Depolarization by transient receptor potential melastatin 4 in pancreatic alpha-cells regulates glucagon secretion

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DEPOLARIZATION BY TRANSIENT RECEPTOR POTENTIAL MELASTATIN 4 IN PANCREATIC ALPHA-CELLS REGULATES GLUCAGON SECRETION

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in The Interdepartmental Program in Veterinary Medical Sciences through the Department of Comparative Biomedical Sciences

by

Piper Lynn Nelson
B.S., Henderson State University, 2008
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ABSTRACT

The Transient Receptor Potential Melastatin 4 protein (TRPM4) is a member of the TRP family of ion channels that is expressed in both electrically excitable and non-excitable cells. Functional studies revealed that TRPM4 significantly impacts Ca\(^{2+}\) signals in both immune and pancreatic \(\beta\)-cells, which is important for cellular processes such as hormone secretion. However, its role in glucagon secreting \(\alpha\)-cells has not been reported. Type 2 Diabetes Mellitus is often associated with increased glucagon levels; yet, the exact mechanism controlling its secretion is not known. In pancreatic \(\alpha\)-cells, an increase in intracellular Ca\(^{2+}\) concentration causes glucagon secretion. We hypothesize that TRPM4 is important for glucagon secretion in \(\alpha\)-cells by controlling intracellular Ca\(^{2+}\) signals. In this study, we investigated TRPM4 expression in the \(\alpha\)-cell lines INR1G9 (hamster) and \(\alpha\)TC1-6 (mouse) and characterized the channel using the patch-clamp technique. By RT-PCR we identified TRPM4-transcripts in both cell lines examined. Furthermore, patch-clamp recordings with increasing intracellular Ca\(^{2+}\) concentrations resulted in a dose-dependent activation of TRPM4-like currents. The greatest depolarizing currents were obtained with 3\(\mu\)M Ca\(^{2+}\) concentration. The current-voltage relationship (I/V) resembled those previously described for TRPM4. In addition, we demonstrated the voltage dependency of the channel, where negative potentials inhibited and positive potentials increased channel activity. Finally, replacement of Na\(^+\) ions in the extracellular solution with \(N\)-methyl-D-glucamine significantly reduced the inward currents and caused a hyperpolarizing shift in the I/V, which affirms that the channel is Na\(^+\) permeable. These data demonstrate that TRPM4 is present and functional in pancreatic \(\alpha\)-cells and suggest a potential role for the channel in glucagon secretion and glucose homeostasis. The role
of TRPM4 in glucagon secretion was assessed using a stable TRPM4 knockdown αTC1-6 cell line. Calcium-imaging and glucagon secretion experiments revealed a relationship between the decreased intracellular Ca\(^{2+}\) concentration and glucagon secretion in TRPM4 knockdown cells compared to controls. These results indicate that depolarization by TRPM4 plays an important role in glucagon secretion and perhaps glucose homeostasis. Elucidation of the glucagon secretion pathway could lead to a treatment for hyperglucagonemia associated with Type 2 Diabetes.
CHAPTER 1
GENERAL INTRODUCTION

1.1 THESIS ORGANIZATION

This thesis is written in the journal style format. It contains an acknowledgements section, a general introduction, one research paper, a general conclusion, and a list of references cited in the general introduction and conclusion. The general introduction includes a hypothesis, objectives, background information, and a literature review. Chapter 2 is a research paper entitled “Regulation of Calcium-entry in Pancreatic α-cells by Transient Receptor Potential Melastatin 4 Plays Vital Role in Glucagon Release” that has been submitted to Molecular and Cellular Endocrinology and is currently under review. The general discussion will address the overall findings of this work. This thesis contains experimental results obtained by the author during her graduate study under the supervision of her co-major professors, Drs. Henrique Cheng and Ji-Ming Feng.

1.2 RESEARCH OBJECTIVES

Based on previous work, we know that TRPM4 is able to control the Ca\(^{2+}\) signals of pancreatic β-cells to regulate insulin release (Cheng et al., 2007; Marigo et al., 2009). Depolarization by TRPM4 controls the opening of voltage-dependent Ca\(^{2+}\) channels allowing for Ca\(^{2+}\)-dependent exocytosis of insulin granules. In an effort to elucidate the mechanism of glucagon secretion, we consider the role of TRPM4 in controlling Ca\(^{2+}\) signals that result in the exocytosis of glucagon granules. We hypothesize that TRPM4 is important for glucagon secretion in α-cells by controlling intracellular Ca\(^{2+}\) signals.
Because TRPM4 has not previously been studied in pancreatic α-cells, we will 1) identify and characterize TRPM4 in pancreatic α-cells then, 2) evaluate the role of TRPM4 in glucagon secretion.

1.3 BACKGROUND AND LITERATURE REVIEW

The family of Transient Receptor Potential (TRP) ion channels was originally identified in the *Drosophila* visual system. From there, eight subfamilies were identified in organisms ranging from *C. elegans* to humans (Fig. 1.1). Most TRP channels share a similar basic structure containing 6 transmembrane domains, a hydrophobic pore region between domains 5 and 6, and the C and N terminal regions on the intracellular side of the membrane. The TRP channels form homomeric or heteromeric tetramers in the cell membrane. However, the ionic permeability and activation vary greatly among the members of each subfamily. Functional studies of the TRP channels have implied physiological as well as pathophysiological roles for many of these channels (for review, see Nelson et al., 2010).

The first subfamily identified is the TRPC (canonical). This subfamily is most closely related to the *Drosophila* TRP channel subfamily and is the most well studied. Acting mainly as Ca$_{2+}$ regulators, the TRPC channels play a major role in the maintenance of cellular Ca$_{2+}$ homeostasis. This is important for processes such as keratinocyte differentiation (Beck et al., 2008), immune response (Sel et al., 2008; White et al., 2006), and muscle contraction (Tsvilovskyy et al., 2009). The vanilloid subfamily (TRPV) is named after their activation by vanilloid and vanilloid-like compounds such as capsaicin. These channels function as cellular heat and pH sensors (Geppetti et al., 2006)
as well as nociceptors (Caterina et al., 1997; Tominaga et al., 1998). The TRPN (no mechanopotential C or NOMPC) has no mammalian members. The ankyrin (TRPA) subfamily has only one mammalian member, which acts as a nocicepter as well as a mechanosensor aiding in the cochlear amplification of sound (Corey et al., 2004; Nagata et al., 2005). The TRPML (mucolipin) channels serve a lysosomal role in mammals, and mutations of the TRPML genes are normally associated with lysosomal storage diseases (Bargal et al., 2000; Bassi et al., 2000; Sun et al., 2000) and hearing loss (van Aken et al., 2008). The TRP channels associated with polycystic kidney disease make up the TRPP (polycystin) subfamily. These channels are located in the plasma membrane and sense mechanical stimulation such as fluid movement (Harris et al., 2006).

Figure 1.1: Transient Receptor Potential channel subfamilies. Modified from Christensen and Corey, 2007 and Hoenderop et al., 2005.
The melastatin (TRPM) subfamily is composed of 8 members that can be divided into subgroups based on their structural similarity. The founding member, TRPM1, is named after a pigment found in melanoma cells. Calcium signaling through TRPM1 is thought to be involved in melanocyte differentiation, migration, and survival (Bellone et al., 2008). Structurally similar to TRPM1, TRPM3 responds to hypotonic conditions resulting in cell swelling and is important for Ca\(^{2+}\) homeostasis in the kidneys (Grimm et al., 2003). Both TRPM6 and TRPM7 are activated by low intracellular Mg\(^{2+}\). TRPM7 is involved in cellular Mg\(^{2+}\) homeostasis (Schmitz et al., 2003); and TRPM6 plays a role in Mg\(^{2+}\) homeostasis specifically in the epithelium of the kidneys and intestines (Rondon et al., 2008; Voets et al., 2004). Deletion of the TRPM7 gene also disturbs normal growth and development in mice and zebrafish (Elizondo et al., 2005; Jin et al., 2008). The activation of TRPM2 by intracellular adenosine-diphosphoribose (ADP-ribose) and reactive oxygen species (Perraud et al., 2001; Sano et al., 2001) suggests a role for this channel in oxidative stress and cell death (Hara et al., 2002). TRPM8 functions as a cellular cold-sensor activated by cold temperatures and menthol (McKemy et al., 2002; Peier et al., 2002). Activation of a truncated form of TRPM8 in the lungs leads to cytokine production (Sabnis et al., 2008).

Of all the TRPM channels, only TRPM4/5 are impermeable to Ca\(^{2+}\). TRPM4/5 share a sequence homology of 45% (Hofmann et al., 2003). They are only permeable to monovalent cations (e.g. Na\(^{+}\) and K\(^{+}\)); however, they are activated by intracellular Ca\(^{2+}\) (Launay et al., 2002; Prawitt et al., 2003). They belong to a group referred to as Ca\(^{2+}\)-activated nonselective (CAN) cation channels. Because they are not permeable to Ca\(^{2+}\), TRPM4/5 play an indirect role in regulating intracellular Ca\(^{2+}\). Intracellular Ca\(^{2+}\) activates TRPM4/5 resulting in Na\(^{+}\) influx followed by membrane depolarization (Launay et al.,
This depolarization controls Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels (SOCs) in non-excitabile cells and voltage-dependent Ca\(^{2+}\) channels (VDCCs) in excitabile cells (Launay et al., 2004; Marigo et al., 2009). The depolarization effect by TRPM4/5 depends on the cell type. In excitabile cells, such as cardiac and skeletal muscle cells, endocrine cells, and neural cells, depolarization causes an increase in Ca\(^{2+}\) entry through VDCCs. Calcium influx through VDCCs depends on 2 factors: driving force of Ca\(^{2+}\) through the channel and activation state of the channel. During depolarization the membrane potential approaches 0 mV. At 0 mV, conditions are optimal for Ca\(^{2+}\) entry through VDCCs, because there is driving force and channel activation. However, in non-excitabile cells such as endothelial cells or immune cells, depolarization inhibits Ca\(^{2+}\) entry through their major Ca\(^{2+}\) channels, SOCs due to a decrease in driving force for Ca\(^{2+}\).

Because Ca\(^{2+}\) is an important second messenger in a number of physiological processes and intracellular Ca\(^{2+}\) activates TRPM4/5, it is likely that these channels are critical for cell physiology. TRPM5 is not as widely expressed as TRPM4; it was first identified in the taste receptors where it is involved in taste perception for sweet, bitter, and umami flavors (Perez et al., 2002; Zhang et al., 2003). TRPM5 has also been found in the olfactory system where it is involved in the processing of olfactory sensory information (Lin et al., 2007). Following its identification in the pancreatic β-cells (Prawitt et al., 2003), Colsoul and colleagues assessed the role of TRPM5 in these cells (Colsoul et al., 2010). They found that inhibition of TRPM5 decreased Ca\(^{2+}\) signals and insulin secretion; however, since TRPM4 is also present in β-cells, it is not known the contribution each one has on insulin secretion.
WideMly expressed throughout the body, TRPM4 is implicated in a variety of functions throughout the mammalian system in both excitable and non-excitable cells. The role of TRPM4 was first described in immune cells where channel inhibition increases Ca\(^{2+}\) entry resulting in an increase in cytokine production by T-cells (Launay et al., 2004). The deletion of TRPM4 in mast cells had a similar effect on Ca\(^{2+}\) signaling leading to cellular degranulation and increased immune response (Vennekens et al., 2007). Other studies found TRPM4 to be responsible for controlling mast cell and dendritic cell migration (Barbet et al., 2008; Shimizu et al., 2009). TRPM4 plays an indirect role in non-excitable vascular smooth muscle cells where inhibition reduces myogenic constriction affecting blood flow to the brain (Earley et al., 2004). The studies of TRPM4’s role in excitable cells are less numerous. The role of TRPM4 in excitable sino-atrial node cells was assessed and is suspected to aid in the pacemaker function (Demion et al., 2007). Another type of excitable cells, the pancreatic β-cells, was also studied. In these cells, inhibition of TRPM4 decreases Ca\(^{2+}\) signals and insulin secretion (Cheng et al., 2007; Marigo et al., 2009). In this system TRPM4 provides the depolarization necessary to open VDCCs thus, it controls Ca\(^{2+}\)-activated exocytosis of insulin granules.

The pancreatic α-cells produce the hormone, glucagon, to counteract the effects of insulin. Glucagon is secreted from the α-cells in response to hypoglycemia. It acts on the liver to induce glycogenolysis and gluconeogenesis. Glucagon receptors are located in the liver, heart, kidney, spleen, stomach, thymus, adipose tissue, and pancreas (Dunphy et al., 1998; Hansen et al., 1995). The precise mechanism for stimulation of glucagon secretion is not known. The pancreatic islets are innervated with sympathetic and parasympathetic
neurons, and the presence of receptors for their respective neurotransmitters in α-cells is suspected (for review, see Ahren, 2000).

There is a disagreement among studies as to whether or not α-cells sense hypoglycemic conditions directly or through paracrine or autonomic signals. The school of thought that believes α-cells directly sense blood glucose levels are left with the task of explaining how high glucose can stimulate insulin secretion in β-cells while inhibiting glucagon secretion from α-cells. Many studies suggest that a voltage-dependent Na\(^+\) channel that inactivates at voltages beyond -50mV is present in α-cells but not in β-cells. It allows the α-cell to secrete glucagon up to a point when glucose metabolism depolarizes the membrane beyond -50mV (Barg et al., 2000; Gopel et al., 2000; MacDonald et al., 2007; Vieira et al., 2007). The group who believes that α-cells only respond to paracrine and autonomic signals of hypoglycemia argue that intravenous administration of glucose to diabetic humans (Greenbaum et al., 2002) and dogs (Braaten et al., 1974) with failing β-cells increases glucagon secretion. However, Salehi and colleagues noted a paradoxical response where glucose stimulates glucagon secretion in an alpha-cell line and isolated islets (Salehi et al., 2006), and others found that there is decreased glucagon response in hyperinsulinemic hypoglycemic infants (Hussain et al., 2005). These studies suggest that autonomic input or paracrine input from functional β-cells is required for proper glucagon response to hyperglycemic conditions.

Exocytosis, like many other physiological processes, is highly dependent on intracellular Ca\(^{2+}\) levels. The exocytosis of insulin granules is regulated by Ca\(^{2+}\) entry through VDCCs under the control of TRPM4 (Cheng et al., 2007; Marigo et al., 2009). TRPM5 has also been suggested to contribute to the Ca\(^{2+}\) oscillations associated with insulin secretion, however the mechanism of TRPM5 action is not clear (Colsoul et al.,
2010). The exocytosis of glucagon granules from pancreatic α-cells is also dependent on intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]). Still, the mechanism for glucagon secretion is unknown. Exocytosis of glucagon granules depends on electrical activity (Barg et al., 2000) and Ca\(^{2+}\) entry through VDCCs (Gromada et al., 1997). But, the source initiating the Ca\(^{2+}\) current is under question. In the α-cell there are several types of VDCCs, the most abundant is the L-type. The identification of the non-L-type VDCCs is still unclear but may be T-, N-, R-, or P-types depending on the species and the particular study (Gopel et al., 2000; Gromada et al., 1997; Vignali et al., 2006). Although most Ca\(^{2+}\) enters through L-type VDCCs, one study suggests that this type of VDCC is not responsible for Ca\(^{2+}\) entry that results in exocytosis, but that N-type VDCCs serve the function of inducing glucagon release except in conditions that increase cAMP (Gromada et al., 1997). Regardless of the type of VDCC, this process relies on depolarization (Barg et al., 2000). The positively charged amino acid, L-arginine, can directly depolarize the membrane increasing the electrical activity and glucagon secretion (Barg et al., 2000; Gromada et al., 1997). Neurotransmitters such as adrenaline also stimulate glucagon secretion by mobilizing Ca\(^{2+}\) from the ER stores via the G\(_s\)-protein coupled receptor pathway that increases cAMP (Gromada et al., 1997). Interestingly, the pancreases of Type 2 diabetic patients have been found to contain higher levels of adrenaline (Ostenson et al., 1993), and its α-cells display an enhanced response to adrenaline (Ahren et al., 1995). This may explain the source of stimulation for the hyperglucagonemia seen in diabetics (Dinneen et al., 1995; Unger, 1978).

Considering the regulatory role that TRPM4 plays on Ca\(^{2+}\) entry in β-cells, we suspect that TRPM4 could also control the Ca\(^{2+}\) entry in α-cells (Fig. 1.2). Both cell types are excitable, contain VDCCs opened by depolarization as their major Ca\(^{2+}\) channel, and
rely on Ca\textsuperscript{2+} entry to stimulate exocytosis of their hormone granules. The mechanism of glucagon secretion remains to be determined but, if revealed, could have a significant impact on the clinical control of hyperglucagonemia in those suffering with Type 2 Diabetes Mellitus.

Figure 1.2: TRPM4 expression in the human pancreatic islet. (Our own unpublished data.)
CHAPTER 2
REGULATION OF CALCIUM-ENTRY IN PANCREATIC ALPHA-CELLS BY TRANSIENT RECEPTOR POTENTIAL MELASTATIN 4 PLAYS VITAL ROLE IN GLUCAGON RELEASE

2.1 INTRODUCTION

Glucagon is a hormone produced and secreted from pancreatic α-cells in response to hypoglycemia. Glucagon acts on the liver to stimulate glycogenolysis and gluconeogenesis. The regulation of glucagon secretion is very important in maintaining glucose homeostasis. Uncontrolled glucagon secretion can lead to hyperglycemia, a problem that would only amplify the hyperglycemic condition often associated with Type 2 Diabetes Mellitus (Baron et al., 1987; Basu et al., 2004; Shah et al., 2000). There are many opposing views on what may be controlling glucagon secretion and what cellular components are involved, but despite the decades of research into the mechanism, the exact pathway remains elusive. For example, Gopel and colleagues recognize that L-type Ca\(^{2+}\) current is not sufficient to sustain electrical activity and secretion itself, so glucagon release requires large depolarizations achieved through voltage-gated Na\(^{+}\) channels (Gopel et al., 2000). They believe that this mechanism allows for K\(_{ATP}\) channels to serve the same function in α-cells that they do in β-cells while still having paradoxical responses to glucose metabolism. However, others disagree with this proposal based on studies with K\(_{ATP}\) channel knockout mice that suggest no role of K\(_{ATP}\) channels in glucagon secretion. Liu and colleagues found adrenaline to directly stimulate glucagon secretion bypassing K\(_{ATP}\) channels (Liu et al., 2004). There is little agreement in the literature as to what ion channels are present in α-cells. Even the glucose-sensing
capability of the α-cell is controversial (Gromada et al., 2007; Miki et al., 2001; Rorsman et al., 2008). There is, however, a general agreement that glucagon secretion results from an increase in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (Gromada et al., 2007).

The Transient Receptor Potential (TRP) family of ion channels in mammals is comprised of 6 subfamilies: TRPV (vanilloid), TRPC (canonical), TRPM (melastatin), TRPP (polycystin), TRPA (ankyrin), and TRPML (mucolipin). The subfamilies vary in their permeability and activation. The melastatin subfamily has 8 members (TRPM1-8). TRPM4 is a 6-transmembrane protein with C- and N-terminals on the intracellular side and a single channel conductance of 25 pS (Launay et al., 2002). It is a Ca\(^{2+}\)-activated non-selective (CAN) cation channel permeable to monovalent cations, namely Na\(^+\) and K\(^+\). Activation of TRPM4 allows Na\(^+\) to enter, leading to cell depolarization. In non-excitable cells, TRPM4 inhibits Ca\(^{2+}\) entry through store-operated channels (Launay et al., 2004), a necessary step for cell migration, appropriate immune response, and many other physiological actions (Barbet et al., 2008). In excitable cells, for example pancreatic β-cells, TRPM4 promotes Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels (VDCCs) (Marigo et al., 2009), which allows for processes such as hormone secretion (Cheng et al., 2007). In β-cells, oscillations in the membrane potential result in oscillations in Ca\(^{2+}\) signals because each depolarization opens L-type Ca\(^{2+}\) channels and Ca\(^{2+}\) influx occurs. As a result, insulin is secreted in a pulsatile fashion (Gilon et al., 2002). Recent studies found that TRPM4 impacts Ca\(^{2+}\) signals in pancreatic β-cells and subsequently insulin secretion (Marigo et al., 2009). Because both pancreatic α- and β-cells are excitable, their mechanism for secretion may be similar. It is known that Ca\(^{2+}\) entry through VDCCs and the resulting increase in intracellular Ca\(^{2+}\) concentration of the α-cell is imperative for glucagon secretion (Barg et al., 2000; Gromada et al., 1997). In the present study, we
hypothesized that TRPM4 is important for glucagon secretion in α-cells by controlling intracellular Ca\(^{2+}\) signals. Therefore, we characterized TRPM4 in the alpha-cell line, αTC1-6, and examined its function in glucagon secretion.

2.2 RESEARCH DESIGN AND METHODS

**Cell Culture:** The mouse pancreatic α-cell line, αTC1-6, was maintained in RPMI 1640 with 10% FBS and aerated with 5% CO\(_2\) and 95% air at 37°C. All experiments were performed with cells from passages 37-45.

**RT-PCR:** RNA was extracted from INS-1, MIN-6, INR1G9 and αTC1-6 cells using the RNAqueous-4PCR® kit according to manufacturer’s instructions (Ambion, Austin, TX, USA). The RNA was purified with DNase 1 treatment. Reverse transcription was performed using MMLV-Reverse Transcriptase and Oligo(dT) primers. PCR was performed using Ambion’s RETROscript® kit and TRPM4 primers with the sequences listed (forward/reverse [5’ to 3’]): GACCTGCTTATTTGGGCTCTG / AGATGGGAGTTGTGCTGTCC mouse; CACCAGCCAGTTGGGCATACT / CGTGAGCAAGATGATGAAGG hamster; TTGGCATACTGGGAGACGCA / GGCCCAAGATCGTCATCGT rat. Mouse GAPDH primers with sequence (forward/reverse [5’ to 3’]) TGCTGAGTATGTGCTGGAGTCTA / AGTGGGAGTTGCTGTGGAAGTCG was used as a positive control for PCR, and ultrapure water was used as a negative sample for RNA.

**Immunocytochemistry:** αTC1-6 cell suspension was fixed in 4% paraformaldehyde for 10 min at RT then seeded on to poly-D-lysine coated coverslips. Next, cells were permeabilized with 0.1% Triton X-100 for 2 min. Cells were then immunostained with a
primary-antibody cocktail [anti-TRPM4 rabbit polyclonal antibody (1:600) + anti-
glucagon mouse monoclonal antibody (1:1000) (Sigma-Aldrich, Saint Louis, MO,
USA)]. After sufficient washing with PBS, binding was detected with a secondary-
antibody cocktail [anti-rabbit-FITC (1:400) (eBioscience, San Diego, CA, USA) + anti-
mouse-Rhodamine Red-X (1:400) (Jackson ImmunoResearch Laboratories, West Grove,
PA, USA)]. Immunofluorescence images were obtained by confocal microscope with the
focus plane cutting through the nucleus. Using the sequential acquisition technique, first
the FITC chromophore was excited with a laser at 488 nm, and emission was collected at
wavelengths from 500-530 nm. Then, Rhodamine Red-X was excited with a laser at 543
nm, and emission was collected at wavelengths from 570-650 nm.

**Electrophysiology**: Cells were maintained in standard modified Ringer’s solution of the
following composition (in mM): NaCl 140, KCl 2.8, CaCl₂ 1, MgCl₂ 2, glucose 4,
HEPES-NaOH 10, pH 7.2 adjusted with NaOH. The standard internal solution contained
(in mM): Cs-glutamate 120, NaCl 8, MgCl₂ 1, Cs-BAPTA 10, HEPES-CsOH 10, pH 7.2
adjusted with CsOH. The internal solution’s buffered Ca²⁺ concentration was adjusted as
necessary with CaCl₂ (calculated with WebMaxC http://www.stanford.edu/~cpatton/
webmaxcS.htm). The Na⁺-free modified Ringer’s solution contained (in mM): N-methyl-
D-glucamine (NMDG) 140, KCl 2.8, CaCl₂ 1, MgCl₂ 2, glucose 4, HEPES-CsOH 10, pH
7.2 adjusted with CsOH. The osmolarity of the solutions were ~300 mOsm/L. TRPM4
currents were recorded in the tight-seal whole-cell configuration mode at 21-25 °C. High-
resolution current recordings were acquired by a computer-based patch-clamp amplifier
system (EPC-10, HEKA, Lambrecht, Germany). Patch pipettes had resistances between
4-7 MΩ. Immediately following establishment of the whole-cell configuration, voltage
ramps of 50 ms duration spanning the voltage range of −100 to +100 mV were delivered
from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 300-600 s. All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions, calculated using Igor PPT Liquid Junction Potential software (Wavemetrics, Portland, OR, USA). The half-maximal excitatory concentration (EC$_{50}$) and Hill coefficient were also calculated using the Igor software with the formula: $f(x) = (Y_{min} + (Y_{max} - Y_{min}) \times (1 / (1 + (K_d / x)^n)))$ where $K_d$=EC$_{50}$ and $n$=Hill coefficient.

**Generation of TRPM4 knockdown population:** Using calcium chloride (0.6M CaCl$_2$), 293T cells were transfected with 3 plasmids containing sequences coding for the lentiviral vector genome (pccL backbone), viral packaging proteins (pdelta8.2R), or viral VSVG (pVSVG) envelope from the vesicular stomatitis virus. The VSVG pseudotype envelope enhances infectivity of lentiviruses produced towards human cells, although mouse and rat cells can similarly be infected by this modified envelope lentivirus. The transient transfection procedure for viral production is performed to maintain safe handling of the virus and avoid generation of self-replicating particles. Lentivirus plasmids were obtained from Sigma-Aldrich (Saint Louis, MO, USA) in a pLKO.1 backbone and contained either nonspecific control (SHC002) or shRNA specific for mouse TRPM4 (SHDNA-NM_175130, TRCN0000068684) under the control of the U6 promoter, plus the puromycin resistance gene. We reconstructed the lentiviral vector in a pCCL backbone plasmid in order to generate a bicistronic construct expressing both puromycin resistance gene and a green fluorescence protein (GFP) reporter separated by an internal ribosome entry sequence, CMV.Puro(r).IRES.GFP. Lastly, using BstBI restriction enzyme cloning, we ligated into the previous vector the PCR-amplified U6p-shRNA constructs (shc002, or shRNA specific for mouse TRPM4). The lentiviral vector therefore contains two expression cassettes, one driven by U6p, and another driven by
CMVp. The first is under the control of the RNA polymerase III promoter, U6, and includes the shRNA specific for mouse TRPM4 or a nonspecific control. Following the shRNA gene is an RNA polymerase II promoter, CMV, which controls transcription of the subsequent puromycin resistance gene and the GFP gene. These 2 genes are separated by an internal ribosome entry sequence to increase ribosome binding so the GFP gene gets translated. The second cassette ends with a woodchuck hepatitis post-transcriptional regulatory element (WPRE), shown to stabilize the RNA and improve viral titers (Klein et al., 2006). The final lentiviral vector, thus, has the configuration: 5’ pCCL--- U6p-shc002 --- CMV-Puro(r).IRES.GFP--WPRE-3’. Lentiviruses are produced in 293T cells and released into the media supernatant, which was collected at 48, 72, and 96 hours post-transfection, filtered using a 0.22 µm membrane, and stored at –80°C in aliquots. For determining viral concentration (titer), 200µl of either pure supernatant (1x), or dilutions of 1:10 or 1:100 were used to infect 293T cells (5x10⁴) seeded in 24-well plates. Infection was performed in the presence of the transduction-enhancing agent Polybrene at 8µg/ml for cells assayed for percentage of cells expressing GFP by flow cytometry 48h after infection. For infecting experimental cells a multiplicity of infection (MOI) of 5 lentiviruses was used to transduce αTC1-6 cells. Cells were selected in 1µg/mL puromycin for one week, and percent of puromycin-resistant cells expressing GFP was determined by flow cytometry using FACScan (BD Biosciences, Franklin Lakes, NJ, USA). Puromycin-selected transduced cells were used for electrophysiology, Ca²⁺-imaging, and glucagon secretion experiments.

**Calcium Imaging:** Control and TRPM4 shRNA αTC1-6 cells were loaded with 5µM Fura-2AM for 30 minutes at 37°C. A Ca²⁺-imaging buffer containing (in mM) NaCl 136, KCl 4.8, CaCl₂ 1.2, MgSO₄ 1.2, HEPES 10, glucose 4, and 0.1% BSA, pH 7.3 was used
for Fura-2AM loading and perfusion throughout imaging experiments. Calcium measurements were obtained using a dual excitation fluorometric imaging system (TILL-Photonics, Gräfelfingen, Germany) controlled by TILLvisION software. Fura-2AM loaded cells were excited by wavelengths of 340 nm and 380 nm. Fluorescence emissions were collected at a wavelength of 540 nm at a frequency of 1 Hz and computed into relative ratio units of the fluorescence intensity derived from the different excitation wavelengths ($F_{340}/F_{380}$).

**Measurement of Glucagon Secretion:** Control and TRPM4 shRNA αTC1-6 cells were plated into 24-well plates at ~4 x 10^5 cells/well and grown for 2-3 days. Measurement of glucagon secretion was accomplished by replacing the culture medium with modified KRB containing (in mM) NaCl 136, KCl 4.8, CaCl$_2$ 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 5, HEPES 10, glucose 1.67, and 0.1% BSA, pH 7.3. After a 15-min equilibration period at 37°C, the KRB was removed from the well and replaced with KRB containing either 1mM L-Arginine, 1µM AVP, 20mM KCl, or 1µM BayK 8644 and allowed to incubate for 30 min. The KRB was then collected and stored at -80°C for glucagon radioimmunoassay. Experiments were performed in quadruplicates and repeated three times. Data was normalized by cell number per well.

**Data analysis:** Patch-clamp recordings are shown as means ± S.E.M., and were plotted using Igor Pro 5 software program (Wavemetrics, Portland, OR, USA). Peak Ca$^{2+}$ data and glucagon secretion values were both analyzed using a two-tailed, unpaired Student’s $t$-test. Statistical significance was established at P<0.05.
2.3 RESULTS

We first examined if the TRPM4 gene was expressed in a mouse pancreatic α-cell line and whether the channel was functional. By RT-PCR, we detected TRPM4 transcripts in the mouse alpha-cell line, αTC1-6 (Fig. 2.1A). The pancreatic α-cell line, INR1G-9, and β-cell lines MIN-6 and INS-1 were used as positive controls (Marigo et al., 2009). Although TRPM4 is expressed in both the hamster and mouse alpha-cell lines, we selected the mouse cell line, αTC1-6, for our studies because of the availability of molecular tools for knockdown experiments. The expression of the TRPM4 protein in αTC1-6 cells was then confirmed by immunocytochemistry. Figure 2.1B shows TRPM4 (left panel) co-localization with glucagon (center panel) in αTC1-6 cells.

Next, we investigated if TRPM4 was functional using the patch-clamp technique. Increases in [Ca$^{2+}$]$_i$ is known to activate TRPM4, a CAN channel (Launay et al., 2002). Therefore, we performed patch-clamp recordings with increasing buffered [Ca$^{2+}$]$_i$ to determine if currents with the characteristics of those previously reported (Launay et al., 2002) for the channel could be detected. An increase in [Ca$^{2+}$]$_i$ resulted in a concentration-dependent activation of TRPM4-like currents with maximal current amplitude obtained with 3µM buffered Ca$^{2+}$ (Fig. 2.2A). The current-voltage (I/V) relationship taken at 600s after establishment of whole-cell configuration resembles those of TRPM4 (Fig. 2.2B) (Launay et al., 2002). The EC$_{50}$ was 0.62µM with a Hill coefficient of 2.72 (Fig. 2.2C). TRPM4 is also a voltage-dependent channel in which hyperpolarization decreases and depolarization increases its open probability (Launay et al., 2002). Hence, we examined, in the α-cell line, its voltage dependency. Patch-clamp recordings with intracellular Ca$^{2+}$ buffered at 1µM and holding potentials (HP) of -60, 0,
+60 mV resulted in a voltage-dependent activation of TRPM4 currents with the smallest current amplitude recorded at -60mV and the greatest at +60mV (Figs. 2.3A and 2.3C). The I/V relationships taken at 300s into the experiments resembled that of TRPM4 (Fig. 2.3B) (Launay et al., 2002). Since TRPM4 conducts Na\(^+\) into cells, we replaced NaCl in the extracellular buffer with NMDG and recorded currents under 1µM intracellular buffered Ca\(^{2+}\) and +60mV HP conditions. The absence of extracellular Na\(^+\) resulted in a significant reduction in the amplitude of TRPM4-like currents (Fig. 2.4A). The I/V
relationship showed a shift in the reversal potential to the left due to hyperpolarization caused by the absence of Na\(^+\) entry (Fig. 2.4B). Based on these observations, we concluded that the observed current is mediated by TPRM4 and from now on we will refer to this as TRPM4 current.

![Graphs A, B, and C](image)

**Figure 2.2: Calcium activates TRPM4 in pancreatic \( \alpha \)-cell line in a dose-dependent manner.** (A) Average inward and outward currents of TRPM4 after intracellular perfusion with increasing buffered Ca\(^{2+}\) concentrations. Traces represent the mean + S.E.M. (n=3-6 cells/concentration) extracted at a holding potential of +60mV. (B) Current-voltage relationship (I/V) under experimental conditions described in (A) taken from representative cells at the peak current amplitude at 600s for each Ca\(^{2+}\) concentration. (C) A dose-response analysis resulted in a half maximal excitatory Ca\(^{2+}\) concentration (EC\(_{50}\)) of 0.62µM and a Hill coefficient of 2.72.
Having characterized TRPM4 in αTC1-6 cells, we examined its role in Ca\(^{2+}\) signaling and glucagon secretion using a stable TRPM4 knockdown population generated with a lentiviral vector carrying shRNA, GFP reporter, and puromycin-resistance genes. Fluorescent-activated cell sorting (FACS) analysis revealed >96.5% GFP expression in the control and TRPM4 knockdown population (Fig. 2.5A-C). Furthermore, we confirmed TRPM4 inhibition by patch-clamp under elevated buffered Ca\(^{2+}\) conditions. A significant reduction in TRPM4 currents in response to 1µM Ca\(^{2+}\) and +60mV HP
occurred in knockdown cells compared to control cells (Fig. 2.6A). These experiments showed that channel knockdown was successful. The I/V relationship of the control cells still resembled that of TRPM4 indicating that transduction with the viral vector did not affect the normal function of the channel (Fig. 2.6B) (Launay et al., 2002).

Glucagon secretion from pancreatic α-cells requires increases in [Ca^{2+}]_i (Gromada et al., 1997). Therefore, we determined the impact of TRPM4 knockdown on Ca^{2+} signals in response to different stimuli. Perfusion of control cells with 1µM arginine vasopressin (AVP), a G_q-protein coupled receptor agonist in α-cells (Yibchok-Anun et al., 2000) caused a sharp increase in intracellular Ca^{2+} followed by a secondary phase (Fig. 2.7A).
Figure 2.5: Transduction of αTC1-6 cells with shRNA specific for TRPM4 using lentiviral vector. Fluorescent activated cell sorting was used to measure the percentage of GFP-positive cells of (A) non-transduced αTC1-6 cells, (B) αTC1-6 cells transduced with non-specific shRNA, and (C) shRNA TRPM4 cells.
However, inhibition of TRPM4 by shRNA significantly decreased the responses to AVP ($P<0.0001$). The effect of direct cell depolarization on $\text{Ca}^{2+}$ signals was examined by perfusing cells with 1mM L-arginine (L-Arg) or 20mM KCl. TRPM4 inhibition decreased $\text{Ca}^{2+}$ entry in response to both L-Arg ($P<0.0001$, Fig. 2.7B) and KCl ($P<0.0001$, Fig. 2.8A). In another experiment, direct activation of L-type $\text{Ca}^{2+}$ channels with 1µM BayK 8644 also elicited a significantly smaller response in TRPM4 knockdown cells ($P<0.0001$, Fig. 2.8B). Histograms show a comparison between basal and peak $\text{Ca}^{2+}$ increase within the same group (Figs. 2.7,2.8). To assess parasympathetic control of $\text{Ca}^{2+}$ signals, we perfused cells with 100µM acetylcholine, but neither control nor the TRPM4 knockdown cells responded to treatment (data not shown).

After confirming the reduced responses in $\text{Ca}^{2+}$ signals with TRPM4 knockdown, we investigated its impact on stimulus-induced glucagon secretion using static incubation
TRPM4 knockdown cells secreted significantly less glucagon compared to control cells when stimulated with 1 µM AVP ($P<0.0001$), 20mM KCl ($P<0.0001$), 1mM L-Arg ($P<0.0001$), and 1µM BayK 8644 ($P=0.0079$).

**Figure 2.7:** Inhibition of TRPM4 decreases the magnitude of stimulus-induced Ca$^{2+}$ signals in pancreatic α-cell line. Average increase in [Ca$^{2+}$]$_i$ during stimulation with 1µM AVP (A) or 1mM L-Arg (B) in control and TRPM4 shRNA cells. Right panel: basal Ca$^{2+}$ (filled bar) and peak Ca$^{2+}$ increase (open bar) for control shRNA and TRPM4 shRNA cells of each treatment group. Values are mean ± S.E.M.; n=69-95 cells per treatment from 3 independent experiments; * $P<0.05$ compared to basal Ca$^{2+}$; # $P<0.05$ comparing peak of control cells to TRPM4 knockdown cells.
We have investigated for the first time the role of TRPM4 in glucagon secretion from pancreatic α-cells. Using RT-PCR and immunocytochemistry, we demonstrated TRPM4 gene expression and protein in the mouse pancreatic α-cell line, αTC1-6. Electrophysiological recordings revealed currents with the characteristics of TRPM4.

Figure 2.8: TRPM4 inhibition decreases stimulus-induced Ca\(^{2+}\) signals. Average increase in [Ca\(^{2+}\)]\(_{i}\) during stimulation with 20mM KCl (A) or 1µM BayK 8644 (B) in control and TRPM4 shRNA cells. Right panel: basal Ca\(^{2+}\) (black bar) and peak Ca\(^{2+}\) increase (white bar) for control shRNA and TRPM4 shRNA cells of each treatment group. Values are mean ± S.E.M.; n=69-95 cells per treatment from 3 independent experiments; * P<0.05 compared to basal Ca\(^{2+}\); # P<0.05 comparing peak of control cells to TRPM4 knockdown cells.

2.4 DISCUSSION

We have investigated for the first time the role of TRPM4 in glucagon secretion from pancreatic α-cells. Using RT-PCR and immunocytochemistry, we demonstrated TRPM4 gene expression and protein in the mouse pancreatic α-cell line, αTC1-6. Electrophysiological recordings revealed currents with the characteristics of TRPM4.
suggesting that the channel is functionally active in αTC1-6 cells. By inhibiting TRPM4, using lentiviral transduction of TRPM4-specific shRNA, we were able to investigate its role in glucagon secretion. We found that TRPM4 inhibition decreased the magnitude of intracellular Ca\(^{2+}\) signals and glucagon secretion in response to several stimuli compared to controls. Similarly, TRPM4 knockdown decreases the responses to stimulation during Ca\(^{2+}\) imaging and insulin secretion experiments from β-cells (Cheng et al., 2007; Marigo et al., 2009). Despite being different cell types, both utilize VDCCs as the main pathway for Ca\(^{2+}\) influx and hormone secretion. The fact that suppression of TRPM4 in α-cells...
with shRNA and a dominant-negative construct in β-cells resulted in similar observations strongly suggests a role for the channel in Ca\(^{2+}\) signaling and hormone secretion from islet cells. In addition to TRPM4, studies with TRPM5 in knockout mice, a closely related channel with similar function have shown loss of high frequency Ca\(^{2+}\) oscillations from islets and hyperglycemia in response to glucose overload (Brixel et al., 2010; Colsoul et al., 2010). TRPM5 activation at least in β-cells has been linked to rapid changes in intracellular Ca\(^{2+}\) concentration (Prawitt et al., 2003). However, its role in α-cells remains to be determined. It is tempting to speculate that both channels might be working in a coordinated fashion to control Ca\(^{2+}\) signals and hormone secretion in response to different agonists. Two other members of the TRP family of ion channels (TRPM2 and TRPM3) were reported to control insulin secretion from β-cells (Togashi et al., 2006; Wagner et al., 2008), but their function in α-cells is unknown.

The biophysical characterization of TRPM4 in our study of αTC1-6 cells revealed Ca\(^{2+}\)-activated currents that are voltage-dependent and promote Na\(^+\) influx. These properties are similar to those described in other cells types (Earley et al., 2004; Launay et al., 2004; Marigo et al., 2009; Nilius et al., 2003) and confirm the presence of TRPM4 in αTC1-6 cells. The EC\(_{50}\) for Ca\(^{2+}\)-induced TRPM4 activation in αTC1-6 was 0.62µM with a Hill coefficient of 2.72. This is in line with our previous finding in the β-cell lines HIT-T15, RINm5F, MIN-6 and β-TC3 (Marigo et al., 2009). In the same report, patch-clamp recordings in a hamster α-cell line revealed transient currents characterized by brief activation followed by channel closure. Our findings in mouse αTC1-6 cells differ from this observation, but are in agreement with the biphasic current pattern reported in most cell types. The difference in TRPM4 activity suggests that in hamster it may be involved in the control of transient Ca\(^{2+}\) increases rather than continuous oscillations.
Inhibition of TRPM4 by shRNA reduced the magnitude of Ca\(^{2+}\) signals in response to all stimuli tested. L-Arginine as well as KCl depolarizes cells directly to open VDCCs. It is reasonable to speculate that TRPM4 activation may occur during Ca\(^{2+}\) influx in addition to release from intracellular stores, which would further contribute to cell depolarization and the elevation in intracellular Ca\(^{2+}\) signals. Stimulation of αTC1-6 cells with AVP, a G\(_q\)-protein coupled receptor agonist in α-cells resulted in decreased Ca\(^{2+}\) signals in TRPM4 knockdown cells. Binding of AVP to its receptor leads to Ca\(^{2+}\) release from the endoplasmic reticulum (ER) followed by a secondary phase due to Ca\(^{2+}\) influx from the extracellular space. In this scenario, TRPM4 activation during Ca\(^{2+}\) release from the ER would lead to depolarization and Ca\(^{2+}\) influx via VDCCs. A similar observation was made during TRPM4 inhibition in β-cells stimulated with AVP (Marigo et al., 2009). When we tested the effect of BayK 8644 on Ca\(^{2+}\) signals there was also a decrease in the magnitude after TRPM4 knockdown. BayK 8644 is known to directly activate L-type VDCCs, however, it can inhibit T-type VDCCs (Wu et al., 1992). Because Ca\(^{2+}\) signals were reduced in TRPM4 knockdown cells, we have to consider the possibility of TRPM4 regulation of other VDCC types in α-cells. In fact, Gromada and colleagues (Gromada et al., 1997) reported that N-type VDCCs are the most important for Ca\(^{2+}\) influx and glucagon secretion. The regulation of N-type VDCCs by TRPM4 remains to be determined. Increases in the intracellular Ca\(^{2+}\) results in glucagon secretion and is an essential step in the pathway leading to exocytosis (Barg et al., 2000; Gromada et al., 1997). The amount of glucagon secreted from αTC1-6 cells was directly related to the magnitude of Ca\(^{2+}\) signals observed in both control and TRPM4 knockdown groups when stimulated with AVP, KCl and L-Arg. However, treatment with BayK 8644 resulted in increased glucagon secretion above basal in both groups. Perhaps direct
opening of L-type VDCCs is sufficient to promote Ca\(^{2+}\) influx to stimulate secretion. Although TRPM4 knockdown resulted in less glucagon release than controls, it agrees with the magnitude of Ca\(^{2+}\) signals during BayK 8644 stimulation. We also investigated the responses to acetylcholine in Ca\(^{2+}\) signaling and glucagon secretion, which is a neurotransmitter used physiologically by the parasympathetic nervous system to elicit glucagon secretion (Verspohl et al., 1990). Yet, neither control nor TRPM4 knockdown cells responded to treatment. We can speculate that the murine α-cell line used in the study may not have the receptors for acetylcholine.

Based on our findings, we propose a model of glucagon secretion in which depolarization by TRPM4 contributes to the secondary phase of Ca\(^{2+}\) influx through VDCCs (Fig. 2.10). Direct depolarization, as with L-Arg and KCl, opens VDCCs allowing Ca\(^{2+}\) influx and glucagon secretion, but it also activates TRPM4. Sodium influx through TRPM4 depolarizes the membrane, resulting in an increase in Ca\(^{2+}\) signals. These events potentiate glucagon secretion. For G\(_q\)-protein coupled receptor agonists (e.g. AVP), Ca\(^{2+}\) release from the ER caused by an increase in IP\(_3\) activates TRPM4 leading to depolarization. The depolarization opens VDCCS allowing Ca\(^{2+}\) influx during the secondary phase and sustained glucagon secretion. The findings in our study provide the foundation for further research into the specific components of the glucagon secretion pathway. Clarifying this mechanism may allow for the control of hyperglucagonemia in Type 2 diabetic patients.

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**Figure 2.10. Role of TRPM4 in stimulus-induced glucagon secretion.** Solid arrows indicate steps in direct depolarization pathway. Dashed arrows indicate steps in Gq-protein coupled receptor pathway. See text for details. AVP, arginine vasopressin; PLC, phospholipase C; IP3, inositol triphosphate; ER, endoplasmic reticulum; K+, potassium ion; L-Arg, L-arginine; VDCC, voltage-dependent Ca2+ channel; TRPM4, transient receptor potential melastatin 4.


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CHAPTER 3
GENERAL CONCLUSION

Prior to this study the presence and function of TRPM4 in pancreatic α-cells was unknown. After identifying TRPM4 and carefully assessing its function in these cells, we were able to establish a relation between the control of Ca\(^{2+}\) entry and the control of glucagon release. We have learned that TRPM4 does affect Ca\(^{2+}\) signaling in the α-cell, and in turn glucagon secretion. Inhibition of TRPM4 in mouse αTC1-6 cells decreased Ca\(^{2+}\) signals and glucagon secretion in response to the stimuli: L-arginine, KCl, arginine vasopressin, and BayK 8644. Each of these stimuli utilizes a pathway that results in an increased [Ca\(^{2+}\)]\(_i\) that stimulates exocytosis of glucagon granules. Based on the data presented in this thesis, we reason that TRPM4 is controlling the [Ca\(^{2+}\)]\(_i\) allowing for regulation of the amount of glucagon release. Depolarization caused by Na\(^+\) entry through TRPM4 directly controls the Ca\(^{2+}\) entry through VDCCs; this indicates TRPM4 as a key player in the glucagon secretion pathway. These findings bring the field one step closer to uncovering the exact mechanism of glucagon secretion. The elucidation of this pathway has the potential to provide many new targets for the treatment of hyperglucagonemia in diabetic patients.


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