{20} phosphorylation. Since KCl (40 mM) induced a profound increase in $[Ca^{2+}]_i$ but failed to induce detectable activation of MLCK, the validity of such method in non-muscle cells is questioned. Further development is needed to improve the assay in non muscle cells and is a current objective of the laboratory.

All the previous data has characterized the effect of gramicidin D on βTC3 cells. Surprisingly, some of the effects of gramicidin D were not mimicked in pancreatic islets and these differences are summarized in Table 5. A major difference was that gramicidin D failed to stimulate insulin secretion from pancreatic islets although it increased $[Ca^{2+}]_i$ in primary β-cells to similar levels induced in βTC3 cells. While βTC3 cells used for secretion studies were of a monolayer cell-type, pancreatic islets are composed of multiple layers of cells. Therefore, due to the presence of gap junctions (proteins known to regulate cell function) it is possible that gramicidin D could not access the primary pancreatic β-cells to the same extent as it affected βTC3 cells. In order to verify such hypothesis, the ability of gramicidin D to stimulate insulin release from dispersed pancreatic β-cells would be tested. A second difference observed was that ouabain stimulated insulin secretion in pancreatic islets but not in βTC3 cells. Concomitant with this observation, a different Ca^{2+}-response was induced by ouabain. While ouabain induced a transient increase in $[Ca^{2+}]_i$ in βTC3 cells, it promoted a gradual increase in $[Ca^{2+}]_i$ in primary β-cells. This observation suggested that these differences were the

result of a deficiency related to the Na^+/K^+ ATPase and was confirmed by the lower level of activity of the Na^+/K^+ ATPase in βTC3 cells compared to pancreatic islets (7 μMole P_i/mg protein/hour vs. 13.3 μMole Pi/mg protein/hour for βTC3 cells and islets, respectively). It is not clear whether this observed decrease in the activity was the result of a loss of protein amount or an impairment of the expressed protein. Taken together, these data suggest the presence of an impaired or ouabain-insensitive Na^+/K^+ ATPase in βTC3 cells. It is possible therefore, that gramicidin D-induced elevation of $[Na^+]_i$ is not efficiently removed by the Na^+/K^+ ATPase, forcing the βTC3 cells to use an alternative pathway of Na^+-efflux through the Na^+/Ca^{2+} exchanger resulting in increased $[Ca^{2+}]_i$. This may contrast with the primary β-cells, in which the effects of gramicidin D to elevate $[Na^+]_i$ can be counteracted more efficiently by the active Na^+/K^+ ATPase.

The presence of an impaired Na^+/K^+ ATPase pump, however, is an insufficient explanation since an increase in $[Ca^{2+}]_i$ in primary β-cells was seen after treatment with gramicidin D (Fig. 38C). Thus an active Na^+/K^+ ATPase is not capable of compensating for gramicidin D induced elevation of Na^+ such that gramicidin D increased $[Ca^{2+}]_i$. It is not clear whether this increase in $[Ca^{2+}]_i$ is the result of membrane depolarization alone and the activation of VSCC or in combination with the activation of the Na^+/Ca^{2+} exchanger in the reverse mode. Therefore, a question to be answered is; Why is the gramicidin D-induced increase in $[Ca^{2+}]_i$ in primary β-cells not accompanied by insulin secretion ? One possible explanation is a different Ca^{2+}-threshold for insulin secretion exists between βTC3 cells relative to primary β-cells. In other words, the increase in $[Ca^{2+}]_i$ induced by gramicidin D was not sufficient to stimulate insulin secretion from primary β-cells but exceeded the threshold required for insulin secretion in βTC3 cells. In fact, it has been shown that different population of primary β-cells required different Ca^{2+} thresholds and show different insulin secretion levels (262). Experiments to test

this hypothesis would be to identify the Ca^{2+}-threshold required for secretion in both primary β-cells and the tumor βTC3 cells using permeabilized cell models. In these experiments, permeablized βTC3 cells and pancreatic islets would be perifused with secretagogues in medium containing different Ca^{2+} concentrations and insulin release would be determined. Under these conditions, the Ca^{2+} concentration of the medium can be incrementally increased using a Ca^{2+} electrode. The data from the current studies predicts that a lower Ca^{2+} threshold exists for the activation of insulin release from βTC3 cells compared to pancreatic islets.

In conclusion, the following study is the first to identify gramicidin as an insulin secretagogue in the tumor cell line βTC3. Gramicidin D-induced insulin secretion is a novel mechanism and involves membrane depolarization leading to the activation of VSCC which promotes Ca^{2+} influx. In addition, this mechanism is thought to involve the activation of the Na^{+}/Ca^{2+} exchanger in the reverse mode to mediate Ca^{2+} influx. The increase in $[Ca^{2+}]_i$ is sufficient to trigger insulin release. This study revealed a difference between βTC3 cells and primary β-cells with respect to Na^{+} homeostasis. The documentation of these differences is important for the future use of this β-cell line as a physiological model of insulin secretion.

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