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Molecular Mechanisms Involved In The Generation Of Calcium Signals In Insulin Secreting Cells.

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ABSTRACT

Intracellular Ca^{2+} regulates diverse cellular functions in almost all the cells. In the pancreatic β-cells, Ca^{2+} signals are critical for insulin secretion. An increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers insulin secretion. Multiple mechanisms increase the $[Ca^{2+}]_i$ in the β-cells, and in this respect depolarization of the plasma membrane and consequent Ca^{2+} entry through the voltage-gated Ca^{2+} channels is particularly important. For depolarization of β-cells the closure of K_{ATP} channels is essential. In addition, cation channels belonging to Transient Receptor Potential (TRP) family are thought to play important roles in causing depolarization. Previous studies have reported that the Transient Receptor Potential Melastatin-like subtype 5 (TRPM5) channel, a Ca^{2+} activated monovalent cation channel, is involved in the stimulus-secretion coupling in the mouse β-cells. We aimed to study the role of the TRPM5 channel in regulating insulin secretion, and $[Ca^{2+}]_i$ in the rat β-cells.

Another focus of this thesis was to evaluate a new human inluinoma cell line as a model for studying Ca^{2+} signaling in the β-cells. Recently a genetically engineered human insulinoma cell line (EndoC-BH1) has been developed. We studied Ca^{2+} signaling in the EndoC-BH1 cells, in an attempt to assess whether these cells could be used as a model for this purpose.

For inhibiting the TRPM5 channels we used triphenyl phosphine oxide (TPPO), a selective and potent pharmacological inhibitor of the channel. We measured insulin secretion from the islets from Sprague-Dawley rats in batch incubations. For measuring $[Ca^{2+}]_i$ from single rat β-cells and EndoC-BH1 cells, we used fura-2 based ratiometric microfluorometry.

We found that TPPO did not inhibit insulin secretion triggered by KCl, or fructose but it significantly reduced insulin secretion in response to glucose, L-arginine, and GLP-1. It also significantly inhibited the K_{ATP} channel-independent insulin secretion by glucose. TPPO significantly inhibited the $[Ca^{2+}]_i$ increase in response to L-arginine. It also inhibited the $[Ca^{2+}]_i$ increase triggered by glucose in a K_{ATP} channel independent mechanism. However, TPPO did not alter the $[Ca^{2+}]_i$ response triggered by KCl, fructose, glucose and GLP-1.

We stimulated the EndoC-BH1 cells with gluocse, GLP-1, KCl, carbachol, L-arginine, and tolbutamide. These agents that are known to increase $[Ca^{2+}]_i$ in the primary β-cells also increased $[Ca^{2+}]_i$ in the these human insulinoma cells. Moreover, we found that GLP-1 was essential for eliciting Ca^{2+} response in the EndoC-BH1 cells upon stimulation by tolbutamide and glucose.

We conclude that in the rat islets, TRPM5 plays an important role in mediating insulin secretion by glucose, and L-arginine, and in potentiating the glucose-induced insulin secretion by GLP-1. We also conclude that the EndoC-BH1cells responds by $[Ca^{2+}]_i$ increase upon stimulation by several well-known agonists.

LIST OF SCIENTIFIC PAPERS

I. **Krishnan K**, Ma Z, Björklund A, Islam M S. Role of transient receptor potential melastatin-like subtype 5 channel in insulin secretion from rat β-cells. (*in press, Pancreas 2014).*

II. **Krishnan K**, Ma Z, Björklund A, Islam M S. Calcium signaling in a genetically engineered human pancreatic β-cell line (*manuscript submitted).*

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Cytoplasm Ca^{2+} concentration
ADP	Adenosine diphosphate
ARG	L-arginine
ATP	Adenosine triphosphate
BSA	albumin from bovine serum fraction V
CCH	Carbachol
CICR	Ca^{2+} induced Ca^{2+} increase
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DZ	Diazoxide
ECM	Extracellular matrix
ER	Endoplasmic reticulum
fura-2 AM	fura-2 Acetoxymentyl ester
GK	Glucokinase
GLP-1	Glucagon like peptide-1
GLUT	Glucose transporter
GSIS	Glucose stimulated insulin secretion
hTERT	Human telomerase reverse transcriptase
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
K_{ATP}	ATP sensitive K^+ channel
KCa	Calcium activated K^+ channels
KCl	Potassium chloride

KRBH	Krebs Ringer bicarbonate HEPES buffer
MC	Mithochondria
NCX	Na^{+}-Ca^{2+} exchanger
PIP2	Phosphatidylinositol 4,5-bisphosphate
PI-PLC	Phosphatidyl inositol specific phospholipase C
PKC	Protein kinase C
PMCA	Plasma membrane Ca2+ ATPase
PP	Pancreatic polypeptide
RPMI 1640	Roswell Park Memorial Institute 1640 medium
RyR	Ryanodine receptor
SCID	Severe combined immunodeficiency
SERCA	Sarco-endoplasmic reticulum Ca2+ ATPase
STIM	Stromal interaction molecule
SUR1	Suplhonylurea receptor 1
SU	Sulphonylurea
SV40LT	Simian Virus 40 large T antigen
TCA cycle	Tricarboxylic acid cycle
TOL	Tolbutamide
TPP	Trphenylphosphine
TPPO	Trphenylphosphine oxide
TRPM3	Transient Receptor Potential Melastatin-like subtype 3
TRPM4	Transient Receptor Potential Melastatin-like subtype 4
TRPM5	Transient Receptor Potential Melastatin-like subtype 5
VGCC	Voltage gated Ca2+ channel

1 Introduction

1.1 Islets of Langerhans

Paul Langerhans, a German pathologist, was the first to describe these microstructures in the pancreas in 1869, but he failed to identify their function. Later in 1893, Edouard Laguesse, a French histologist, coined the name ‘islets of Langerhans’ and suggested that these could produce hormones that lowers hyperglycemia.

Pancreas houses two different types of tissues, namely the exocrine and the endocrine tissues. Islets of Langerhans are the endocrine tissues, and they are more number in the body and tail regions of the pancreas. Islets contain at least five types of cells including the α-cells that secrete glucagon, β-cells that secrete insulin, δ-cells that secrete the growth hormone inhibiting hormone, somatostatin, pancreatic polypeptide producing (PP) cells, and ghrelin producing epsilon cells (1). β-cells sense the change in plasma nutrient concentration and in responses secrete insulin to maintain glucose homeostasis in the blood.

Failure of the β-cells to secrete adequate insulin results in diabetes, a heterogeneous metabolic disorder associated with high blood glucose. Diabetes is classified mainly into two types, namely the type-1 diabetes mellitus and the type-2 diabetes mellitus. Type-1 diabetes is an autoimmune disorder in which β-cells are killed by the immune system. Type-2 diabetes is associated with progressive β-cell dysfunction in secreting insulin due to aging or developing insulin resistance. Studying the physiology of β-cells may give a clearer picture about the pathogenesis of diabetes, and may also be useful in identifying therapeutic targets for the treatment of the disorder.

1.2 Ca^{2+} as a signaling ion

An increase in the free cytoplasm Ca^{2+} concentration ($[Ca^{2+}]_i$) plays pivotal role in many cellular processes that include triggering insulin exocytosis in β-cells (2), fertilization of eggs (3), and enhancing ATP synthesis in the mitochondria

(4). Many *in vitro* experiments on resting β-cells showed that the concentration of Ca^{2+} in the cytoplasm is ~ 20-100 nM, whereas the extracellular concentration is ~1-2 mM. A local increase in $[Ca^{2+}]_i$ could be a trigger for a global $[Ca^{2+}]_i$ increase (5). The entry, duration of presence, and exit of the Ca^{2+} from the cytoplasm is regulated by many channels, pumps, ion exchangers, Ca^{2+}-binding proteins, and other organelles (6).

1.3 Ca^{2+} signaling in the β-cells

Channels present on the plasma membrane like the receptor operated Ca^{2+} channels, store operated Ca^{2+} channels, and voltage gated Ca^{2+} channels (VGCC) regulate the Ca^{2+} entry from extracellular space in to the β-cells. The Ca^{2+} influx, through the L-type VGCCs in response to extracellular glucose is a predominant and a well-established mechanism (7). $[Ca^{2+}]_i$ is also regulated by store operated Ca^{2+} entry, in which the reduction in the Ca^{2+} in the ER (Ca^{2+} store) is sensed by the stromal interaction molecule (STIM), that translocates itself to the plasma membrane to activate the Orai1 channel to allow Ca^{2+} entry (8).

In the G-protein coupled receptor (GPCR) signaling, agonists like acetylcholine bind to the GPCRs to activate phospholipase C (PLC) followed by hydolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 binds to the 1,4,5-inositol trisphosphate receptor (IP_3R) activating the channel to mobilize Ca^{2+} from the ER Ca^{2+} stores into the cytoplasm (9). IP_3R and ryanodine receptors (RyR) are activated or inhibited by Ca^{2+} depending on the concentration of $[Ca^{2+}]_i$ (10). The Ca^{2+} entry through the VGCCs activates the RyRs to mobilize Ca^{2+} from the endoplasmic reticulum to further amplify the Ca^{2+} signals; this process is called Ca^{2+} induced Ca^{2+} release (CICR)(11).

Sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) replenishes the Ca^{2+} stores in the endoplasmic reticulum (12). The mitochndrial Ca^{2+} buffering (13; 14) and efflux of Ca^{2+} across the plasma membrane through plasma membrance Ca^{2+} ATPase, sodium-calcium exchangers also regulate the $[Ca^{2+}]_i$ (15), thereby preventing the Ca^{2+} toxicity in the β-cells. The activation of several TRP

channels also regulates the $[Ca^{2+}]_i$ either by providing background depolarizing current or by permeating Ca^{2+} ions through them (6).

1.4 Stimulus-secretion coupling in the β-cells

The adult human islets comprise 70-80 % of β-cells that play critical role in the maintenance of blood glucose level. The β-cells also act as sensors for fatty acids, amino acids, neurotransmitters, growth factors incretins and other hormones. Insulin exocytosis in β-cells require an increase in the $[Ca^{2+}]_i$. Stimulus-secretion coupling in the pancreatic β-cells includes a combination of metabolic and electrical signaling cascades. After consumption of a meal, blood glucose concentration increases. Glucose is carried in to the β-cells through specific glucose transporters (GLUT 2 in rodents, and GLUT 1 and 3 in humans). Glucose is metabolized via glycolysis in the cytoplasm to produce adenosine triphosphate (ATP) and pyruvic acid. This pyruvic acid in turn is metabolized via the tricarboxylic acid (TCA) cycle in the mitochondria to produce ATP. The combined ATP production from glycolysis and TCA cycle increases the ATP/ADP ratio in the cytoplasm (16; 17).

ATP sensitive K^+ channels (K_{ATP}) that are present on the plasma membrane is the primary sensor that couples the metabolic changes exerted by glucose to electrical activity, and finally to secretion of insulin from the β-cells. K_{ATP} channel comprises of sulphonylurea receptor (SUR1), and four pore forming subunits $K_{IR}6.2$, member of inward rectifier superfamily (7).

The increase in the ATP/ADP ratio in the cytoplasm causes closure of the K_{ATP} channels that prompts cell membrane depolarization; the change in membrane potential activates the voltage gated Ca^{2+} channels (VGCCs) that allows entry of Ca^{2+} into the cytoplasm. The increase in the $[Ca^{2+}]_i$ near the plasma membrane triggers insulin exocytosis (Fig. 1).

Upon reduction in plasma glucose concentration, the decrease in the ATP/ADP ratio increases the opening probability of the K_{ATP} channels causing repolarization that acts as a feedback mechanism to stop insulin secretion. Diazoxide, opens the K_{ATP} channels to prevent depolarization of plasma

membrane, which makes it a therapeutic tool for treating hyperinsulinism (18). Closure of the K_{ATP} channels is an undisputed signaling event in plasma membrane depolarization. However, the K_{ATP} channel closure alone is not adequate to depolarize the membrane. An additional inward depolarizing currents caused by other ion channels present on the membrane is essential to depolarize the plasma membrane. It is thought that some members of the TRP family of channels may account for these inward depolarizing currents.

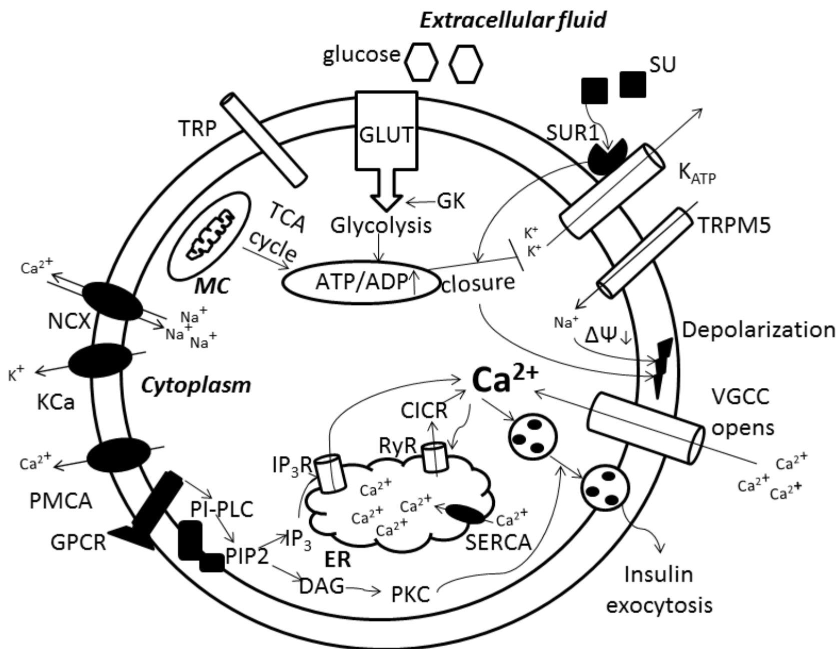

Fig 1. stimulus-secretion coupling in the β-cell. *The figure shows the cell organelles, ion channels, pumps, exchangers, and transporters involved in the regulation of stimulus-secretion coupling. ADP-Adenosine diphosphate; ATP- Adenosine triphosphate;CICR-Ca^{2+} induced Ca^{2+} increase; DAG-diacylglycerol; ER-Endoplasmic reticulum; GK-Glucokinase; GLUT- Glucose transporter; GPCR – G protein coupled receptors; IP_3- inositol 1,4,5-trisphosphate; IP_3R- inositol 1,4,5-trisphosphate receptor; K_{ATP}-ATP sensitive K^+ channel; KCa-Calcium activated K^+ channels; MC-Mithochondria; NCX- Na^+-Ca^{2+} exchanger; PIP_2- Phosphatidylinositol 4,5-*

bisphosphate; PI-PLC- phosphatidyl inositol specific phospholipase C; PKC-protein kinase C; PMCA- Plasma membrane Ca^{2+} ATPase; RyR-Ryanodine receptor; SERCA-Sarco-endoplasmic reticulum Ca^{2+} ATPase; SU-Sulphonylurea; SUR1-Suplhonylurea receptor 1; TCA cycle- tricarboxylic acid cycle; TRPM5- Transient Receptor Potential Melastatin-like subtype 5; VGCC-Voltage gated Ca^{2+} channel.

1.5 K_{ATP} channel independent insulin secretion

The closure of the K_{ATP} channels and depolarization of the plasma membrane are important events in stimulus-secretion coupling. In glucose stimulated insulin secretion (GSIS), there are other coupling factors apart from K_{ATP} channels in bringing about plasma membrane depolarization and insulin secretion. In the presence of diazoxide, which is known to activate the K_{ATP} channels and hyperpolarizes the plasma membrane (19), glucose fails to, depolarize the plasma membrane, and secrete insulin from the mouse islets. However, in the presence of high concentration of K^+, glucose potentiates insulin secretion even in the presence of diazoxide (20) proving that glucose can amplify insulin secretion without involving the K_{ATP} channel activity. This validates that glucose initiates insulin secretion via a K_{ATP} channel dependent manner, and further amplifies insulin secretion in a K_{ATP} independent manner.

1.6 TRP channels

Transient receptor potential (TRP) channels were first discovered in *Drosophila*. A mutation of an ion channel in the photoreceptor cells elicited a transient change in membrane potential in response to continuous light and thus the name transient receptor potential channels (21). So far 28 TRP (27 in human) channels have been found and they are classified into two groups and seven sub families. Group A contains five subfamilies TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), and TRPN. Group B contains two subfamilies TRPP (polycystic) and TRPML (mucolipin) (22). The transmembrane topology of TRP channels show that TRP channels have six transmembrane sections with a pore forming unit between the 5th and 6th transmembrane domain. TRP channels are involved in perceiving various types of sensory reception that includes mechanoreception, thermoreception, chemoreception, and

photoreception. β-cells are found to express at least 13 TRP channels and among them TRPM2, TRPM3, TRPM4 and TRPM5 are thought to play important roles in insulin secretion in the β-cells in the rodents (23)

1.6.1 TRPM5 gene and its expression

The human TRPM5 gene was first described in 2000 by Enklaar and his co-workers (24; 25). The human TRPM5 gene is located on the chromosome 11, and it contains 24 exons composed of 1165 amino acids whereas, the mouse TRPM5 gene is located on chromosome seven, and composed of 1158 amino acids.

1.6.2 TRPM5 channel associated functions

TRPM5 is a monovalent cation channel that is permeable to Na^+, K^+, Cs^+ and Li^+. The channel is activated by voltage, $[Ca^{2+}]_i$, phosphoinositide-4,5-bisphosphate (PIP_2) and heat (15 and 35ºC) (26). TRPM5 has been best studied in the taste cells where it is associated with sensing sweet, umami and bitter tastes (27; 28). The expression of TRPM5 in the stomach and small intestine are thought to be associated with chemosensation in the post ingestion process (29). TRPM5 is also shown to be play important roles in sensing of odors in mice (30).

1.6.3 Pharmacological tools for the study of TRPM5 channel

A major challenge in determining the physiological role or the TRPM5 channel is the lack of availability of potent and specific pharmacological tools. Flufenamic acid, a non-steroidal anti-inflammatory drug, inhibits TRPM5, however, it also inhibits the closely related TRPM4 channel with a 10 fold higher affinity (31). Quinine, a natural alkaloid and a bitter tastant, inhibits TRPM5 channel (32), along with inhibiting other ion channels (33; 34). Spermine, a polyamine, inhibits TRPM7 along with TRPM4 and TRPM5 in the micromolar range (35; 36).

Triphenyl phosphine oxide (TPPO) inhibited TRPM5 currents in a concentration dependent manner, in HEK 293 cells transfected with human TRPM5

(hTRPM5) or mouse TRPM5 (mTRPM5) gene. TPPO at 100 μM concentration did not inhibit the closely related hTRPM4b, hTRPV1, hTRPA1, and TRPM4, which shows TPPO's high selectivity for the TRPM5 channel. Triphenyl phosphine (TPP) that has similar geometric shapes, volume and surface area to that of TPPO, did not inhibit the TRPM5 channels. TPPO features high negative charge above the oxygen atom (Fig. 2) that is thought to be the prime factor involved in the inhibition of TRPM5 channel (37). TPPO has made it convenient to study the roles of the TRPM5 channel in stimulus-secretion coupling in the β-cells.

Fig.2. The figure shows the chemical structure of triphenyl phosphine (TPP) (left) and triphenyl phosphine oxide (TPPO) (right).

1.6.4 Role of TRPM5 in $[Ca^{2+}]_i$ increase and insulin secretion

TRPM5 channels provide inward depolarizing current that is believed to contribute to depolarization and thereby Ca^{2+} influx in to the cytoplasm. TRPM5 knockout mice exhibited the lack of glucose induced fast oscillation in membrane potential and $[Ca^{2+}]_i$. The islets from these mice secreted less insulin in response to glucose stimulation (38). In the same knockout mice model, TRPM5 mediated depolarization is proposed to be coupled with nutrient and L-arginine mediated insulin secretion in the β-cells (39; 40). Until now, understanding of the physiological role of TRPM5 channel in insulin secretion is dominated by data obtained from knockout mice from a single source (38-40). It should also be noted that drawing conclusion about the role of the TRPM5 channel, derived from a model devoid of TRPM5 gene from the embryonic stage can be deceiving. To further elucidate the role of TRPM5 in insulin secretion in response to different stimuli, more studies using different approaches and different animal models are needed. In our studies, using trphenylphosphine

oxide (TPPO), a selective TRPM5 channel inhibitor, we have examined the role of TRPM5 channel in mediating insulin secretion and $[Ca^{2+}]_i$ levels in the rat islets and primary rat β-cells respectively.

1.7 Genetically engineered human β-cells

Insulinoma cell lines offer many possibilities to study the signaling mechanisms, and stimulus-secretion coupling in the context of the pathophysiology of diabetes. For decades, the availability of wide range of insulinoma cell lines from rodents (41) played important roles in the β-cell research. These cell lines are used to overcome the limited availability of primary rodent or human β-cells. However, it should be noted that these cell lines are not from human origin. Conclusion from the data obtained from rodent cell lines can be misleading in understanding the physiological processes of β-cells in humans. Recently, a new functional human β-cell model has been established (42). These cells are obtained by transfecting the oncogene SV40LT in human fetal pancreatic buds followed by grafting the transfected buds into severe combined immunodeficiency (SCID) mice. The transfected buds formed insulinomas that were expanded to generate the EndoC-BH1 cell lines (42). It is necessary to use such human insulinoma cells for Ca^{2+} studies to better understand the Ca^{2+} signaling and regulation of insulin secretion in the human β-cells. Until now, Ca^{2+} signaling has not been reported in these cells. In this thesis, we have studied whether the EndoC-BH1 can used as a model to study Ca^{2+} signaling and stimulus-secretion coupling in β-cells.

2 Materials and methods

2.1 Cells

2.1.1 EndoC-BH1 cells

For experiments involving human cell lines we used the EndoC-BH1 cells, which was a gift from Philippe Ravassard and Raphael Scharfmann, Institut du cerveau et de la moelle, Biotechnology & biotherapy, France. EndoC-BH1 is a genetically engineered human β-cell line generated by transducing human fetal pancreatic buds by a lentiviral vector system followed by grafting in to severe combined Immuno-deficient (SCID) mice. The resulted pancreatic cells that expressed the oncogene Simian Virus 40 large T antigen (SV40LT) were transduced with human telomerase reverse transcriptase and then grafted into other SCID mice. The resulting β-cells were expanded in cultures to form cell lines (42).

2.1.2 Culturing EndoC-BH1 cells

The EndoC-BH1 cell were grown on matrigel/fibronectin-coated flasks. The cell culture support contained Dulbecco's Modified Eagle Medium (DMEM) high glucose (25 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml), fibronectin (2 µg/ml), and extracellular matrix (ECM) (1 % V/V). The volume of cell culture support was 2.5 ml for 25 cm^2 flask. The culture flask was coated with the cell culture support, and incubated for one hour in 5 % CO_2 at 37 °C, before the cells were seeded. EndoC-BH1 cells were cultured in DMEM with glucose (5.5 mM), albumin from bovine serum fraction V (BSA) (2%w/v), 2-mercaptoethanol (50 µM), nicotinamide (10 mM), transferrin (5.5 µg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were cultured in humidified incubator in 5% CO_2 at $37^{o}C$. The medium were changed once in a week. The cells were passaged once every second week. The cells were seeded on glass coverslips that were already coated with the cell culture support. They were cultured for 3 days, and 12 hours prior to using them for measuring $[Ca^{2+}]_i$. They were changed to glucose-starving

medium containing DMEM with low glucose (2.8 mM), BSA (2%w/v), 2-mercaptoethanol (50 µM), nicotinamide (10 mM), transferrin (5.5 µg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml), and streptomycin (100 µg/ml).

2.2 Preparation of islets from rats

Male Sprague-Dawley rats were purchased from Scanbur (Stockholm, Sweden). The rats were maintained in a 12 h (0600–1800 h) light/dark cycle with free access to water and standard laboratory chow. They were 2-3 months old and they weighed 250–350 g at the time they were used for experiments. The animals were killed by inhalation of CO_2. Collagenase (0.1 % w/v) was injected into the pancreatic duct. The pancreas was then removed. Islets were isolated by collagenase digestion in Hank's balanced salt solution followed by centrifugation, density gradient separation with Histopaque 1077, and Histopaque 1119. Islets were then handpicked under a stereomicroscope, and transferred to the petri dishes containing Roswell Park Memorial Institute 1640 (RPMI-1640) medium, glutamine (2 mM), FBS (10 % v/v), benzylpenicillin (100 units/ml), streptomycin (100 µg/ml), and glucose (11 mM). They were then cultured free-floating for 24 h in an incubator at 37 °C in 5 % CO_2. On the day of the experiments, the islets were transferred to petridishes containing 5 ml of Krebs-Ringer bicarbonate HEPES (KRBH) buffer containing HEPES (10 mM), BSA (0.2 %), and glucose (3.3 mM). The islets were preincubated in this solution for 30 min at 37 °C in 5 % CO_2.

2.2.1 Dispersion of rat islets

Islets were dispersed by treating with trypsin EDTA (0.05 % w/v) for 3 min at 37 ºC. The islets were then placed on ice to stop the trypsin effect, and then pipetted up and down ~ 20 times. The cells were cultured on glass coverslips for 24 hours in a humidified incubator in 5 % CO_2 at 37 ºC in RPMI-1640 medium supplemented with FBS (2.5 % v/v), penicillin (50 units/ml), HEPES (10 mM), and streptomycin (50 µg/ml).

2.3 Ethical considerations

All studies involving rat islets were approved by the Northern Stockholm ethical committee on experimental animal care, and performed in accordance with the guidelines of the Karolinska Institutet, and Swedish national board for laboratory animals.

2.4 List of pharmacological tools used

Name of the compund	Effects	Concentration used	Paper
Carbachol (cch)	Muscarinic agonist	100 µM	I,II
Diazoxide (Dz)	Opens K_{ATP} channels	100 µM	I
Glucagon like peptide-1 (GLP-1)	Incretin hormone that potentiates insulin secretion and $[Ca^{2+}]_i$ increase in the presence of glucose	50 nM	I,II
L-Arginine (Arg)	Depolarizes plasma membrane	20 mM	I,II
Potassium Chloride (KCl)	Depolarizes plasma membrane	25 mM	I,II
Tolbutamide (Tol)	Closes K_{ATP} channels	100 µM and 500 µM	II
Triphenyl phosphine oxide (TPPO)	Inhibits TRPM5 channel	100 µM	I

2.5 Measurement of insulin secretion from the islets

Islets of approximately similar sizes handpicked under microscope were chosen for all the experiments. In separate experiments we measured the insulin contents of the islets, and expressed the released insulin as percentage of the total insulin content. We found that insulin content of the islets were not highly variable. Islets of similar sizes were incubated in groups of three in 300 µl KRB buffer containing glucose (3.3 or 16.7 mM) with or without other pharmacological agents, for one hour at 37 °C on a shaking water bath. After incubation of the islets, the supernatant was removed, and stored at -20 ºC. Total insulin content was measured after sonication of the islets for 10–15 s, followed by extraction of insulin overnight at 4 °C in acid-ethanol (70 % v/v). Immunoreactive insulin was measured by radioimmunoassay using polyclonal insulin antibodies (Fitzgerald Inc, USA), and rat insulin as standard (43).

2.6 Measurement of insulin secretion from the EndoC-BH1 cells

The EndoC-BH1 cells were seeded in a 24 well plate that were first coated with the cell culture support. The cells were seeded at a density of 226 000 cells/cm^2. After seeding, the cells were grown for 48 h in DMEM medium with glucose (5.5 mM), BSA (2%w/v), 2-mercaptoethanol (50 µM), nicotinamide (10 mM), transferrin (5.5 µg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml). After that, the cells were incubated overnight in the glucose-starving medium containing DMEM with low glucose (2.8 mM), BSA (2%w/v), 2-mercaptoethanol (50 µM), nicotinamide (10 mM), transferrin (5.5 µg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml). On the day of the experiments, the cells were incubated in KRBH buffer supplemented with glucose (0.5 mM) for one hour, and then with an increasing concentrations of glucose 0.5, 2.8, 5.6, 11, 16.7 and 25 mM glucose. The cells were incubated in 37°C at 5% CO_2 for one hour. After the incubation, the supernatant was removed, and stored at -20 ºC. Total insulin content was measured after extraction of insulin overnight at 4 °C in acid-ethanol (70 % v/v). Immunoreactive insulin was measured by radioimmunoassay using polyclonal insulin antibodies (Fitzgerald Inc, USA), and human insulin as standard.

2.7 Measurement of $[Ca^{2+}]_i$ by microfluorometry

Cells grown on glass coverslips were incubated for 40 min in 2 ml of the 'loading buffer' consisting of RPMI-1640 medium, and BSA (2 %) along with fura-2-acetoxymentyl ester (fura-2 AM) (1 µM) (life technologies, Stockholm, Sweden). The glass coverslips were then incubated for another 15 min in modified KRBH buffer containing NaCl (140 mM), KCl (3.6 mM), NaH_2PO_4 (0.5 mM), $MgSO_4 \cdot 7H_2O$ (0.5 mM), $CaCl_2$ (1.5 mM), HEPES (10 mM), glucose (3 mM) and BSA (0.1%) (pH 7.4) to let the endogenous esterases hydrolyze the AM ester. The coverslip was then mounted at the base of an open perifusion chamber, which was then placed on the stage of an inverted microscope (CK 40, Olympus Inc, Germany). The modified KRBH buffer containing different pharmacological agents was perfused by a peristaltic pump. A water bath and a thermistor connected to the perifusion chamber were used to control the temperature of the solution in the perifusion chamber. It takes about 58 seconds for the new solution to reach the perfusion chamber.

For measurement of $[Ca^{2+}]_i$ from single β-cells, we chose only the large cells to exclude the non-β-cells as much as possible. The fluorescence was measured by dual wavelength excitation fluorometry. The monochromator (PhotoMed DeltaRam) has a diffraction grating that spatially separates the colors of white light emitted from xenon lamp. The generated lights of wavelengths 340 nm and 380 nm were directed onto the cells by a dichroic mirror. The emitted light chosen by a 510 nm filter was detected by the photomultiplier tube detector, and monitored by the Felix32 software (Photon Technology International, Inc).

The block diagram of the fluorescence microscope system is shown in fig. 3. The cells loaded with fura-2 were excited at 340 nm and 380 nm alternately. The ratios between the emitted fluorescence intensities upon excitations at 340 nm and 380nm (F340/F380) were calculated by the Felix32 software. One F340/F380 data point per second was recorded. The background fluorescence was measured by removing the cell from the area of the study. The background was subtracted from the traces before calculation of $[Ca^{2+}]_i$. For calibration, Rmin and Rmax were determined from external standards containing fura-2 free

acid, and sucrose (2 M) (44). The K_d for Ca^{2+}-fura-2 was taken as 225 nM. $[Ca^{2+}]_i$ was calculated from F340/F380, Rmin and Rmax as described before (45).

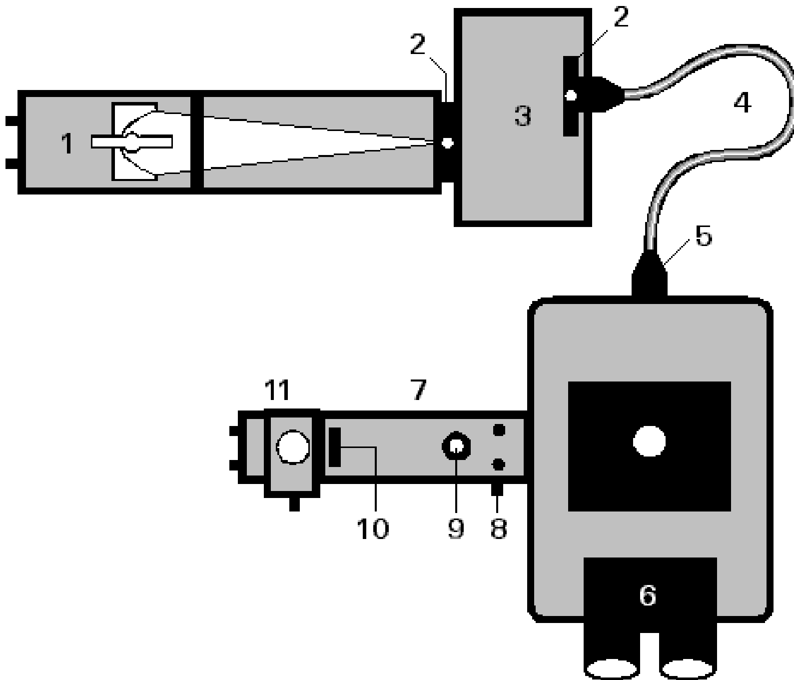

Fig.3. The model of the fluorescent microscopy apparatus used to measure $[Ca^{2+}]_i$ *: 1- Arc lamp housing; 2-Adjustable slits; 3-Excitation monochromator; 4-Liquid light guide; 5-Liquid light guide adapter; 6-Microcope; 7-Microscope photometer; 8-Adjustible iris; 9-viewing eyepiece; 10-filter holder; 11-photmultiplier tube detector.*

2.8 Statistical Analysis

Results were expressed as means ± SEM. Student's paired or unpaired t-test, or Chi-square test, as appropriate, were used for statistical tests of significance. *P*-value < 0.05 was considered as significant.

3 Results and Discussion

The detailed description of results, and discussion of their interpretation are in the published paper, and the manuscript that makes up this thesis. In this section, I shall briefly mention some of the key experiments from the manuscripts.

3.1 Study I

3.1.1 Role of the TRPM5 channels in insulin secretion and Ca^{2+} signaling in the rat β-cells.

We studied the role of the TRPM5 channel in insulin secretion from rat islets and Ca^{2+} signaling from rat pancreatic β-cells. From the preliminary experiments done in our lab, we found that TPPO did not affect the tolbutamide induced $[Ca^{2+}]_i$ increase, and $[Ca^{2+}]_i$ increase in response to depolarization by KCl. This shows that TPPO does not affect the activity of the VGCC's. TPPO, did not increase $[Ca^{2+}]_i$ by itself in the primary rat β-cells making this compound a suitable pharmacological tool to study the TRPM5 channel.

TPPO inhibited the glucose induced insulin secretion from the rat islets. Our results were in line with previous reports that showed impaired insulin secretion in TRPM5 knockout mice (38; 40). However, in our study TPPO did not affect the glucose induced $[Ca^{2+}]_i$ increase. The difference in the experimental conditions could account for this apparent discrepancy. The insulin secretion was measured from whole islets over a period of one hour, whereas $[Ca^{2+}]_i$ was measured from single β-cells for only 500 s. Colsoul *et al* (2010) (38), showed that the absence of TRPM5 reduced the frequency of glucose induced Ca^{2+} oscillations in the intact mouse islets. In our Ca^{2+} experiments we used single β-cells which usually do not show Ca^{2+} oscillations as seen in the intact islets.

One of the major finding in our study was that TPPO reduced both the K_{ATP} channel-independent insulin secretion and $[Ca^{2+}]_i$ increase in response to glucose (Fig. 4a, 4b, and 4c), as tested by the commonly used KCl plus diazoxide protocol (46). Thus, TRPM5 channels seem to play an important role in the insulin secretion by glucose, by the K_{ATP} channel independent mechanism.

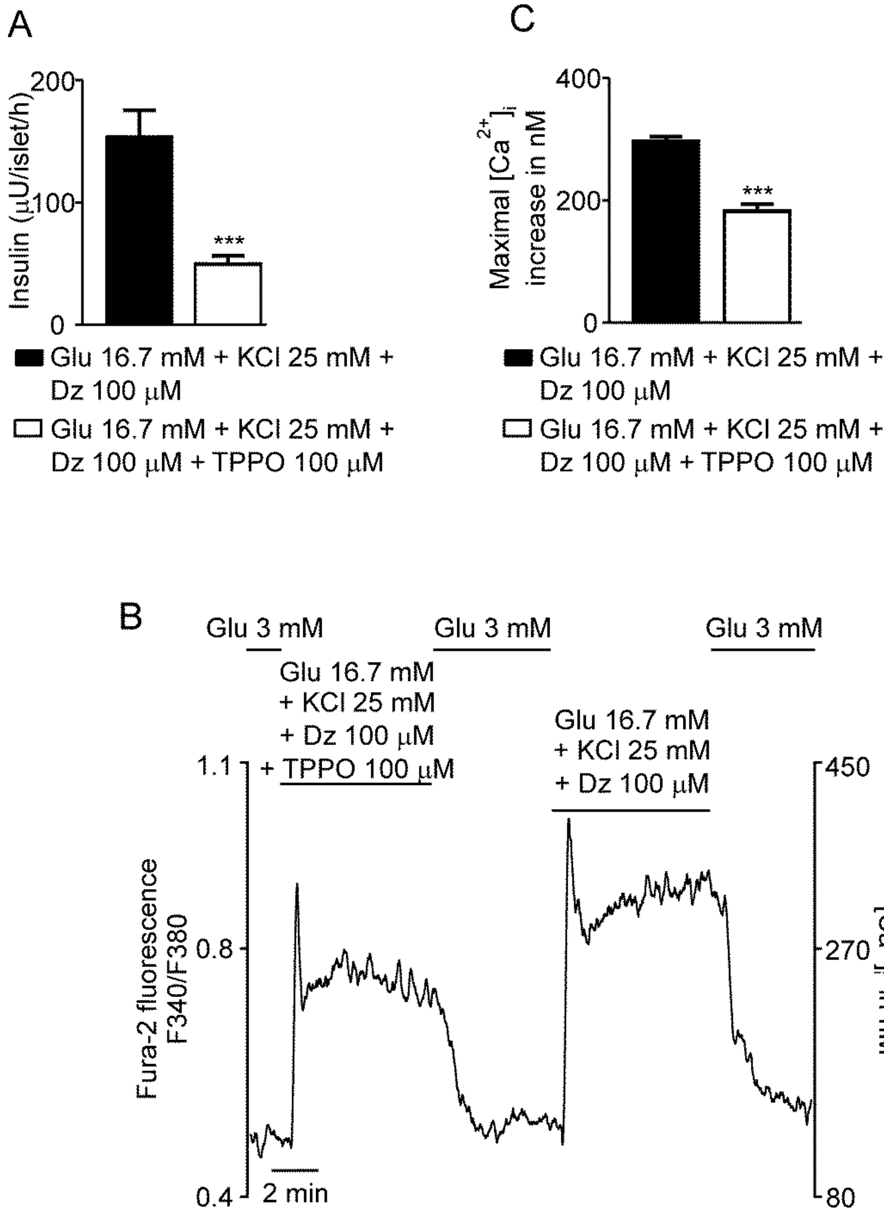

Fig. 4. ***TPPO inhibited the K_{ATP} channel-independent insulin secretion and $[Ca^{2+}]_i$ increase by glucose.*** ***(A)*** *Islets were stimulated by glucose (16.7 mM) plus KCl (25 mM) with diazoxide (Dz) (100 µM), in the presence of TPPO (100 µM) (white bar), or in the absence of TPPO (black bar). TPPO inhibited the K_{ATP} channel independent insulin secretion by glucose ($P < 0.005$, $n = 12$).* ***(B)*** *The trace shows the changes in $[Ca^{2+}]_i$ in a single rat β-cell stimulated first with glucose (16.7 mM), plus KCl (25 mM) plus*

diazoxide (100 μM), in the presence of TPPO (100 μM), and after washout, with glucose (16.7 mM), KCl (25 mM), and diazoxide (100 μM). ***(C)*** *The bars show the maximal* $[Ca^{2+}]_i$ *increase obtained by stimulation by glucose (16.7 mM) plus KCl (25 mM) with diazoxide (100 μM), in the presence of TPPO (100 μM) (white bar), or in the absence of TPPO (black bar). The data were derived from experiments shown in Fig 4B. TPPO inhibited the* K_{ATP} *channel independent* $[Ca^{2+}]_i$ *increase by glucose (*$P < 0.05$*,* $n = 8$*).*

The presence of fructose together with a stimulatory concentration of glucose, further amplifies the insulin secretion (47). TRPM5 has been shown to be involved in this process in the mouse islets (39). In our experiments TPPO did not inhibit either fructose mediated insulin secretion or $[Ca^{2+}]_i$ increase suggesting that in the rat islets, TRPM5 was not involved in insulin secretion in response to this hexose. In this respect, our results contradict the results reported by Kyriazis *et al* (2012) (39), who demonstrated that TRPM5 is involved in insulin secretion in response to fructose in mouse β-cells. The reason for this discrepancy is unclear. These authors have studied insulin secretion using islets from TRPM5 knockout mice, while we have used a pharmacological tool to study insulin secretion form rat islets. Thus, species differences, and differences in the experimental approach, may partly explain the differences in the results obtained.

Another important finding in our study was that TRPM5 was involved in L-arginine induced insulin secretion and $[Ca^{2+}]_i$ increase. L-arginine is a commonly used tool for *in vitro* mechanistic studies of stimulus-secretion coupling and $[Ca^{2+}]_i$ signaling in the β-cells (48). It is postulated that L-arginine, which is a cationic amino acid, depolarizes the plasma membrane upon entering into the β-cell, without requiring metabolism of the amino acid. However, this view may be over simplistic. We found that TPPO markedly reduced, but did not completely inhibit the L-arginine induced insulin secretion and $[Ca^{2+}]_i$ increase (Fig. 5a, 5b,and 5c). These results indicate that while L-arginine alone can increase $[Ca^{2+}]_i$ partially, maximal increase of $[Ca^{2+}]_i$ by the amino acid requires a second event, namely, the activation of the TRPM5 channel by the initial increase of $[Ca^{2+}]_i$ caused by L-arginine.

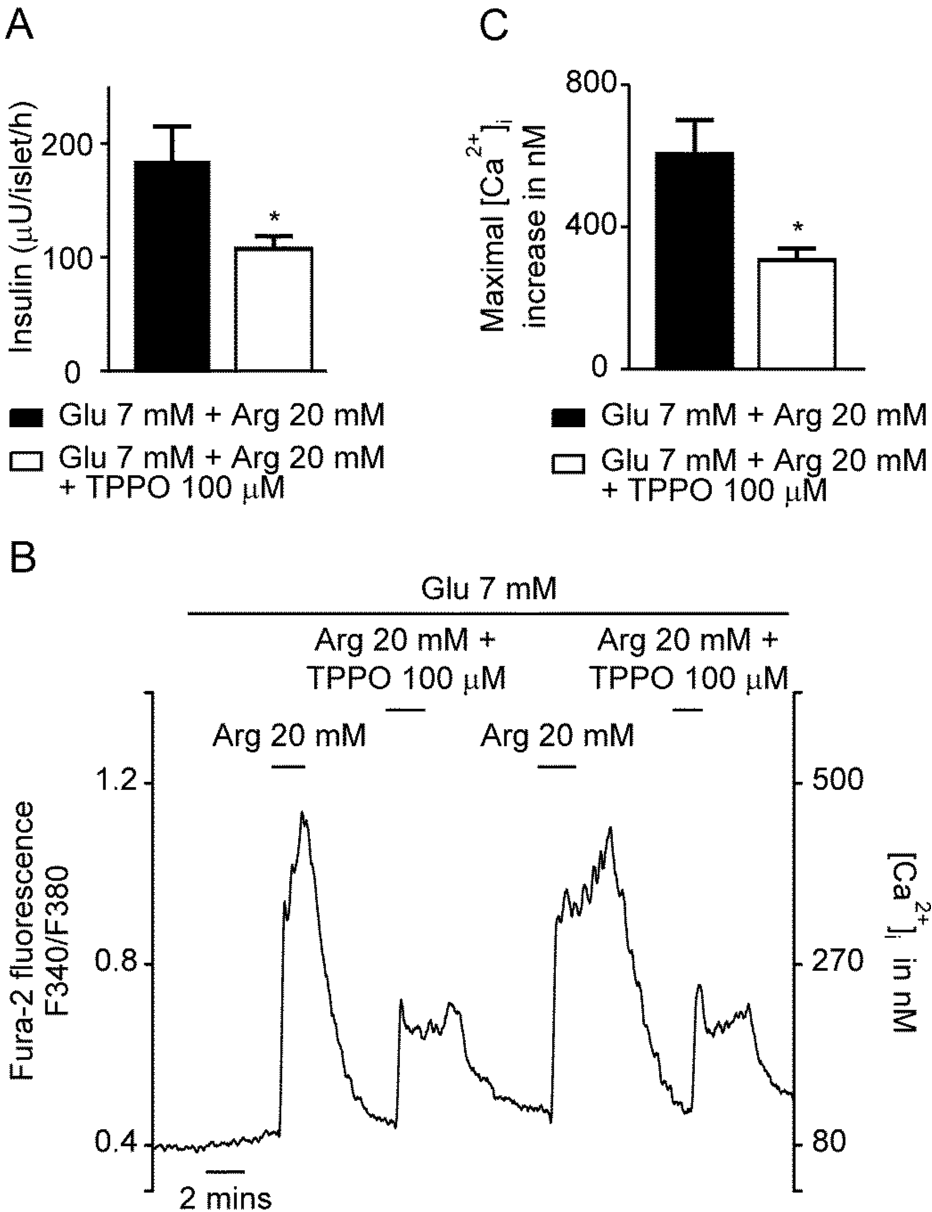

Fig. 5. TPPO inhibited insulin secretion, and $[Ca^{2+}]_i$ increase by L-arginine. ***(A)*** *Rat islets were stimulated by L-arginine (Arg) (20 mM) without TPPO (black bar), and with TPPO (100 μM) (white bar) in the presence of glucose (7 mM). TPPO inhibited L-arginine-stimulated insulin secretion by ~ 40 % ($P < 0.05$, $n = 8$).* ***(B)*** *The trace shows the changes in $[Ca^{2+}]_i$ in a single rat β-cell stimulated by L-arginine (20mM), first without TPPO, and then after washout, with TPPO (100 μM). Glucose (7 mM) was*

present throughout the experiment. TPPO inhibited the L-arginine-induced $[Ca^{2+}]_i$ response in a reversible manner in all experiments. ***(C)*** *The figure shows the maximal $[Ca^{2+}]_i$ increase obtained by L-arginine (20 mM) without TPPO (black bar), or with TPPO (100 µM) (white bar). TPPO inhibited L-Arginine-induced $[Ca^{2+}]_i$ increase by ~ 48 % ($P < 0.05$, $n = 5$). The data were derived from experiments similar to one shown in Fig.5B.*

GLP-1 makes the β-cells competent to stimulation by glucose, GLP-1 has been shown to exert its insulinotropic effect via various mechanisms, including depolarizing plasma membrane by closure of the K_{ATP} channels (49), Ca^{2+} influx through Ca^{2+} channels present on the endoplasmic reticulum (50), and activation of non-specific cation channels (51). In this context, another important finding in our study was that TPPO significantly inhibited the GLP-1 potentiated GSIS (Fig. 6a). This suggests that TRPM5 may be involved in mediating the action of this incretin hormone. It is noteworthy that GLP-1 stimulates Na^+ entry through non-selective cation channels in the plasma membrane (52), which could be TRPM5. We also found that while TPPO inhibited the insulin secretion, it did not inhibit the $[Ca^{2+}]_i$ response triggered by glucose plus GLP-1(Fig 6b, and 6c). This is consistent with the fact that GLP-1 mediates its actions primarily by activating the cAMP dependent pathways, rather than the Ca^{2+} signaling pathways. It remains a possibility that GLP-1 elicited a local Ca^{2+} signaling which was not detectable by our method, which measured the global $[Ca^{2+}]_i$ changes.

In summary, Our results suggest that in the rat islets TRPM5 is involved in mediating insulin secretion in response to glucose, GLP-1, and high concentration of L-arginine, but not in response to fructose. We speculate that TRPM5 is one of the many putative channels that provide the so called inward depolarizing “leak current”. Thus, increased activity of the TRPM5 could make the β-cells more readily electrically excitable by the incretin hormones.

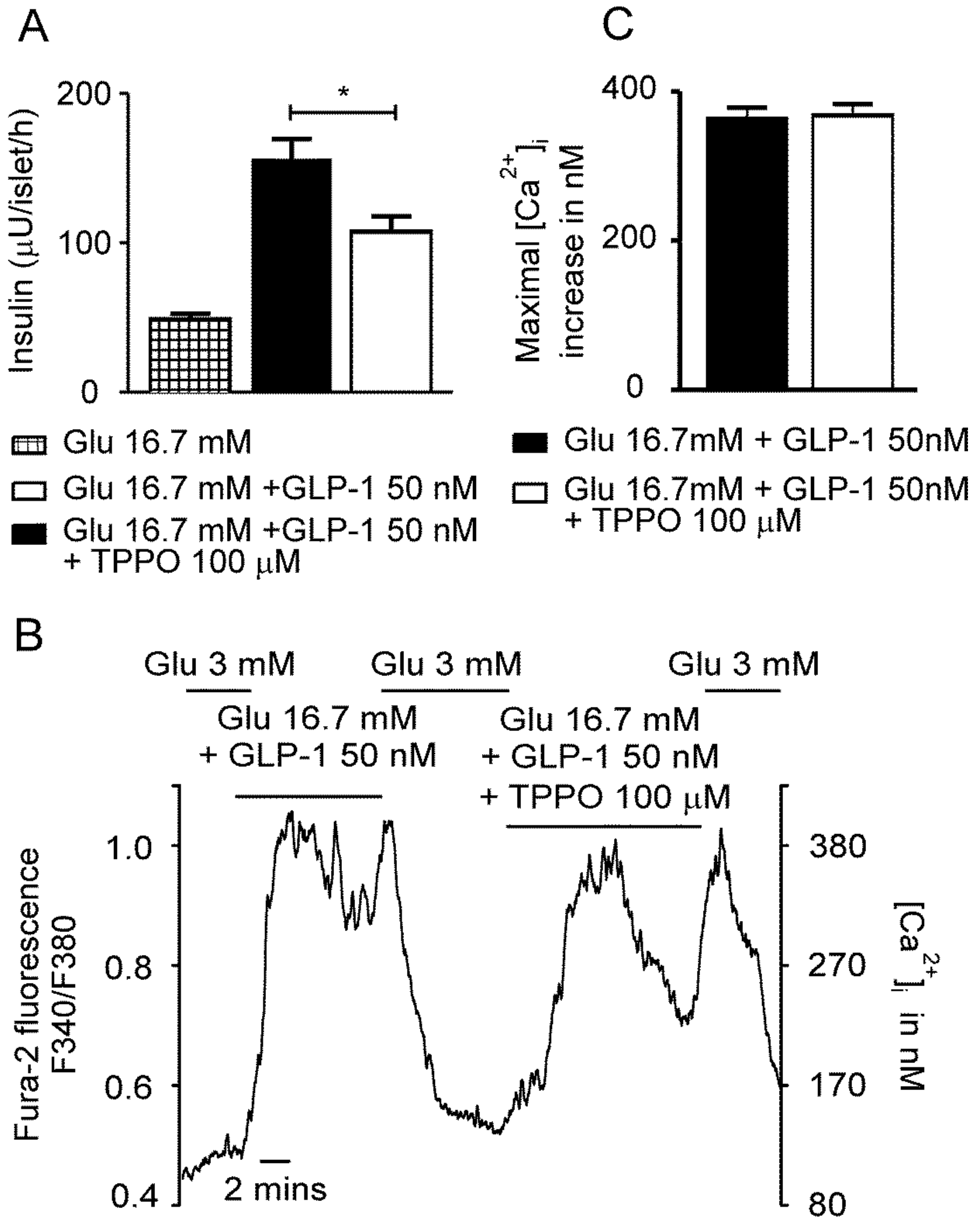

Fig. 6. ***Effect of TPPO on GLP-1-induced insulin secretion and [Ca^{2+}]$_i$ changes. (A)*** *Rat islets were stimulated by glucose (16.7 mM), and GLP-1 (50 nM) without TPPO (white bar), and with TPPO (100 µM) (black bar). GLP-1 increased insulin secretion by ~3 fold compared to that by 16.7 mM glucose alone (checked bar). Stimulation of*

*insulin secretion by GLP-1 (white bar) was inhibited by TPPO (black bar) ($P < 0.05$, $n = 8$). **(B)** The trace shows $[Ca^{2+}]_i$ changes in a β-cell elicited by glucose (16.7 mM) plus GLP-1 (50 nM) without TPPO in the first stimulation, and then after washout, by the same agents in the presence of TPPO (100 µM). (C) The figure shows the maximal $[Ca^{2+}]_i$ increase obtained by glucose (16.7 mM) plus GLP-1 (50 nM) without TPPO (100 µM) (black bar), or with TPPO (100 µM) (white bar). TPPO did not inhibit the $[Ca^{2+}]_i$ increase by glucose plus GLP-1 ($P > 0.05$, $n = 7$). The data were derived from experiments similar to one shown in Fig. 6B.*

It should be noted that we used only one pharmacological tool, namely TPPO, which is relatively new. It is possible that this pharmacological tool may have other effects which we still do not know. For instance, it may inhibit glucose metabolism. However this possibility is less likely, because glucose increased $[Ca^{2+}]_i$ in the presence of TPPO. It is however unclear why TPPO inhibited insulin secretion in spite of the fact that it did not inhibit $[Ca^{2+}]_i$. One possibility is that glucose induced $[Ca^{2+}]_i$ increase does not activate the TRPM5 in the presence of TPPO leading to sub-optimal depolarization, both $[Ca^{2+}]_i$ and depolarization are necessary for optimal insulin secretion.

In spite of the fact that our conclusion is based on the results obtained by using one pharmacological tool, our results are convincing given that previous studies also reported reduced insulin secretion in TRPM5 knockout mice.

3.2 Study II

3.2.1 Insulin secretion and Ca^{2+} signaling in a genetically engineered human pancreatic β-cell line

The development of genetically engineered human insulinoma cell lines that can be easily cultured and propagated has opened up possibilities for studying signal transduction in the β-cells. In this study, we demonstrate insulin secretion in EndoC-BH1 cells in response to glucose, GLP-1 and we show for the first time that $[Ca^{2+}]_i$ can be measured from these cells by using fura-2 based microfluorometry. We have shown that several physiological or pharmacological agonists that are known to increase $[Ca^{2+}]_i$ in the primary β-cells also increase $[Ca^{2+}]_i$ in the EndoC-BH1 cells.

3.2.2 Insulin secretion in EndoC-BH1 cells

Glucose stimulated the insulin secretion in a concentration dependent manner in the EndoC-BH1cells (Fig 7a). These cells secreted more insulin in response to glucose (16.7 mM) and GLP-1 (50 nM) compared to glucose (16.7 mM) alone (Fig. 7b), suggesting that the metabolic coupling pathways involved in glucose and GLP-1 stimulated insulin secretion were intact in these cells.

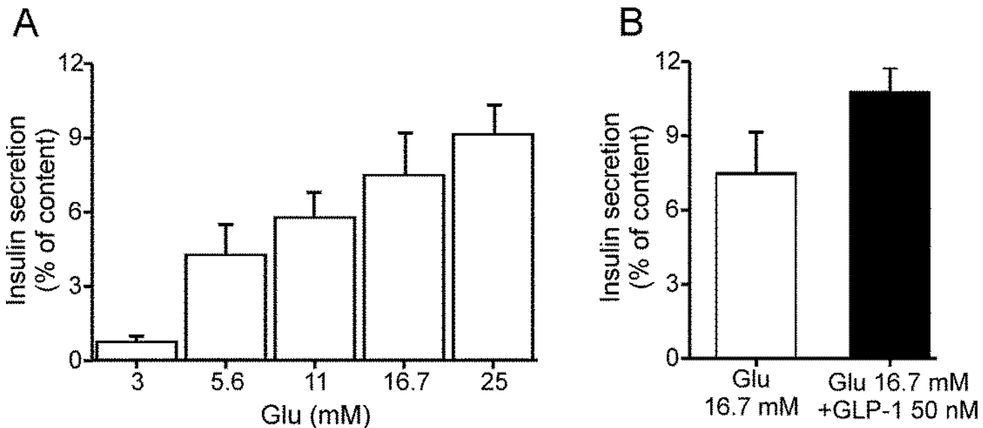

Fig. 7. Glucose stimulated insulin secretion in the EndoC-BH1cells. *(**A**) The figure shows the magnitude of insulin secretion from EndoC-BH1cells in response to various glucose (Glu) concentrations.(**B**) Insulin secreted in response to glucose (16.7 mM) (white bar) and glucose (16.7 mM) plus GLP-1 (50 nM) (black bar).*

3.2.3 $[Ca^{2+}]_i$ signaling in the EndoC-BH1 cells

KCl, which is known to depolarize the plasma membrane and activate VGCCs increased $[Ca^{2+}]_i$ in the fura-2 loaded EndoC-BH1cells proving the presence of functional VGCCs in these cells. They also responded to the cholinergic agent carbachol indicating that the PI-PLC-IP3 pathway for Ca^{2+} signaling was intact in these cells. The cationic amino acid L-arginine also increased $[Ca^{2+}]_i$ in the EndoC-BH1 cells.

Sulphonylureas are known to increase the $[Ca^{2+}]_i$ primarily by inhibiting the K_{ATP} channels and thereby causing depolarization followed by activation of VGCC. We found that, Tolbutamide, a commonly used sulphonylurea, increased

$[Ca^{2+}]_i$ in the presence of GLP-1 (Fig. 8a) indicating that EndoC-BH1 cells possess functional K_{ATP} channels and voltage-gated Ca^{2+} channels. GLP-1 was essential for eliciting Ca^{2+} response in the EndoC-BH1 cells upon stimulation by glucose (Fig. 8b). These observations are consistent with the fact that GLP-1 makes β-cells competent to glucose (49). However, we found that even in the presence of GLP-1 the $[Ca^{2+}]_i$ response to glucose in the EndoC-BH1 cells was small compared to that reported in the primary human β-cell (53). It should be noted that, Insulin secretion in response to glucose is poor (or absent) in most of the rodent insulinoma cell lines available. These observations prove that the ion channels and the signaling molecules that are essential for the regulation of the membrane potential and Ca^{2+} signaling in the primary β-cells are present also in the EndoC-BH1 cells. We conclude that EndoC-BH1 cells could be used as a model to study stimulus-secretion coupling and Ca^{2+} signaling in the human β-cells.

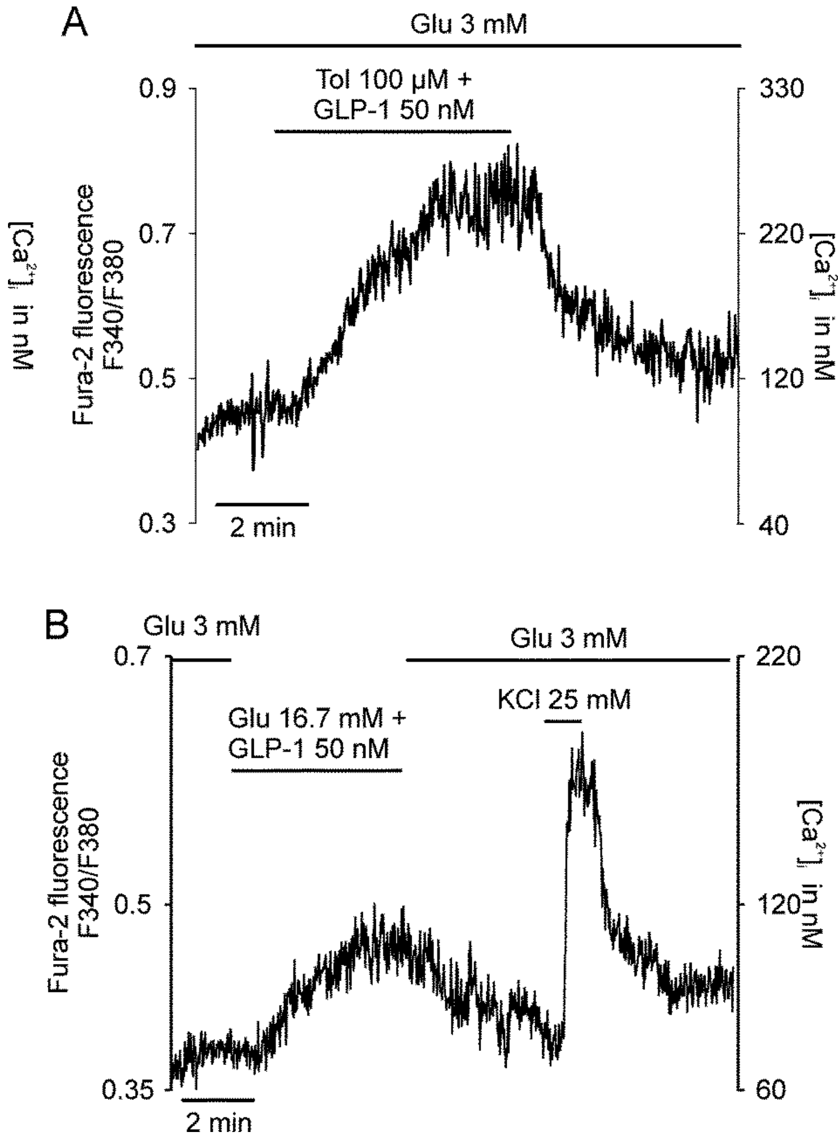

***Fig. 8. GLP-1 makes EndoC-BH1 cells competent** (**A**) The representative trace shows increase in $[Ca^{2+}]_i$ when stimulated with tolbutamide (100 µM) plus GLP-1 (50 nM) (n = 5). (**B**) The representative trace shows increase in $[Ca^{2+}]_i$ when stimulated with glucose (16.7 mM) plus GLP-1 (50 nM), KCl (25 mM) was used as a positive control. (n = 17).*

4 Conclusions

- The TRPM5 channel is involved in insulin secretion and $[Ca^{2+}]_i$ increase in response to glucose, L-arginine and GLP-1, but not in response to fructose.

- The genetically engineered human β-cell line (EndoC-BH1) could be used as a model for studying stimulus-secretion coupling and Ca^{2+} signaling in the human β-cells.

5 Future perspectives

It is important to study the molecular mechanisms of the regulation and function of other TRP channels present in the β-cells in mediating insulin secretion. Future research could focus on developing more potent and selective pharmacological tools for the TRP channels. The use of such tools may provide answers for many unsolved questions. It is possible that TRP channels could be possible therapeutic targets for the treatment of type-2 diabetes.

The EndoC-BH1 cells are of human origin, these cells have good insulin secreting properties in response to glucose. Future research could be intensified to find out whether these cells could be used for β-cell transplantation.

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