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Roles of the Transient Receptor Potential Channels and the Intracellular Ca^{2+} Channels in Ca^{2+} Signaling in the β-cells

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If we knew what it was we were doing,
it would not be called research, would it?
Albert Einstein

To my beloved family,

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Södersjukhuset, Stockholm

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1 Abstract

Previous studies from our group reported that pancreatic β-cells express ryanodine receptors (RyRs) that can mediate Ca^{2+}-induced Ca^{2+} release (CICR). The full consequences of the activation of RyRs on Ca^{2+} signaling in these cells, however, remained unclear. An important open question was whether activation of the RyRs leads to activation of any Ca^{2+} channels in the plasma membrane, and thereby depolarizes membrane potential. One main aim of the thesis was to address this question. As a corollary, we have also looked for the existence of functional TRPV1 channels, and have elucidated the molecular mechanisms that underlie the $[Ca^{2+}]_i$-elevating effect of ADP ribose in these cells.

We used methods such as measurement of the $[Ca^{2+}]_i$ in single cells loaded with fura-2, patch clamp technique, Western blot analysis, immunohistochemistry, a variety of pharmacological tools, and a series of carefully designed protocols. In most experiments, we used S5 cells, derived from the rat insulinoma cell line INS-1E, but we also used primary β-cells from mice, rat, and human.

Activation of the RyRs by 9-methyl 5,7-dibromoeudistomin D (MBED) increased the $[Ca^{2+}]_i$ with an initial peak, followed by a decline to a plateau phase, and regenerative spikes superimposed on the plateau. The initial $[Ca^{2+}]_i$ increase was due to the activation of the RyRs in the ER, since it was abolished by thapsigargin, but was present when extracellular Ca^{2+} was omitted or when Ca^{2+} entry was blocked by SKF 96365. The plateau phase was due to Ca^{2+} entry across the plasma membrane, since it was abolished by omission of extracellular Ca^{2+}, and blocked by SKF 96365. The plateau phase was not solely dependent on the filling state of the ER, since it was not abolished by thapsigargin. Inhibition of the voltage-gated Ca^{2+} channels by nimodipine did not inhibit the plateau phase. Several agents that block TRP channels, e.g. La^{3+}, Gd^{3+}, niflumic acid, and 2-APB, inhibited the plateau phase. It was also inhibited by membrane depolarization. We conclude that the plateau phase was due to activation of some TRP-like channels. Activation of RyRs by MBED also induced membrane depolarization. The spikes required Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channels, as they were abolished by nimodipine. The spikes resulted from CICR, since they were inhibited in a use-dependent way by ryanodine, and abolished after depletion of the ER by thapsigargin. Thus, activation of RyRs activated TRP-like channels, depolarized the plasma membrane, activated L-type voltage-gated Ca^{2+} channels and triggered CICR.

During the course of this thesis we reported that TRPM2 is present in the INS1-E cells and the human β-cells. We studied whether TRPM2 was involved in the Ca^{2+} entry triggered by the activation of RyRs. N-(p-amylcinnamoyl) anthranilic acid (ACA), an inhibitor of TRPM2, did not inhibit the MBED-induced $[Ca^{2+}]_i$ entry. ADP ribose (ADPr), when applied intracellularly, is an agonist of TRPM2. We found that extracellularly applied ADPr increased $[Ca^{2+}]_i$ in the form of an initial peak followed by a plateau that depended on extracellular Ca^{2+}. EC_{50} of ADPr was ~30 μM. NAD^+, cADPr, a phosphonate analogue of ADPr (PADPr), 8-bromo-ADPr or breakdown products of ADPr did not increase $[Ca^{2+}]_i$. Inhibitors of TRPM2, e.g. flufenamic acid, niflumic acid, and ACA did not affect the ADPr-induced $[Ca^{2+}]_i$ increase. Two specific inhibitors of the purinergic receptor P2Y1, e.g. MRS 2179 and MRS 2279 completely blocked the ADPr-induced $[Ca^{2+}]_i$ increase. The $[Ca^{2+}]_i$ increase by ADPr required activation of PI-PLC, since the PI-PLC inhibitor U73122 abolished the $[Ca^{2+}]_i$ increase. The ADPr-induced $[Ca^{2+}]_i$ increase was through the IP_3 receptors, since it was inhibited by 2-APB, an inhibitor of the IP_3 receptors. ADPr increased $[Ca^{2+}]_i$ in the transfected human astrocytoma cells that expressed the P2Y1 receptors, but not in the wild type astrocytoma cells. We conclude that extracellular ADPr is an endogenous and specific agonist of P2Y1 receptors.

Capsaicin and AM404, two specific agonists of TRPV1, increased $[Ca^{2+}]_i$ in the INS-1E cells. Capsazepine, a specific antagonist of TRPV1, completely blocked the capsaicin-induced $[Ca^{2+}]_i$ increase. Capsaicin elicited inward currents that were abolished by capsazepine. TRPV1 protein was detected in the INS-1E cells and human β-cells by Western blot. However, no TRPV1 immunoreactivity was detected in the human islet cells and human insulinoma by immunohistochemistry. Capsaicin did not increase $[Ca^{2+}]_i$ in primary β-cells from rat or human. We conclude that INS-1E cells express functional TRPV1 channels.

In summary, we have shown that (1) RyR activation leads to activation of TRP-like channels in the plasma membrane, membrane depolarization, activation of L-type voltage-gated Ca^{2+} channels and CICR. (2) ADPr is a specific and endogenous low affinity ligand for the P2Y1 receptors. (3) Functional TRPV1 channels are expressed in the INS-1E cells, but not in the primary β-cells.

Keywords: Ca^{2+} signaling, signal transduction, islets of Langerhans, β-cells, ryanodine receptors, Ca^{2+}-induced Ca^{2+} release, TRP-channels, TRPV1, capsaicin, P2Y1 receptors, and ADP ribose.

2 Erratum

Paper IA: page 302, paragraph 2, line 10: -40 mV, not -40 mM.

Paper IB: page 4, line 38: -40 mV, not -40 mM,
page 3, line 4; page 4, line 13; page 13, figure legend 1, line 6: Wistar rat, not Wister rat.

Paper II: fig. 3C: The concentrations are in µM, not mM.

3 List of publications

I. **Jabin Gustafsson, A.**, Ingelman-Sundberg, H., Dzabic, M., Awasum,J., Hoa, N.K., Östenson, C-G., Pierro, C., Tedeschi, P., Woolcott, O.O., Chiounan, S., Lund, P.-E., Larsson, O., and Islam M.S. Ryanodine receptor-operated activation of TRP-like channels can trigger critical Ca^{2+} signaling events in pancreatic β-cells.

A. FASEB Journal express article:
FASEB J. 2005 Feb;19(2):301-3.

B. Full paper:
DOI: 10.1096/fj.04-2621fje
http://tinyurl.com/trp-like
Epub 2004 Nov 30.

II. **Jabin Gustafsson, A.**, Muraro, L., Dahlberg, C., Migaud, M. Chevallier, O., Hoa, N.K., Krishnan, K., Li, N., and Islam, M.S., 2011. ADP ribose is an endogenous ligand of P2Y1 receptor. Mol Cell Endocrinol 333:8-19.

III. **Jabin Fågelskiöld, A.**, Kannisto, K., Boström, A., Hadrovic. B., Farre, C., Eweida, M., Wester, K., and Islam, M.S. Insulin-secreting INS-1E cells express functional TRPV1 channels (submitted).

Other papers published during the PhD period, but not included in the thesis:

Original papers:

Woolcott,O.O., **Gustafsson,A.J.**, Dzabic,M., Pierro,C., Tedeschi,P., Sandgren,J., Bari,M.R., Hoa, N.K., Bianchi,M., Rakonjac,M., Rådmark,O., Östenson,C.G., and Islam,M.S., 2006. Arachidonic acid is a physiological activator of the ryanodine receptor in pancreatic beta-cells. Cell Calcium, 39:529-537.

Bari, M.R., Akbar, S., Eweida, M., Kühn, F.J.P., **Gustafsson, A.J.**, Lückhoff, A., and Islam, M.S., 2009. H_2O_2-induced Ca^{2+} influx and its inhibition by N-(p-amylcinnamoyl) anthranilic acid in the beta-cells: involvement of TRPM2 channels. J Cell Mol Med, 13:3260-3267.

Reviews:

Gustafsson A.J., Islam, M.S., 2005. Cellens kalciumjonsignalering – från grundforskning till patientnytta. (Cellular calcium ion signaling - from basic research to benefits for patients) Läkartidningen, 102:3214-3219.

Book chapter:

Gustafsson A.J., Islam, M.S., 2007. Islets of Langerhans - cellular structure and physiology in Chronic Allograft Failure: Natural History, Pathogenesis, Diagnosis, and Managements. Editor: Ahsan, N., Landes Bioscience.

4 List of abbreviations

ACA	*N*-(p-amylcinnamoyl) anthranilic acid
ADPr	Adenosine diphosphate ribose
AM	Acetoxymethyl ester
AM404	*N*-(4-hydroxyphenyl)-arachidonoylamide
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cADPr	Cyclic adenosine diphosphate ribose
cAMP	Adenosine 3´5´-cyclic monophosphate
CICR	Ca^{2+}-induced Ca^{2+} release
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
GLP-1	Glucagon-like peptide
HBSS	Hank's balanced salt solution
I_{CRAC}	Ca^{2+} release activated Ca^{2+} current
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
K_{ATP} channel	ATP-sensitive potassium channel
KRBH	Krebs Ringer bicarbonate HEPES buffer
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
Orai1	A pore forming subunit of the mammalian CRAC channel
P2Y1	Purinergic receptor type 2Y1
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PI-PLC	Phosphoinositide-specific phospholipase C
PMCA	Plasma membrane Ca^{2+} ATPase
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RyR	Ryanodine receptor
SERCA	Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SOCE	Store-operated Ca^{2+} entry
STIM1	Stromal interaction molecule 1
TRP	Transient receptor potential
TRPM2	Transient receptor potential melastatin 2
TRPV1	Transient receptor potential vanilloid 1

Contents

5 Introduction and Background

5.1 The islets of Langerhans

The islets of Langerhans, named after the German pathologist Paul Langerhans, is a critical organ unique in that it is split into about a million units hidden in the pancreas. In 1869 Langerhans described small, clearer areas in the pancreas that stained differently from the rest of the pancreas. He thought that these structures were lymphatic tissues. Others thought that these could be embryonic remnants. These were named "islets of Langerhans" by the French histologist Gustave-Edouard Laguesse 24 years later. He suggested that the structures formed the endocrine part of the pancreas with a possibility to produce a hormone with glucose-lowering effect (1).

The islets have an essential role in regulation of the glucose homeostasis. The glucose concentration in the plasma is kept in a narrow interval irrespective of food intake or starving situation, by a fine-tuning system where the plasma glucose-lowering hormone insulin is antagonized by glucagon. The location of the islets is advantageous, since the hormones are secreted into the portal vein enabling direct control of the hepatic function. Impaired function or destruction of the cells in the islets underlies pathogenesis of different forms of diabetes, which is a public health problem throughout the world.

In humans, islets of Langerhans are spherical clusters of cells with a diameter between ~50-250 μm (2). The total number of islets varies depending on age, body mass index, size of the pancreas, and conditions such as pregnancy (3). They are in a higher number in the tail than in the head and body of the pancreas (4). The number of islets increases as the diameter of the islets decreases (5). Most of the islets are of small diameter, i.e. ~50-100 μm. However, medium sized islets with a diameter of ~100-200 μm contribute most to the total islet volume at all ages with the exception of the newborn, where it is the opposite (5). The islets of patients who have diabetes can be very large, up to ~350 μm in diameter, because of oedema and deposition of amyloid (2).

There are three major types of cells in the islets, i.e. the α-, β-, and δ-cells. In addition, there are other minor cell types, e.g. the pancreatic polypeptide-secreting (PP)-cells, the ε-cells, and the dendritic cells. Most of the cells (70-80%) in the adult human islets are insulin-secreting β-cells. Among the remaining are 15-20% glucagon-secreting α-cells, 5-10% δ-cells, 1% ε-cells (6), and 1% PP-cells. δ-cells secrete somatostatin and possibly gastrin. ε-cells secrete ghrelin, which stimulates growth hormone release and apetite (7). In each islet there are 5-20 dendritic cells, which express class II antigen with phagocytotic capacity (8). In addition to insulin, the β-cells secrete islet amyloid polypeptide (IAPP). However, all β-cells do not secrete IAPP since only 54% of β-cells stain for IAPP (2).

The islets coordinate their work even though they are structurally separated. A β-cell communicates with another through paracrine mechanisms or via a local vascular system within the islet. There is electrical synchronization between β-cells through gap junctions. Also, the β-cells communicate with non-β-cells via gap junctions. The gap junctions are made of connexin36, which is important for the oscillation of insulin secretion (9;10).

Today, there are about 285 million people in the world with overt islet failure (11). By the year of 2030, the number is likely to increase to 438 million. A gradual decrease in function of the islets takes place over years, and it is not until as much as 90% of the islets have stopped to function or are destroyed that any decline in health is noticed.

5.2 Insulin secretion

When studying β-cells, it is common to assume that they have a resting state when they do not secret insulin, and a stimulated state when they do. However, under physiological conditions, large insulin secretion occurs even under the fasting state, and secretion increases after food intake. In human, about 75% of the insulin secretion occurs in the form of oscillations with an interpulse interval of about five minutes (12;13). The pulsatile pattern of insulin secretion, which has many physiological advantages, is lost in patients with type 2 diabetes. The insulin secretion is regulated by the amplitude rather than the frequency of insulin oscillation. The synchronization signals for insulin secretion from a large number of islets are unclear, but neural networks are thought to be important in this process.

After a meal, the concentrations of nutrients including glucose, amino acids, and free fatty acids in the plasma increase, and the amplitude of insulin pulses increases. To trigger insulin secretion, glucose needs to be metabolized by glucokinase. Some mutations in the glucokinase gene can cause maturity onset diabetes of the young (MODY) (14). Metabolism of pyruvate and ATP production in the mitochondria are essential for glucose-stimulated insulin secretion. Several other factors generated from the mitochondria also potentiate insulin secretion. Some uncommon forms of diabetes are due to mutations or deletions in mitochondrial DNA.

A $[Ca^{2+}]_i$ increase is an essential trigger for insulin exocytosis. Insulin secretion is also regulated by neurotransmitters, and incretin hormones secreted from the gut. Glucagon like peptide 1 (GLP-1) is one important incretin hormone that augments insulin secretion, somatostatin secretion, and inhibits glucagon secretion. Furthermore, it promotes β-cell survival and proliferation. These actions of GLP-1 are mediated by Ca^{2+} as well as cyclic AMP (cAMP), and other signaling pathways. Thus, the insulin secretion is a highly controlled process that involves multiple nutrients, neurotransmitters, and hormones.

5.3 Ca^{2+} signaling in the β-cells

The calcium of importance for intracellular signaling is the ionized form of calcium, Ca^{2+}, inside the cell. Changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) induces signals for various cellular processes. Many Ca^{2+}-binding proteins, membranes, channels, pumps, stores, and other organelles are involved in the generation, and shaping of the Ca^{2+} signals. The Ca^{2+} signals in the β-cells control exocytosis of insulin. In a "resting" β-cell in vitro, the $[Ca^{2+}]_i$ is ~ 20-100 nM, and outside the cells the Ca^{2+} concentration is 10 000 times higher.

To avoid toxicity, the $[Ca^{2+}]_i$ must return to a resting level, and this is achieved by the plasma membrane Ca^{2+} ATPases (PMCA) that pumps out Ca^{2+} from the cytoplasm. Also, there are Na^+/Ca^{2+} exchangers for lowering $[Ca^{2+}]_i$. The ATP-driven pumps have high affinity but low capacity, compared to the exchangers that take care of the large Ca^{2+} loads. In Ca^{2+} signaling the $[Ca^{2+}]_i$ increases, and returns to the resting level shortly after. The $[Ca^{2+}]_i$ increase is likened to pressing the on-button, and a decrease in $[Ca^{2+}]_i$ means that the off-button is pressed.

Other molecules that are involved in Ca^{2+} signaling include Ca^{2+} binding proteins, Ca^{2+} channels, Ca^{2+} mobilizing messengers, and Ca^{2+}-sensing molecules. Calmodulin is a Ca^{2+} binding protein present in almost all cells. It contains a single polypeptide chain of 150 amino acids with four Ca^{2+} binding sites. Calmodulin constitutes about 1% of the total protein mass of the β-cells. Besides $[Ca^{2+}]_i$ regulation, calmodulin mediates many Ca^{2+} regulated processes in the cell, and works as a multipurpose intracellular Ca^{2+} receptor. The binding of Ca^{2+} enables calmodulin to bind to various target proteins, and alter their activity. Ca^{2+}/calmodulin binds to, and activates the PMCA that pumps Ca^{2+} out of the cell. Most effects of Ca^{2+}/calmodulin are mediated by the Ca^{2+}/calmodulin-dependent kinases.

In the plasma membrane there are different Ca^{2+} channels: voltage-gated, receptor-activated, and channels belonging to the "transient receptor potential" (TRP) family. Glutamate receptors and purinergic receptors of P2X type are examples of receptor-activated channels that are present in many cells. IP_3 is the most well characterized Ca^{2+} mobilizing intracellular messenger. Others are cADPr and nicotinic acid adenine dinucleotide phosphate (NAADP). Together, all the molecules involved in Ca^{2+} signaling in the β-cells orchestrate the $[Ca^{2+}]_i$ to fine-tune the insulin secretion.

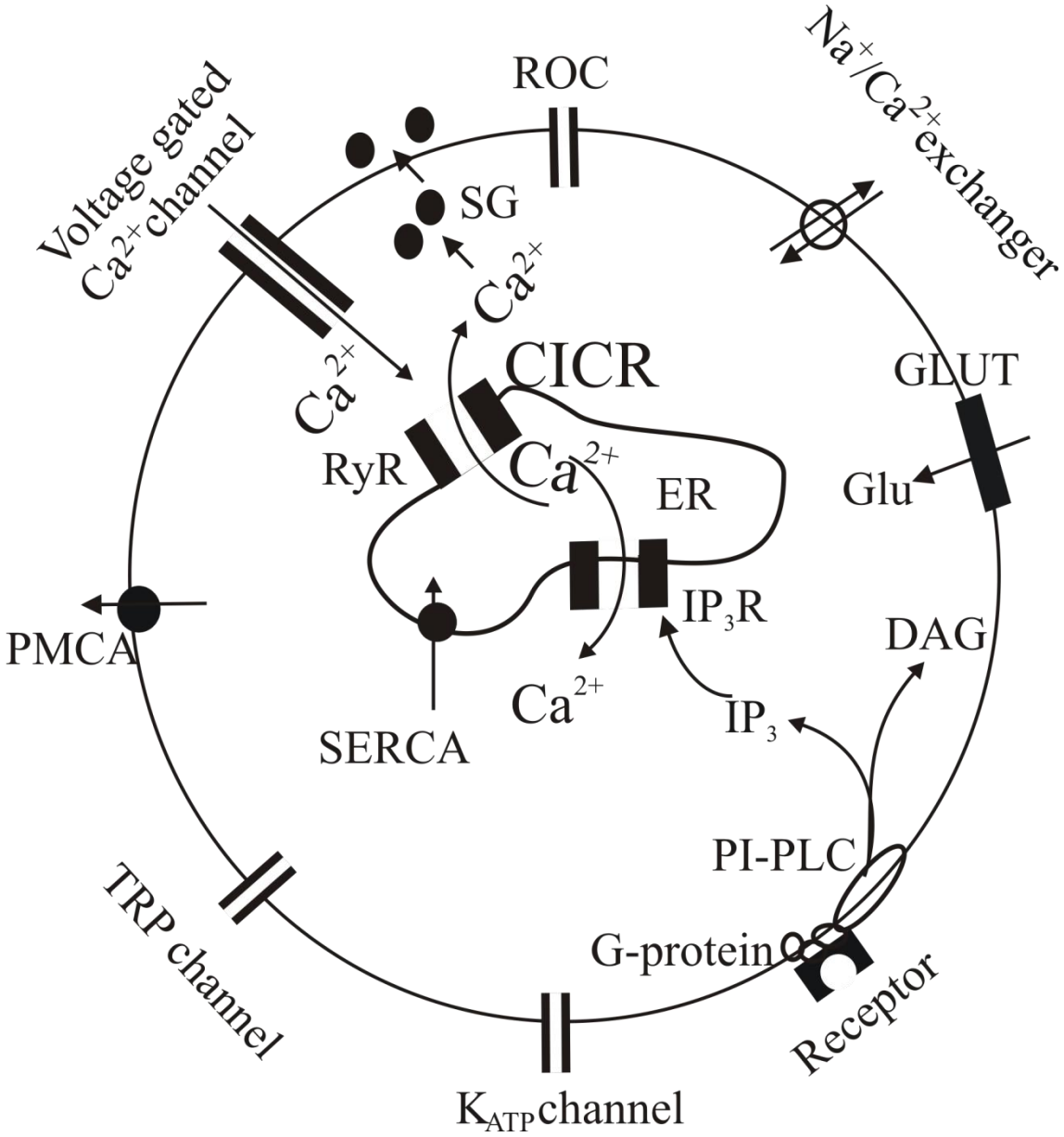

Figure 1. The figure shows some of the molecules involved in Ca^{2+} signaling in the β-cell. The figure also shows a mechanism for Ca^{2+} induced Ca^{2+} release (CICR). DAG = diacylglycerol; ER = endoplasmic reticulum; Glu = glucose; GLUT = glucose transporter; IP_3 = inositol 1,4,5-trisphosphate; IP_3R = inositol 1,4,5-trisphosphate receptor; RyR = ryanodine receptor; SG = secretory granulae; PI-PLC = phosphatidyl inositol specific phospholipase C; PMCA = plasmamembrane Ca^{2+}ATPase; ROC = receptor activated channel; SERCA = sacro(endo)plasmic reticulum Ca^{2+}ATPase.

5.3.1 Ca^{2+} oscillations

The $[Ca^{2+}]_i$ increase often takes place in the form of oscillations. Low concentration of an agonist leads to a low frequency, whereas a higher concentration leads to a higher frequency of the oscillations. The advantage of Ca^{2+} oscillations compared to continuously increased $[Ca^{2+}]_i$ is that the cells are not damaged by Ca^{2+} when the $[Ca^{2+}]_i$ oscillates. There is also a less likelihood of desensitization of the intracellular Ca^{2+} sensors. The β-cells interpret the Ca^{2+} signals by the degree of $[Ca^{2+}]_i$ increase or the frequency of Ca^{2+} oscillations. It has been shown that Ca^{2+} oscillations increase the efficiency, and the information content of Ca^{2+} signals that lead gene expression. In β-cells, at least three different types of Ca^{2+} oscillation have been described (15). The mechanism involved in the formation and decoding of Ca^{2+} oscillations is an active research field. Perturbed oscillations may be a cause for impaired insulin release that is normally pulsatile possibly because of the Ca^{2+} oscillations (16).

5.4 Stimulus-secretion coupling in the β-cells

The main triggers for insulin secretion from the β-cells are nutrient-induced $[Ca^{2+}]_i$ increases. Glucose is transported into the β-cell through a facilitative glucose transporter (GLUT1 and 3 in humans, and GLUT 2 in rodents) (17;18). Glycolysis, and metabolism in the mitochondria increases ATP/ADP ratio (19;20). The cytoplasmic ATP/ADP ratio acts as intracellular messenger that couples nutrient metabolism to electrical activity of β-cells. In this respect, the ATP-sensitive potassium channel (K_{ATP} channel) acts as a sensor of cellular metabolism. K_{ATP} channels of β-cells consist of two subunits, the channel subunit KIR6.2, and the sulfonylurea receptor SUR1. These channels are inhibited by, and are targets for the insulin-lowering sulfonylurea drugs (21). The K_{ATP} channels can be activated by agents such as MgADP and diazoxide, by involvement of the two nucleotide binding folds (NBF) 1, and 2 of SUR1 (22). This leads to hyperpolarization of the plasma membrane. When plasma glucose concentrations are reduced, a decreased ATP/ADP ratio leads to opening of the K_{ATP} channels, and causes repolarization. In this way, insulin secretion, and hypoglycaemia is prevented. Closure of the K_{ATP} channels is an initial signaling event leading to membrane depolarization. It should be emphasized, however, that closure of the K_{ATP} channels alone is not sufficient to depolarize the cell. That needs a co-existing inward depolarizing current. These depolarizing currents through as yet unknown channels depolarize the plasma membrane when the K_{ATP} channels are closed. It has been suggested that some TRP channels may account for these currents.

5.5 Transient receptor potential channels

The TRP channels were discovered in the photoreceptor cells of blind fruit flies (23). The light-induced change of membrane potential in these cells was transient rather than sustained. This was due to a mutation of a channel, thus called the transient receptor potential channel. There are 28 (27 in human) TRP channels, and one or other TRP channels are present in almost all cells. They are diverse when it comes to the regulation and function. The TRP channels are tetrameric ion channels that may form both homo-

and heterotetramers, and this gives possibilities for formation of many different channels. TRP channels mediate many sensory functions. The channels are divided into two groups according to their molecular similarities. Group 1 has five subfamilies. There are seven TRP channels related to the classical or canonical channel (TRPC). These channels are the most related to the original TRP channels. There are six TRP channels related to the vanilloid receptor (TRPV), and eight TRP channels related to the melastatin subfamily (TRPM). There are also TRPA channels, with many ankyrin repeats, and TRPN channels (24).

Group 2 TRP channels consist of two subfamilies: TRPP and TRPML. Mutations in the TRPP channels cause autosomal dominant polycystic kidney disease. Mutation in TRPML causes the neurodegenerative disorder mucolipidosis type IV.

We studied the mechanisms of RyR-activated membrane depolarization. TRP channels are known to mediate membrane depolarization in many cells (25;26). Therefore, we studied whether TRP channels were involved in the RyR-activated membrane depolarization in the β-cells.

5.5.1 TRP channels in the β-cells

At the beginning of this thesis, there was scanty information in the literature about TRP channels in the β-cells. During subsequent years, research from many groups has shown that many TRP channels are present in the β-cells. These are TRPC1-6 (27-29), TRPM2-5 (30-34), and TRPV1, 2, and 4 (35-37). Two of these have been dealt with in this thesis, and these will be discussed further. It is possible that some of the TRP channels mediate the inward depolarizing currents in the β-cells. The depolarization leads to activation of voltage-gated Ca^{2+} channels, and influx of Ca^{2+}.

5.5.1.1 TRPM2 channels

The type 2 melastatin-like transient receptor potential (TRPM2) is a chanzyme, forming a non-selective cation channel permeable to Na^+, K^+, and Ca^{2+} (38). The C-terminal of TRPM2 has an ADPr pyrophosphatase domain (38;39). TRPM2 expression is highest in the brain, but several peripheral cell types also express TRPM2 (40).TRPM2 functions as a cellular redox sensor, and TRPM2 activation leads to apoptosis and cell death (41;42).

TRPM2 is also activated by ADP ribose, NAD^+, nitric oxide, arachidonic acid, temperatures >35 ºC and Ca^{2+}. TRPM2 can be activated by Ca^{2+} released from the intracellular stores (43). *N*-(p-amylcinnamoyl) anthranilic acid (ACA) is an inhibitor of TRPM2, but it is not so specific. Flufenamic acid, the antifungal agents miconazole and clotrimazole are also inhibitors of TRPM2 (44;45). Whether 2-APB inhibits TRPM2 is controversial (46;47) Our group has shown that in the human islets, there are at least two main isoforms of TRPM2 channels: one is the full-length form (TRPM2-L) and the other is a nonfunctional form because of C-terminal truncation (TRPM2-S) (48). TRPM2 is mainly located in the plasma membrane and allows Ca^{2+} entry. However, TRPM2 is also located on the lysosomal membranes Activation of TRPM2 releases Ca^{2+} from the lysosomes (49). We have shown that functional TRPM2 channels are present in the INS-1E cells and the human β-cells (fig. 2) (30).

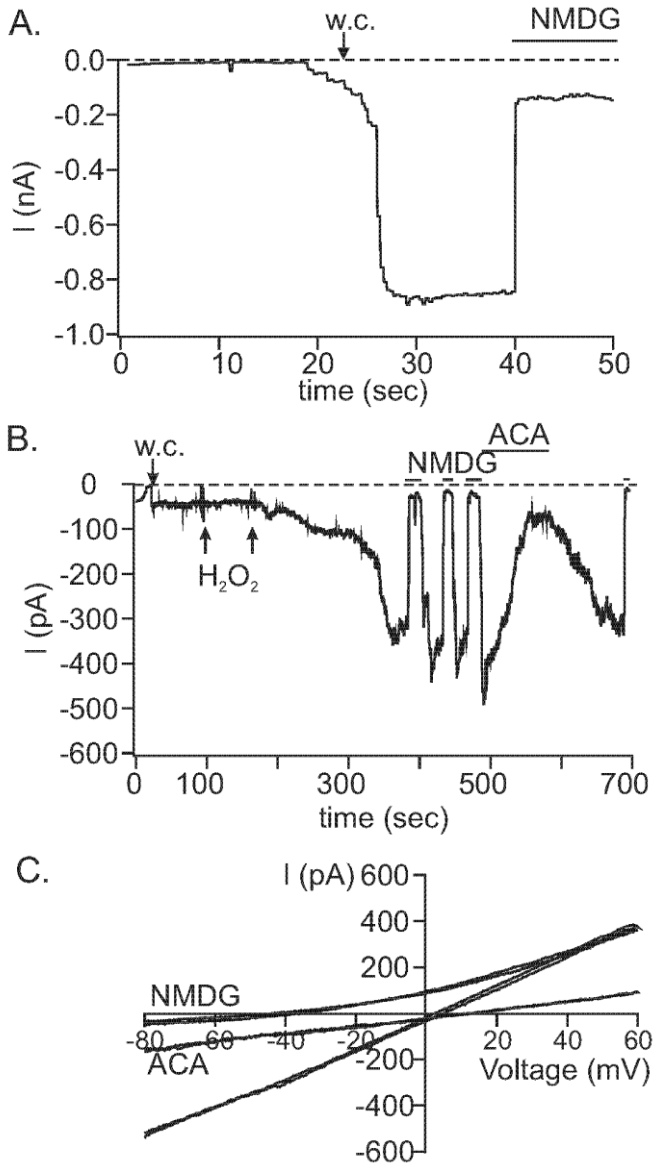

Figure 2. Whole-cell currents induced by ADPr and H_2O_2 in INS-1E cells. The figure is reproduced from Bari *et al* 2009 with permission. The whole-cell configuration was attained at the point indicated with "w.c.". Recordings were performed at room temperature and the holding potential was -60 mV. Bars indicate times where the standard bath solution was changed to a solution containing ACA, a TRPM2 inhibitor, or N-methyl-D-glucamine ($NMDG^+$), which is impermeable to TRPM2. Whole-cell current was recorded in the presence of intracellular ADPr. The pipette solution contained 0.6 mM ADPr and 1μM Ca^{2+} (A). Whole cell currents recorded without ADPr and after application of 1-2 μl 30% H_2O_2 directly into the recording chamber. The estimated final concentration of H_2O_2 in the chamber was ~10 mM. The pipette solution contained 1 μM free Ca^{2+} (B). Current-voltage relationship of H_2O_2-induced currents as derived from (B), recorded during voltage ramps from -90 to +60 mV of 400 ms duration (C).

5.5.1.2 TRPV1 channels

TRPV1 is a non-selective cation channel that mediates peripheral nociception and pain sensation. It is abundant in the trigeminal and the dorsal root ganglia. TRPV1 positive afferent neurons have been claimed to play a critical role in local islet inflammation in autoimmune diabetes pathoetiology (50). One group has reported that TRPV1 protein is expressed in the rat insulinoma cell lines RIN and INS-1 (35). Whether TRPV1 exists in the primary β-cells remains controversial. TRPV1 immunoreactivity has been described in primary β-cells of Sprague-Dawley rats by one group (35), but not in those of Zucker diabetic rats (51) or NOD mice (50). It is not known whether TRPV1 is present in the human β-cells.

Capsaicin, resiniferatoxin, temperature >43 ºC and low pH are some of the activators of TRPV1 (52). Capsaicin is the pungent component of chili pepper. It produces burning pain, desensitisation and degeneration of a specific subset of sensory fibres that are also sensitive to chemical irritants and noxious heat. This explains the burning sensation of chili pepper intake. Due to desensitization of nociceptive terminals, capsaicin also exhibits analgesic properties. Capsaicin is lipophilic and binds to the intracellular part of TRPV1 and thereby activates the TRPV1 channel (53;54).

TRPV1 is expressed in nerve fibres in the islets of Langerhans of rats and mice (50;51). Akiba *et al* have reported that TRPV1 protein is expressed in the rat insulinoma cell lines RIN and INS-1, and that insulin secretion is increased by capsaicin in the RIN cells (35). We have studied whether TRPV1 activation leads to $[Ca^{2+}]_i$ increase or induces currents in the β-cells.

We have used capsaicin and AM404 as agonists of TRPV1. The active metabolite of paracetamol, AM404 activates TRPV1 at analgesic doses of paracetamol (55;56). After ingestion, paracetamol is metabolized into, among others, *p*-aminophenol. AM404 is formed by conjugation of *p*-aminophenol and arachidonic acid. AM404 is formed in the brain by the action of fatty acid amide hydrolase (FAAH) (57). The TRPV1 antagonist capsazepine is a synthetic analogue that competitively inhibits capsaicin binding (58). It also blocks TRPV1 activation induced by low pH (59). We used capsaicin, AM404 and capsazepine as tools for identifying the TRPV1 channel in the β-cells.

It is not fully established whether TRPV1 exists in the primary β-cells. Therefore, we studied the effect of capsaicin on $[Ca^{2+}]_i$ in primary rat and human primary β-cells. Immunohistochemistry was used to study the expression of TRPV1 protein in the human islet cells and the human insulinoma cells.

5.6 The role of the endoplasmic reticulum in Ca^{2+} signaling

Like many other cells,the β-cells have several Ca^{2+} stores. Among these, the endoplasmic reticulum (ER) is the best characterized. The ER is best known for its role in the protein synthesis, but it is also a sophisticated instrument for Ca^{2+} signaling. The Ca^{2+} concentration in the ER of resting β-cells is high, about 250 µM. On the ER membranes,

there are Ca^{2+} channels and Ca^{2+} pumps that regulate the luminal $[Ca^{2+}]_i$. ER is filled with Ca^{2+} by sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA). There is a large amount of Ca^{2+} binding proteins in the ER. Calsequestrin is one such Ca^{2+} binding protein in the ER lumen. It has a high capacity and low affinity for binding Ca^{2+}. Thus, the ER has many important players that regulate the $[Ca^{2+}]_i$ inside the lumen and release Ca^{2+} in response to various signals.

5.7 Ca^{2+} channels in the ER

5.7.1 Activation of RyRs and IP_3Rs

There are two main families of Ca^{2+} channels in the ER: the inositol 1,4,5-trisphosphate receptors (IP_3Rs), and the RyRs. The latter name is derived from the plant alkaloid ryanodine, which binds to the receptor with nanomolar affinity, and activates the channel. Whereas submicromolar concentrations of ryanodine lock the channel in a long-lived open state, micromolar concentrations inhibit the channel. Insulin secretion is stimulated by low concentrations (~1 nM) of ryanodine (60). While IP_3 activates the IP_3 receptor, the ryanodine receptor is activated by several mechanisms. Fructose 1,6 diphosphate, arachidonic acid, cyclic adenosine diphosphate ribose (cADPr), long chain Acyl CoA, and ATP are some of the activators or positive modulators of RyRs (61-63). Caffeine is a widely used pharmacological activator of RyRs (64). But caffeine has many non-specific effects. Our group has shown that caffeine inhibits the K_{ATP} channels, elevates the cAMP concentration, and inhibits the L-type voltage-gated Ca^{2+} channels (65). Caffeine also inhibits store-operated Ca^{2+} entry (66).

5.7.2 MBED

Instead of caffeine, we have used 9-methyl 5,7-dibromoeudistomin D (MBED) as a RyR activator. MBED is derived from the natural product eudistomin D, isolated from the marine tunicate *Eudistoma olivaceum* (67). MBED has caffeine-like properties, but it is a more specific, and more potent activator of RyRs, and is thus more suitable for mechanistic studies of these channels (68). It has been suggested that MBED binds to a different site than ryanodine on the RyRs, since MBED does not inhibit ryanodine binding to the receptor (68). The effects of MBED on RyRs have been known for about 20 years, and so far no non-specific effect has been reported. We have reported that MBED does not inhibit cAMP-phosphodiesterases, IP_3Rs, voltage-gated Ca^{2+} channels or K_{ATP} channels in the β-cells (69).

5.7.3 Isoforms of RyRs and IP_3Rs

Both IP_3Rs and RyRs are present in many cells, and are regulated by positive feedback, whereby the released Ca^{2+} can bind to the channel, and increase the Ca^{2+} release. There are three isoforms of both channels. The rat insulinoma cell line INS-1 express mRNA for IP_3R1, IP_3R2, and IP_3R3, and IP_3R1 is in abundance (70). mRNA for all the three

isoforms is also found in rat pancreatic islets, rat insulinoma RINm5F cells, and mouse insulinoma βHC9 cells, but in these cells IP_3R1 is in greater abundance (71).
RyR1 and RyR2 are mainly expressed in the skeletal muscles and heart, respectively, while the RyR3 is expressed in the brain, the smooth muscles and the epithelial cells (72). All the three isoforms of RyRs, i.e. RyR1, RyR2, and RyR3, are present in human islets (73). RyR2, but not RyR1 has been detected by RT-PCR in INS-1 cells, and rat islets (29). The RyR2 is mainly located on the ER/SR membranes (74). One group has shown that the RyR2 is also expressed on the plasma membrane in the β-cells (75).

5.8 Store-operated Ca^{2+} entry

Store-operated Ca^{2+} entry (SOCE), also called capacitative Ca^{2+} entry, is a process whereby the Ca^{2+} entry across the plasma membrane is closely coordinated with the depletion of ER Ca^{2+} stores (76). It is conserved from lower organisms such as yeast, worms, and flies to human. SOCE has been described in β-cells (77). In β-cells, as in many other cells, an important molecule involved in SOCE is the stromal interaction molecule 1 (STIM1) that acts as the Ca^{2+} sensor in the ER. Mammals also have a related gene that encodes STIM2. STIM2 is also a Ca^{2+} sensor, but with a different sensitivity for the ER Ca^{2+} concentration than STIM1 (78). An intraluminal EF-hand domain of STIM1 senses the Ca^{2+} concentration in the ER lumen. STIM1 is transported to the plasma membrane upon ER Ca^{2+} pool depletion. Orai1, also called CRACM1, is the pore-forming subunit of a store-operated Ca^{2+} channel in the plasma membrane. This channel conducts a highly Ca^{2+}-selective, non voltage-gated, inwardly rectifying current, called Ca^{2+} release activated Ca^{2+} current (I_{CRAC}) (79). According to one report, I_{CRAC} is inhibited when the RyRs are inhibited (80). It is unknown whether STIM1 interacts with Orai1 in the β-cells. Interaction between STIM1, Orai1, and TRPC might be of importance in SOCE (81). One study has shown a connection between the IP_3Rs and some TRP channels in the plasma membrane (82). Thus, multiple mechanisms may underlie different forms of SOCE.

5.9 Voltage-gated Ca^{2+} channels

In β-cells, the most important Ca^{2+} channels are the ones that are activated upon plasma membrane depolarization. Ca^{2+} entry through voltage-gated Ca^{2+} channels triggers exocytosis of insulin (83). There are ten voltage-gated Ca^{2+} channels coded by three gene families: The Ca_v1 family has electrical properties of L-type, i.e. they require high voltage for activation, and are open for a longer period ("Large and Long"). These channels are inhibited by dihydropyridine antagonists. Glucose-induced insulin release is inhibited to 80-100% by dihydropyridine antagonists (84;85). The main form of L-type voltage-gated Ca^{2+} channels in the β-cells is $Ca_v1.3$ (α_{1D}). It is activated at a lower membrane potential (~-55mV) compared to $Ca_v1.2$ (α_{1C}).

The Ca_v2 family mediates currents of N-, P/Q- or R-type. The P/Q-type Ca^{2+} channels are also coupled to insulin secretion, and account for 45% of integrated whole-cell Ca^{2+} current in human β-cells. R-type Ca^{2+} channels are not present in human β-cells (85), but

may be involved in insulin secretion through central neurons or GLP-1-producing L-cells in the gut (86). There is also the Ca_v3 family of ion channels that is activated by low voltage and have electrical properties of T-type, with smaller and shorter lasting currents ("tiny and transient"). The T-type current in human β-cells is mediated by $Ca_v3.2$ (α_{1G}), and is involved in insulin release induced by 6 mM but not by 20 mM glucose (85). Neither Ca_v2 nor Ca_v3 are blocked by dihydropyridine antagonists. We have shown that L-type voltage-gated Ca^{2+} channels are activated by membrane depolarization after RyR activated Ca^{2+} entry.

5.10 Ca^{2+}-induced Ca^{2+} release

Ca^{2+}-induced Ca^{2+} release (CICR) is an intracellular signaling phenomenon, where a $[Ca^{2+}]_i$ increase triggers Ca^{2+} release from the ER. CICR was first described in the heart muscle cells, where a small Ca^{2+} entry trough the L-type voltage-gated Ca^{2+} channels caused a large Ca^{2+} release from the SR. This phenomenon takes place in many excitable cells, e.g. muscle cells, nerve cells, and the β-cells. CICR induces synchronous, transient rises in the $[Ca^{2+}]_i$ that amplifies the Ca^{2+} signals. Both IP_3Rs and RyRs are Ca^{2+}-gated Ca^{2+} channels in the ER (87). In the β-cells, activation of RyRs or IP_3Rs amplifies Ca^{2+}-dependent exocytosis of insulin by CICR (88;89). GLP-1, a blood-glucose-lowering incretin hormone, increase the cAMP in the cytoplasm of the β-cells, and facilitates CICR by cAMP-dependent phosphorylation of the RyRs (90;91). CICR is also stimulated by activation of RyRs by cAMP-regulated guanine nucleotide exchange factors (Epac) in the human β-cells (92).

5.11 Pyridine nucleotide-derived molecules and Ca^{2+} signaling

Several reports have demonstrated that glucose elevation increases cyclic ADPr (cADPr) and NAADP concentration in the β-cells. cADPr is known to stimulate insulin secretion in β-cells by Ca^{2+} release from the intracellular Ca^{2+} stores, and has also been shown to activate the TRPM2 channels (31). NAADP releases Ca^{2+} from acidic Ca^{2+} stores, and from insulin secretory vesicles (74). A group of voltage-gated ion channels called two-pore channels (TPCs) are located on the lysosomal membranes, and are activated by nanomolar concentrations of NAADP, while micromolar concentrations of NAADP inhibit them (93).

ADPr is formed from β-NAD^+, and NAADP is formed from $NADP^+$ by ADP ribosyl cyclases, including CD38 (94). CD38 and its homologues have NADase, ADP-ribosyl cyclase, and cADPr hydrolase activities (95). ADPr constitutes more than 99% of the products produced by the action of CD38 (96-98). ADPr is also produced by hydrolysis of cADPr, and from NAD^+ by NAD glycohydrolases, (95). Furthermore, poly (ADPr) glycohydrolase can produce ADPr from poly (ADPr) (99;100).

Since CD38 is located with its catalytic site oriented extracellularly in the plasma membrane (101;102), ADPr produced by CD38 and related enzymes is likely to be released extracellularly. Extracellular release of ADPr has been shown in cortical

astrocytes (103). Synaptosomes have been reported to have NADase activity, giving rise to speculations that ADPr could be a neurotransmitter (104). ADPr is shown to be released during nerve stimulation (105).

CD38 and related enzymes are also present in the β-cells, and they are thought to play some roles in mediating insulin secretion (106). The role of CD38 in insulin secretion is generally attributed to RyR activation by cADPr and NAADP (107). It remains unclear whether extracellular ADPr can signal by acting on cell surface receptors or whether it must enter into the cell. The entrance of ADPr is thought to be via CD38, but the transport rate is slow, and this mechanism is not universal (108;109). Ecto-nucleotide pyrophosphatases degrade ADPr to AMP (110;111). The conversion of ADPr to AMP can also be catalysed by apyrase, and AMP is further metabolized to adenosine by 5´nucleotidase (112;113). Extracellular ADPr is thus a well-suited nucleotide for signaling by activating cell surface receptors. Our studies show that ADPr increase $[Ca^{2+}]_i$ by activation of purinergic receptors of type P2Y1 in the INS-1E cells as well as in the rat and human β-cells.

5.12 Purinergic receptors

Receptors for purine nucleotides and nucleosides are present in numerous tissues. The purinoceptors are classified into P1, which are more specific for adenosine and AMP than for ADP and ATP. The adenosine/P1 purinoceptors are in turn divided into A1, A2a, A2b, and A3 (114). The A1 and A3 subtypes inhibit adenylate cyclase, while the A2 subtypes activates adenylate cyclase (115;116).

The P2 purinoceptors are, in contrast, more specific for ATP and ADP than for adenosine and AMP. They are divided into P2X and P2Y subtypes, which can be discriminated by their response profiles to different ATP-analogues (117). P2X receptors are intrinsic ion channels (not G-protein coupled) permeable to Na^{2+}, K^{+}, and Ca^{2+} (118). P2X receptors in the β-cells are of subtype P2X1, P2X3, P2X4, P2X6, and P2X7 (119-122).

There are eight human P2Y receptors: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 (123-125). The missing numbers in the sequence are receptors that are cloned from non-mammalian vertebrates or receptors under characterization. The P2 receptor subtypes in β-cells are P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12 (126). The P2Y1 receptor has been shown to be involved in insulin secretion, but both stimulation of insulin secretion and inhibition of secretion have been reported (127). Some studies have claimed that P2Y purinoceptors can constitute new targets for antidiabetic drugs (128;129). P2Y receptors are G-protein coupled, and often activate the PI-PLC pathway leading to IP_3 production (130).

5.13 The signaling enzyme PI-PLC

Phosphatidylinositol specific phospholipase C (PI-PLC) constitutes a family of key enzymes in the Ca^{2+} signaling. There are eleven isoforms of PI-PLC, and they are divided into four families: β, γ, δ, and ε. G-protein coupled receptors activate PI-PLCβ, and receptor protein-tyrosine kinases activate PI-PLCγ (131). Thus, there are many growth factors that activate PI-PLCγ. PI-PLCδ is activated by Ca^{2+}, and PI-PLCε is activated by GTP-Ras (132). PI-PLCε is involved in activation of the GLP-1-receptor-induced facilitation of CICR (133).

The PI-PLC enzymes cleave phosphatidyl inositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a second messenger that binds to the IP_3 receptor in the ER, and triggers release of Ca^{2+} into the cytoplasm. DAG has two signaling roles: it can be cleaved to release arachidonic acid that either works as a messenger in its own right, or is used in the synthesis of eicosanoids, such as prostaglandins, prostacyclins, tromboxanes, and leukotrienes. DAG activates protein kinase C (PKC), and the activation is usually Ca^{2+} dependent (134;135). When the $[Ca^{2+}]_i$ increases, PKC translocates from the cytosol to the cytoplasmic face of the plasma membrane, where it is activated by Ca^{2+}, DAG, and negatively charged membrane phospholipids such as phosphatidylserine.

6 Aims of the thesis

The aims of this thesis were to study:

1. the consequences of activation of RyRs on $[Ca^{2+}]_i$ in pancreatic β-cells.

2. whether functional Ca^{2+} permeable TRP-like channels operate in the β-cells.

3. whether activation of the RyRs leads to the activation of plasma membrane ion channels, and depolarization of the membrane potential.

4. the molecular mechanisms by which ADPr increases $[Ca^{2+}]_i$ in the insulin-secreting cells.

5. whether functional TRPV1 channels are present in the insulin-secreting cells.

7 Methods

7.1 Cells

In most of the experiments, we used the rat insulinoma cell line INS-1E (subclone S5). INS-1E cells are widely used as a model for β-cells. The S5 cells were derived from INS-1E cells in our laboratory, and they differ from the INS-1E cells in that they are adjusted to grow in 2.5% FBS while they require a higher concentration of β-mercaptoethanol. The advantage of using insulinoma cell lines instead of primary β-cells is that the cell lines consist of pure insulin-secreting cells, whereas cells prepared from islets contain a mixture of cells, which cannot be easily identified under microscope. For the experiments, cells of round shape that looked like differentiated β-cells were chosen. Such cells constitute only about 10-20% of the cells in the microscope field. The handling of the cells is described in the methods section of each paper.

The use of primary β-cells and human islets for experiments was approved by local ethics committee. Primary β-cells were prepared from the Wistar rat islets. Primary β-cells from CD1 mice of 12-16 weeks of age were used for insulin secretion studies. The procedures for isolation of islets and preparation of β-cells are described in the attached papers. Human islets were obtained from islet transplantation programmes, and single cells were prepared as described in the papers. In paper II, we used 1321N1 human astrocytoma cells that stably overexpress human recombinant P2Y1 receptors, and wild type (WT) astrocytoma cells that do not express any P2Y1 receptors.

7.2 Chemical tools used

Compound	**Effects**	**Side effects**	**Used concentration**	**Paper**
ADP	Activates purinergic receptors		30 µM	II
ADP ribose (ADPr)	Activates TRPM2		30 µM	II
2-aminoethoxy-diphenyl borate (2-APB)	Inhibits I_{CRAC} and activates TRPV1-3 Inhibits IP_3R and some TRP channels, including TRPC1,3-6	Inhibits SERCA (136)	30 µM	I
Arachidonic acid	Activates many TRP channels and RyRs		5 µM	III

8-Bromo-ADPr	ADPr antagonist		30 µM	II
cADPr	Activates RyR (?)	Activates TRPM2	30 µM	II
Capsaicin	Activates TRPV1		300 nM	III
Capsazepine	Inhibits TRPV1		10 µM	III
Carbachol (cch)	Muscarinic agonist		10-100 µM	I, II, III
2-chloro N6-methyl-(N)-methanocarba-2-deoxyadenosine-3,5-bisphosphate (MRS 2279)	Inhibits P2Y1 receptor	Not reported	10 µM	II
2´Deoxy-N6-methyladenosine 3,5-bisphosphate (MRS 2179)	Inhibits P2Y1 receptor	Inhibits P2X1 receptor (137)	1-10 µM	II
Diazoxide	Opens K_{ATP} channels		100 µM	I
Gadolinium chloride ($GdCl_3$)	Blocks several TRP channels, including TRPC1,3,6, TRPM3,4, TRPV4, TRPP1, TRPML1	Inhibits voltage-gated Ca^{2+} channels and stretch-activated channels (138;139)	10 µM	I
Lanthanium chloride ($LaCl_3$)	Blocks several TRP channels, including TRPC3-7, TRPV2,4-6, TPRM4,7, TRPP1, TRPML1	Activates TRPC3 and 5 in µM concentrations.	100 µM	I
9-methyl 5,7-dibromoeudistomin D (MBED)	RyR activation	Not reported	50 µM	I
Nimodipine	Blocks L-type voltage-gated Ca^{2+} channels		5 µM	I
NAD^+	Activates TRPM2 through conversion to ADPr		30 µM	II

Niflumic acid	Inhibits TRP channels, including TRPC4,6, TRPM2,3, and TRPV4 (140;141)		50 µM	I
Nimodipine	Blocks L-type voltage-gated Ca^{2+} channels		5 µM	I
N-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404)	Activates TRPV1		5 µM	III
N-(p-amylcinna-moyl) anthranilic acid (ACA)	Inhibits TRPM2	Inhibits TRPM8 and TRPC6 (142)	20 µM	II
N-propargyl-nitrendipene (MRS 1845)	Blocks SOCE		5 µM	I
O-acetyl adenosine diphosphate ribose (OAADPr)	Actylated analogue of ADPr, activates TRPM2 (143)		10 µM	II
PADPr	Stable analogue of ADPr		100 µM	II
p-aminophenol	Metabolite of paracetamol		5 µM	III
Potassium chloride (KCl)	Depolarization of plasma membrane		25 mM	I, II, III
Ruthenium red (RR)	Blocks RyRs and TRP channels, including TRPC3, TRPV1-6, TRPM3,6, and TRPA1	Many nonspecific effects	10 µM	I
Ryanodine	Activates (nM) and inhibits (µM) RyR		50 µM	I
SKF 96365	Inhibits several TRP cannels including TRPC6, 7 and TRPV2.	Inhibits voltage-gated Ca^{2+} channels and SOCE.	10 µM	I

Thapsigargin	Inhibits SERCA		125-500 nM	I, II, III
U73122	Inhibits PI-PLC		10 μM	II

7.3 Measurements of $[Ca^{2+}]_i$ by microfluorometry

It is nowadays common to use fluorescence techniques to measure $[Ca^{2+}]_i$ in single living cells in real time. The measurement is done by use of a variety of fluorescent Ca^{2+} indicators. For measurement of $[Ca^{2+}]$i, fura-2 is the most commonly used indicator. The fluorescence ratio between the free and the Ca^{2+}-bound forms of fura-2 enables one to calculate the $[Ca^{2+}]_i$. Since fura-2 is cell-impermeant, an acetoxymethyl (AM) ester is coupled to the carboxylate groups of fura-2 to enable penetration through the cell membrane. Once inside the cell, the AM-group is hydrolyzed by the intracellular esterases, and fura-2 becomes Ca^{2+}-sensitive.

The fluorescence of fura-2 at 340 nm increases about threefold and at 380 nm decreases about tenfold upon Ca^{2+}-binding. The emission maximum of fura-2 is at 510 nm. $[Ca^{2+}]_i$ is calculated from *F*340/*F*380 according to Grynkiewicz *et al* (144). R_{max} and R_{min} were determined in our studies by using external standards containing fura-2 free acid and sucrose (2 M) (145). The method is described in detail in paper I, II, and III.

7.4 Electrophysiology

Patch clamp-recordings enable measurement of the electrical potential or the electrical current across the cell membrane. It is possible to isolate currents through a specific class of channels by adjustment of the ionic composition of the extracellular and intracellular solutions, application of pharmacological inhibitors etc. There are several modes of patch clamp, depending on whether single channels or a group of channels are going to be studied.

All patch clamp experiments start in the cell-attached patch mode. A tight contact between the recording pipette and the cell is accomplished by light suction to the pipette interior. A high shunt resistance (> 1 gigaohm) is produced. This is called a giga-seal. The cell is still intact. We used the pore-forming agent amphotericin B to perforate the cell membrane. This is called perforated patch whole-cell configuration. Physical contact with the cell interior is thus established. The advantage of the perforated-patch whole cell method is that there is no washout of intracellular compounds, since the pores only allow passage of small monovalent ions but not larger molecules or ions such as Ca^{2+} (146). In this way, it is a more physiological configuration than the standard whole-cell configuration, where the membrane rupture is achieved by a pulse of negative pressure by gentle suction.

For current measurements, we used a fully automated patch clamp workstation (Port-a-patch, Nanion, Munich, Germany) equipped with an HEKA EPC 10 amplifier (HEKA, Lambrecht/Pfalz, Germany). The planar patch clamp glass chip containing a micron sized

aperture was primed by adding 5 µl of internal and external solution to the respective sides of the chip. The PatchControl software (Nanion Technologies, Munich, Germany) applied a suction protocol to automatically capture a cell, obtain a giga-seal between the glass substrate and the cellular membrane, and eventually obtain whole cell voltage clamp configuration. Details are written in the respective papers.

7.5 Measurement of insulin secretion

The use of islets from mice was approved by the local ethics committee. Islets from mice pancreas were isolated as described by Kelly *et al* (147). After 24 h incubation and recovery from the isolation procedure, the cells were dispersed by trypsin (0.25%) for 8 min to obtain single cells. Total separation of the cells was verified microscopically, and the cells were transferred to multi-well plates (2×10^5 cells/well). For attachment, the cells were incubated for 24 h in 11 mM glucose. A washing procedure repeated three times with KRBH containing 3.3 mM glucose, and 15 min of preincubation in 3.3 mM glucose preceded the stimulation. According to the different treatments tested, the wells were divided into 4 groups. Group 1 was incubated with 3.3 mM glucose, group 2 with 16.7 mM glucose, group 3 with 3.3 mM glucose and 80 µM ADPr, and group 4 was incubated with 16.7 mM glucose and 80 µM ADPr. Insulin concentration in the collected samples was measured by ELISA using a commercial kit (Crystal Chem Inc).

7.6 Whole-blood flow cytometric assays

The experiments were approved by local ethics committee. We tested blood from three individuals between the ages of 24 and 42. Venous blood was collected by venepuncture. Within 5 min of blood collection, the blood samples were processed for flow cytometric measurements. We used whole-blood flow cytometry to evaluate the effect of ADPr on platelet shape change, aggregability (fibrinogen binding), and secretion (P-selectin expression). Whole-blood flow cytometric assays of platelet P-selectin expression and fibrinogen binding have been described previously (148). Platelets were gated by their characteristic light scattering signals, and the gated cells were confirmed with fluorescein isothiocyanate (FITC) conjugated anti-CD42a (GPIX) monoclonal antibody (MAb) Beb1 (Becton Dickinson, San Jose, CA, USA). Please see details in paper II on how the platelet shape change was monitored. Platelet shape change was expressed as percentage calculated according to the following formula: % of platelet shape change=100×((platelet counts within the innergate after stimulation-platelet counts within the innergate before stimulation)/(platelet counts within the inner gate before stimulation)).

7.7 Western blot analysis

Western blot was used to study the expression of TRPV1 protein in the INS-1E cells and in the human islets. The primary anti-TRPV1 antibody used was affinity-purified rabbit polyclonal IgG antibody (BIOMOL international, U.K., BML-SA564-0050, Lot # P9604a, cat. no. SA-6564). The antibody was directed against the peptide sequence DASTRDRHATQQEEV, which represents the amino acid residues 824-838 in the C-

terminal region of the rat TRPV1. The specific blocking peptide antigen (TRPV1 blocking peptide, BIOMOL international, U.K., BML-SA564-0050, Lot #P9604a, SA-564) was used to test the specificity of the antibody. Please see detailed information of the procedures in paper III.

7.8 Immunohistochemistry

Immunohistochemistry was used to detect TRPV1 protein in the human islet cells and the human insulinoma cells. Human pancreas resection specimens were collected from the Laboratory of Pathology at the Uppsala University Hospital, Sweden. They were from surgical specimens that were stored in the biobank after approval from the local ethics committee. The samples were fixed in formalin and embedded in paraffin wax. Sections from the tissue microarray blocks were cut at 4 μm thickness and immunostained. Primary antibodies and a dextran polymer visualization system (UltraVision LP HRP polymer®, Lab Vision) were incubated for 30 min each at RT. Diaminobenzidine (Lab Vision) was used as chromogen, and slides were developed for 10 min. For details of the immunostaining procedures, please see paper III.

We used eight different antibodies that were affinity purified rabbit polyclonal IgG antibodies raised against synthetic peptides corresponding to either the C-terminus or the N-terminus of TRPV1. The antibodies were from: 1. Biosensis (cat. no. R-076-100), 2. Alomone (cat. no. ACC-03), 3. Sigma (cat. no. V2764), 4. and 5. Santa Cruz Biotechnologies (cat. no. Sc-20813 and Sc-28759), 6. and 7. Chemicon (cat. no. AB5889 and AB5370P) and 8. the human protein atlas project (HRPK2180179, not published in the Protein Atlas, yet).

7.9 Statistical analysis

The data were expressed as means ± SEM. When comparison between two groups was made, Student's unpaired t-test was used, and when comparison was made within groups paired *t*-test was used. The *p*-value was considered as significant when <0.05. The concentration-response curves were made by using Graph Pad software.

8 Results and discussion

The detailed results of experiments and discussions of their interpretation and importance are in the two published papers and one manuscript that constitute this thesis. In the following paragraphs, I shall briefly mention only the results of some of the key experiments.

8.1 RyRs operate activation of TRP-like channels

RyRs amplify Ca^{2+} signals by CICR and thereby increase insulin secretion (63;74;89;149). However, any possible role of RyRs in triggering Ca^{2+} entry through the plasma membrane remained unknown. Many groups have used caffeine to activate RyRs, but caffeine inhibits many ion channels, enzymes, and receptors. It also inhibits K_{ATP} channels, voltage-gated Ca^{2+} channels, and store operated Ca^{2+} channels (65;66). We used a more specific agonist of RyR, namely MBED, to study the consequences of RyR activation in the β-cells.

In paper I, we showed that MBED activated RyRs in the β-cells and elicited a pattern of $[Ca^{2+}]_i$ increases that could be divided into three distinct components. First, there was an initial peak, which declined to a plateau phase with regenerative spikes superimposed on the plateau (fig. 3). We found that the different phases of $[Ca^{2+}]_i$ increases were due to different underlying mechanisms. The initial peak was present even when the extracellular Ca^{2+} was omitted, but was abolished when the ER Ca^{2+} pools were depleted by thapsigargin, a specific inhibitor of the SERCA (150). Thus, the initial peak was due to a transient Ca^{2+} release from the ER caused by RyR activation. These results were in accordance with earlier studies (69;151).

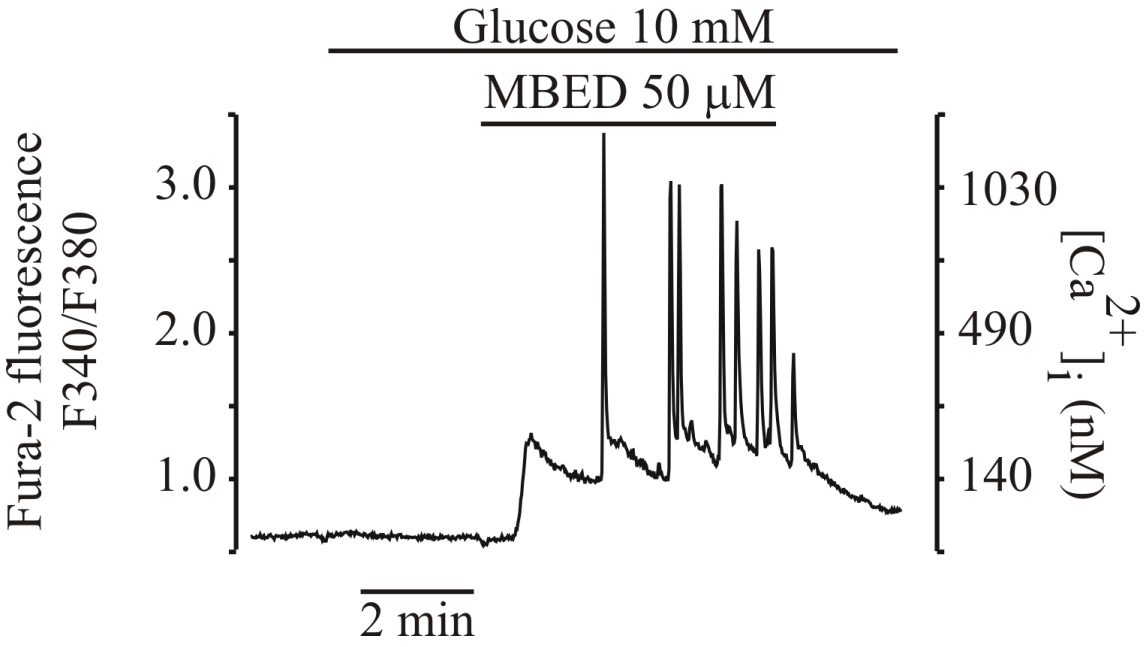

Figure 3. Activation of RyRs elicited a characteristic pattern of $[Ca^{2+}]_i$ changes. The figure is reproduced from Jabin Gustafsson *et al* 2004. MBED activated the RyRs in INS-1E cells, which resulted in a characteristic pattern of changes in $[Ca^{2+}]_i$. After addition of MBED (50μM) in the presence 10 mM glucose, there was an initial rapid rise of $[Ca^{2+}]_i$, which declined to a plateau. A series of large $[Ca^{2+}]_i$ spikes were superimposed on the $[Ca^{2+}]_i$ plateau.

The plateau phase was the most important finding in this study. It was abolished by omission of extracellular Ca^{2+}, and by SKF 96365, an inhibitor of SOCE and several TRP channels (152;153). Hence, we concluded that RyR activation also led to a prolonged $[Ca^{2+}]_i$ increase that was due to Ca^{2+} entry through some TRP-like Ca^{2+} channels in the plasma membrane. In comparison to the carbachol-induced capacitative Ca^{2+} entry (SOCE), the RyR-operated Ca^{2+} influx was much larger. When the cells were treated with thapsigargin, there was still a $[Ca^{2+}]_i$ plateau after stimulation by MBED. This suggests that the Ca^{2+} influx through the plasma membrane was not entirely dependent on the filling state of the ER. Instead, protein-protein interactions and conformational coupling could possibly be the link between activation of RyRs in the ER and the activation of Ca^{2+} channels in the plasma membrane. Such gating of the putative TRP channels by RyRs has previously been reported in other systems (154;155).

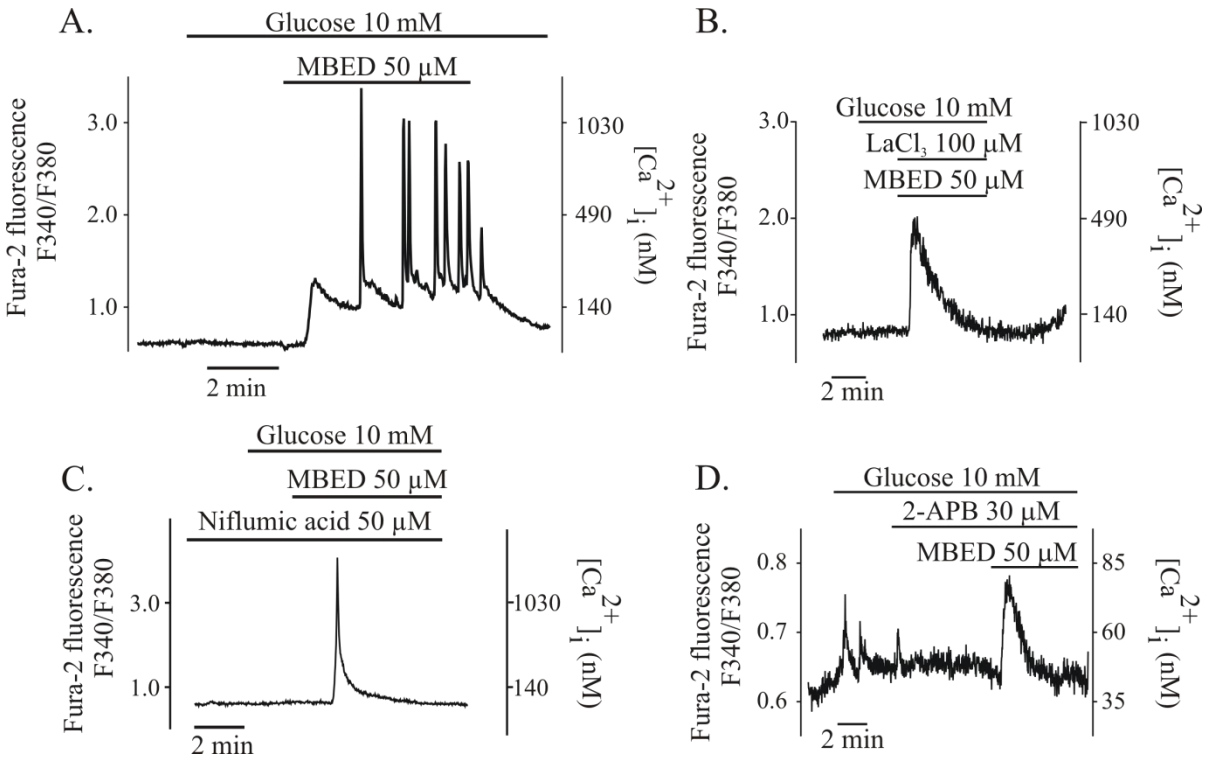

Figure 4. $LaCl_3$, niflumic acid, and 2-APB inhibited the $[Ca^{2+}]_i$ plateau that followed the activation of RyRs. The figures are reproduced from Jabin Gustafsson *et al* 2004. Activation of RyRs by MBED (50 µM) in the continued presence of $LaCl_3$ (100 µM) (B) or niflumic acid (50 µM) (C) caused the initial rise of $[Ca^{2+}]_i$, but the plateau phase of $[Ca^{2+}]_i$ increase was inhibited. The plateau phase was also inhibited by 2-APB (30 µM) (D).

More recently, Rosker *et al* have reported that the Ca^{2+} entry in the plateau phase is mediated by RyR2 located on the plasma membrane (75). However, the reported currents do not mimic any earlier reported currents of RyRs (156;157). It has been suggested that the channels described by Rosker *et al*, may represent a novel, non-selective ion-channel (158). In our study, the Ca^{2+} entry was blocked by SKF 96365, a compound that does not block RyRs. Therefore, it is unlikely that the Ca^{2+} entry was due to RyR in the plasma membrane. To rule out whether the plateau phase was due to activation of voltage-gated Ca^{2+} channels, nimodipine was used. But nimodipine did not inhibit the Ca^{2+} plateau. La^{3+}, Gd^{3+}, SKF 96365, niflumic acid, and 2-APB are non-selective inhibitors of different channels including several TRP channels (159;160), and they all inhibited the Ca^{2+} plateau. These results suggest that the plateau phase was due to activation of some Ca^{2+} channels belonging to the TRP family.

The third pattern of changes in $[Ca^{2+}]_i$ was the regenerative spikes that were superimposed on the plateau phase. Our results demonstrated that after activation of the RyR, the plasma membrane was depolarized to about -40 mV as a result of Ca^{2+} entry through the putative TRP-like channels. Such depolarization in turn activated the L-type voltage-gated Ca^{2+} channels. Since the spikes were inhibited by nimodipine, they required Ca^{2+} entry through the L-type voltage-gated channels. Also, the spikes were caused by CICR through the RyRs, as evidenced by the fact that high concentrations of ryanodine inhibited the spikes.

After the paper was published, we studied whether the plateau phase was due to the activation of TRPM2 or TRPV1, two TRP channels that we identified in the INS-1E cells. But neither ACA, a specific inhibitor of TRPM2, nor capsazepine, a specific inhibitor of TRPV1, were able to inhibit the plateau phase (data not shown). Thus, the identity of the TRP-like channels that mediate the Ca^{2+} entry in response to the activation of the RyRs remains unclear. Transcripts for several TRP channels have been found in the β-cells. Also, the possibility of different types of TRP forming homo- and heterotetramers yields many optional channels (161).

Under physiological conditions, the glucose metabolism is sensed by RyRs through molecules such as cADPr and fructose 1,6 diphosphate, among others. When the RyRs are activated, this will lead to $[Ca^{2+}]_i$ increase by activation of the putative TRP channels, membrane depolarization, and activation of L-type voltage-gated Ca^{2+} channels. The $[Ca^{2+}]_i$ increase will trigger exosytosis of insulin (fig. 5). It is of great importance that the role of TRP channels in Ca^{2+} signaling in the β-cells is elucidated and its physiological importance further investigated.

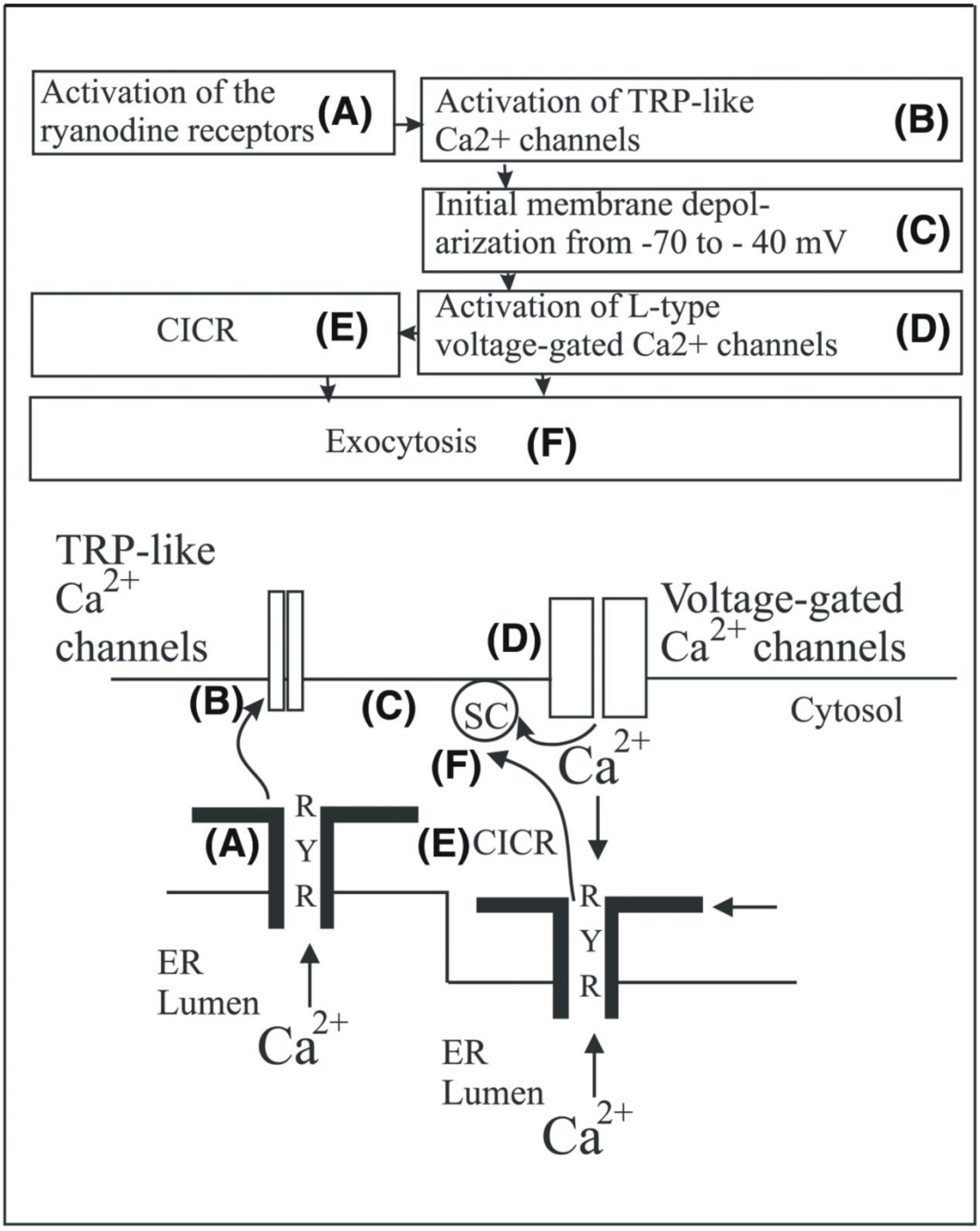

Figure 5. Schematic diagram of hypothesized involvement of RyRs and TRP-like channels in Ca^{2+} entry and membrane depolarization in β-cells. The figure is reproduced from Jabin Gustafsson *et al* 2005: The cartoon illustrates a sequence of events, whereby activation of RyRs (A) leads to the activation of TRP-like channels (B), an initial -membrane depolarization to about -40 mV (C), activation of the L-type voltage-gated Ca^{2+} channels (D), CICR (E), and exocytosis of insulin (F).

8.2 Extracellular ADPr activates P2Y1 receptors

Our study on the effect of ADPr on $[Ca^{2+}]_i$ in the β-cells was a side track from the main focus of this thesis. During our search for TRP channels in the β-cells we used ADPr as a tool to activate TRPM2, and to our surprise we found that extracellular ADPr increases $[Ca^{2+}]_i$ in the β-cells. This effect of ADPr was so obvious that we decided to identify the cell surface receptor involved in mediating the $[Ca^{2+}]_i$ response. ADPr increased $[Ca^{2+}]_i$ in a concentration-dependent manner (EC_{50} of ~ 30 μM). The $[Ca^{2+}]_i$ increase was observed in the INS-1E cells, as well as in the primary rat and human β-cells. Our first suspicion was that commercially available ADPr might contain ADP as a contaminant, which could elicit the observed $[Ca^{2+}]_i$ increase. Therefore, we synthesized highly purified ADPr that was free from ADP, but still similar $[Ca^{2+}]_i$ increase by ADPr was observed. The concentration of ADPr required for $[Ca^{2+}]_i$ increase in our experiments was much higher than that of ADP, the cognate agonist of P2Y1 receptors. The EC_{50} for ADP-induced activation of the P2Y1 receptor is 1 μM (162). However, ADPr concentrations in the range of 30 μM have been used in the past to demonstrate biological effects of ADPr in different tissues (112;163-166). It is possible that the concentration of ADPr at its local sites of actions is in the micromolar range, but we do not have any proof for that.

NAD^+, cADPr or breakdown products of ADPr did not increase $[Ca^{2+}]_i$. Neither PADPr, a phosphonate analogue of ADPr, nor 8-bromo-ADPr, increased $[Ca^{2+}]_i$. Non of them altered the ADPr-induced $[Ca^{2+}]_i$ changes.

ADPr increased $[Ca^{2+}]_i$ in the form of an initial peak followed by a plateau that depended on extracellular Ca^{2+}. Such biphasic $[Ca^{2+}]_i$ increase resembles the $[Ca^{2+}]_i$ changes upon activation of receptors coupled to PI-PLC. When the ER Ca^{2+} pool was depleted by thapsigargin, the $[Ca^{2+}]_i$ increase was abolished, indicating that the $[Ca^{2+}]_i$ rise was due to release of Ca^{2+} from the ER. Furthermore, this $[Ca^{2+}]_i$ increase was abolished by the PI-PLC inhibitor U73122, and by 2-APB, which inhibits the IP_3 receptor. These results suggest that the ADPr-induced $[Ca^{2+}]_i$ increase was due to activation of the PI-PLC-IP_3 pathway.

When Ca^{2+} was omitted from the extracellular medium, the plateau phase of the ADPr-induced $[Ca^{2+}]_i$ increase was abolished, indicating that this phase was due to Ca^{2+} entry from outside the cell. The plateau phase was not inhibited by inhibitors of TRPM2, namely flufenamic acid, niflumic acid, and ACA. Inhibition of the L-type voltage-gated Ca^{2+} channels also did not inhibit the plateau phase. These results indicate lack of involvement of both TRPM2 channels and L-type voltage-gated Ca^{2+} channels in mediating the Ca^{2+} entry.

The most important findings in this study were that MRS 2179 and MRS 2279, two specific inhibitors of the purinergic receptor P2Y1 (167;168), completely blocked the ADPr-induced $[Ca^{2+}]_i$ increase (fig. 6). MRS2279 only inhibits P2Y1, but MRS2179 also inhibits P2X1 and P2X3 (137). These results are strong evidence for the involvement of the P2Y1 receptor in the ADPr-induced $[Ca^{2+}]_i$ increase.

To further establish that we were dealing with P2Y1 receptors, we used 1321N1 human astrocytoma cells that stably overexpress human P2Y1 receptors. ADPr increased $[Ca^{2+}]_i$ in these cells, but did not increase $[Ca^{2+}]_i$ in the wild type astrocytoma cells that do not express P2Y1 receptors. Biological effects of ADPr-induced $[Ca^{2+}]_i$ increase were tested in the platelets, which express native P2Y1 receptors. ADPr induced platelet shape change as a result of $[Ca^{2+}]_i$ increase through P2Y1 activation.

The role of P2Y1 in insulin secretion is controversial. Depending on experimental conditions, cell types used, choice of P2Y1 agonist and its dosage, P2Y1 activation can either increase or inhibit the insulin secretion (169-172). The $[Ca^{2+}]_i$ increase leading to the insulin secretion is mainly due to Ca^{2+} entry through the voltage gated Ca^{2+} channels (85). In our study ADPr did not alter the basal or glucose-induced insulin secretion. We conclude that ADPr is a novel endogenous and specific agonist of P2Y1 receptors that increases the $[Ca^{2+}]_i$ in the insulin-secreting cells (fig. 7). The physiological importance of this finding needs further investigations.

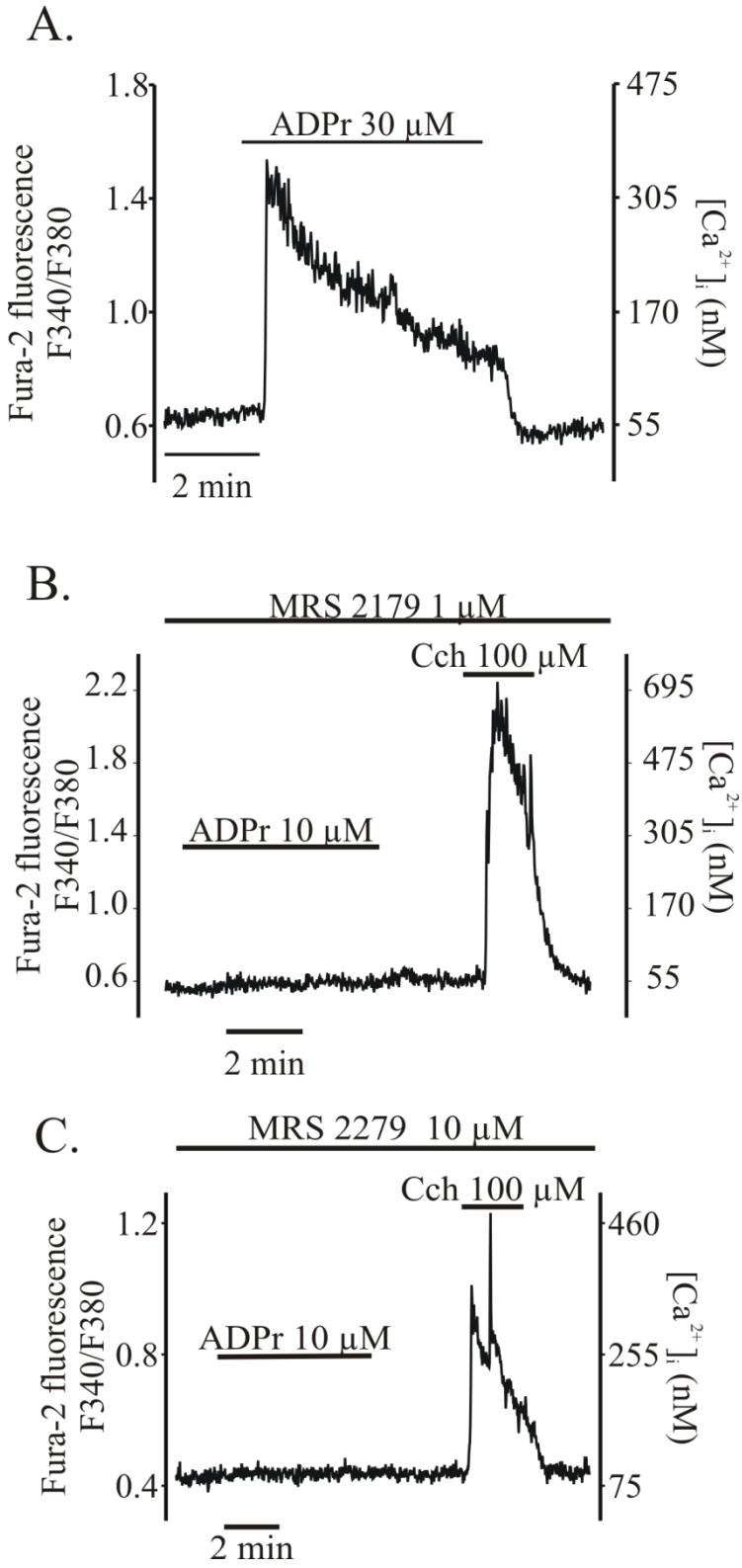

Figure 6. ADPr-induced $[Ca^{2+}]_i$ increase was due to the activation of P2Y1 receptors. The figure is reproduced from Jabin Gustafsson *et al* 2011. The INS-1E cells were incubated for 10 min with either MRS 2179 (1 and 10 µM) (B) or MRS 2279(10 µM) (C). The inhibitors were also present in the perfusion during the experiment. Both MRS2179 and MRS2279 completely inhibited the $[Ca^{2+}]_i$ increase by ADPr (10 µM). Fig. A is a control experiment that shows ADPr-induced $[Ca^{2+}]_i$ increase in the absence of the inhibitors. MRS2179 and MRS2279 did not block the carbachol- induced $[Ca^{2+}]_i$ increase. The traces are representatives of at least three experiments each.

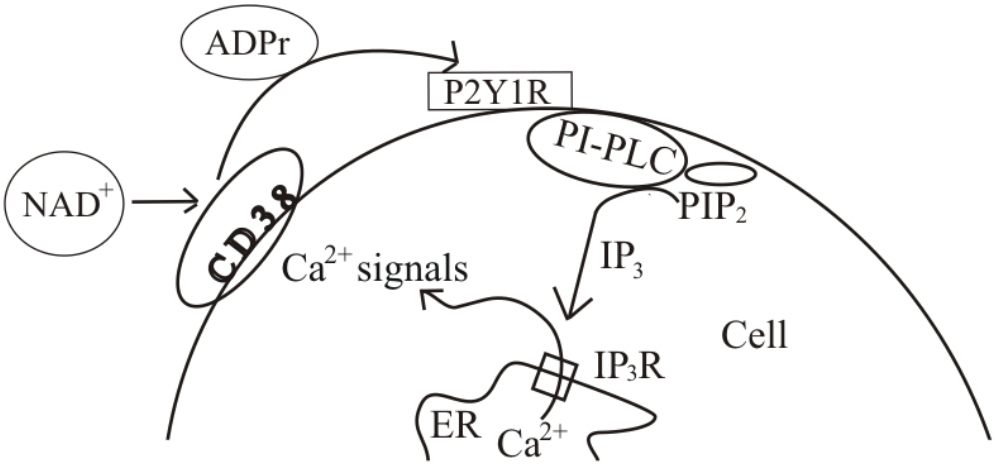

Figure 7. Schematic figure of ADPr as a ligand of the P2Y1 receptor. Extracellular ADPr activates the P2Y1 receptor (P2Y1R) and the PI-PLC, leading to the formation of IP_3 produced from PIP_2. The IP_3R is activated, and Ca^{2+} is released from the ER.

8.3 INS-1E cells express functional TRPV1 channels

In paper III, we studied whether β-cells have functional TRPV1 channels. We tested whether TRPV1 activation leads to $[Ca^{2+}]_i$ increase. Capsaicin, a specific agonist of TRPV1, increased $[Ca^{2+}]_i$ in the INS-1E cells in a concentration-dependent manner. The $[Ca^{2+}]_i$ increase was dependent on extracellular Ca^{2+}.These results indicated that we were dealing with Ca^{2+} channels in the plasma membrane. AM404, another known TRPV1 agonist, also increased $[Ca^{2+}]_i$ in the INS-1E cells. However, the precursors *p*-aminopenol and arachidonic acid did not increase $[Ca^{2+}]_i$. Capsazepine, a specific inhibitor of TRPV1, completely blocked both the capsaicin-induced and the AM404-induced $[Ca^{2+}]_i$ increase. These results together suggest that TRPV1 channels are located in the plasma membrane in the INS-1E cells, and causes Ca^{2+} entry and $[Ca^{2+}]_i$ increase upon activation.

Capsaicin elicited inward currents in the INS-1E cells, and the currents were inhibited by capsazepine. Since the permeability of TRPV1 is higher for Ca^{2+} than for Na^+ (PNa^+/PCa^{2+} = 1:9) (24), Ca^{2+} was probably the main carrier of the current in our experiments. This is consistent with microfluorometry experiments where capsaicin induced robust increase in $[Ca^{2+}]_i$.

The expression of TRPV1 protein in the INS-1E cells and the human islets was detected by Western blot analysis. The bands that were seen at ~94 kDa in the INS-1E cells and at ~96 kDa in the human islets were compared with the expected molecular weight of TRPV1 estimated from the mRNA. According to the comparison, the bands represented TRPV1. Our results were also in accordance with several earlier studies (173-177).

The existence of TRPV1 in primary β-cells is debated. Akiba *et al* have demonstrated TRPV1 immunoreactivity in primary β-cells from Sprague-Dawley rats, but they did not report the effect of capsaicin in these cells (35). Gram *et al* reported TRPV1 immunoreactivity in the nerve fibres in the islets, but not in the β-cells (51). In our study, we used primary β-cells from Wistar rat, but capsaicin did not induce any $[Ca^{2+}]_i$ increase in these cells. These results suggest that primary β-cells do not have TRPV1 channels.

The existence of TRPV1 in human β-cells is questionable. In our study, capsaicin did not increase $[Ca^{2+}]_i$ in human β-cells. Also, no TRPV1 immunoreactivity was detected in the human islets or human insulinoma cells. We used eight different antibodies that all detected TRPV1 immunoreactivity in the dorsal root ganglion cells, which were used as controls. Thus, TRPV1 is not expressed in the human β-cells, at least not at as high level as in the dorsal root ganglion cells. We conclude that functional TRPV1 channels are expressed at high level in the INS-1E cells, but not in the primary β-cells from rat or human.

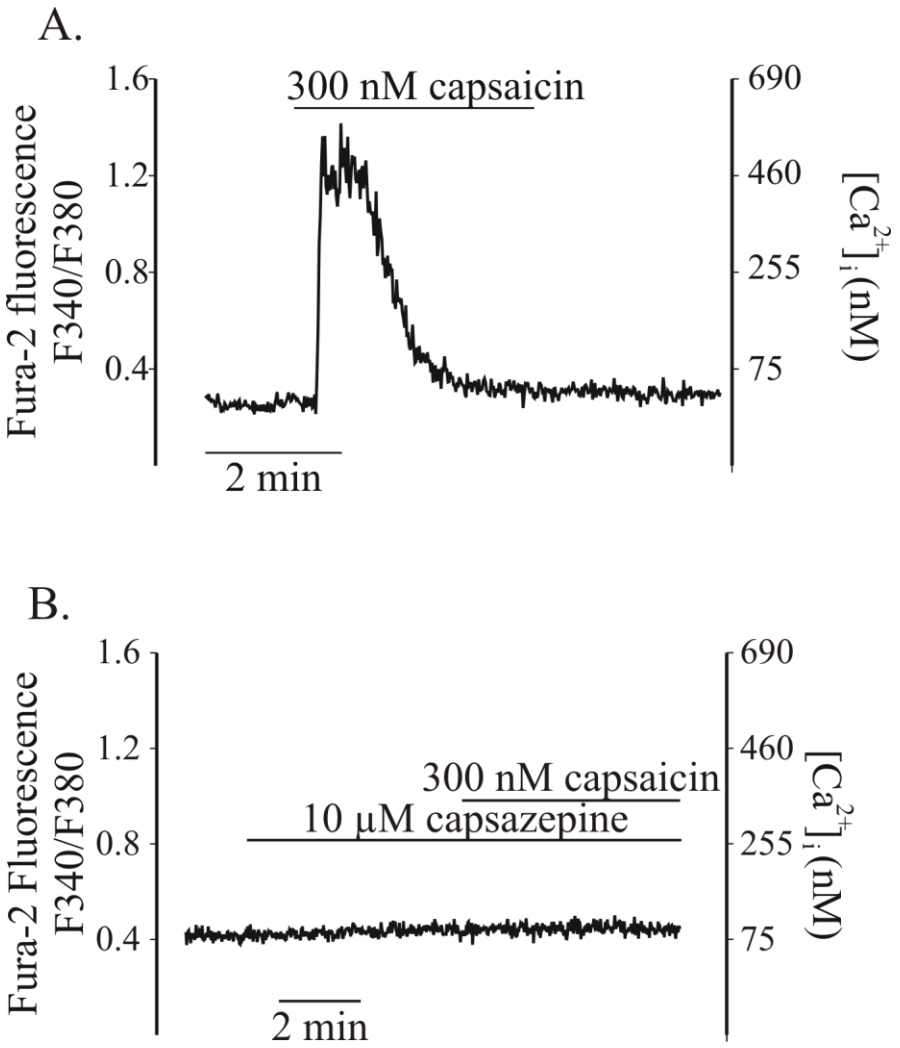

Figure 8. Effect of capsaicin and capsazepine on $[Ca^{2+}]_i$ in the INS-1E cells. The figure is reproduced from Jabin Fågelskiöld *et al* 2011. Capsaicin (300 nM) increased $[Ca^{2+}]_i$ (A). In the presence of capsazepine (10 μM), capsaicin failed to increase $[Ca^{2+}]_i$ (B).

9 Conclusions

1. The activation of RyRs induces a series of distinct signaling events, which include release of Ca^{2+} from the ER, activation of putative Ca^{2+}-permeable TRP-like channels in the plasma membrane, membrane depolarization, Ca^{2+} entry through the voltage-gated Ca^{2+} channels, and regenerative CICR.

2. Extracellular ADPr increases $[Ca^{2+}]_i$ in the insulin-secreting cells by activation of the P2Y1 purinergic receptors.

3. Functional Ca^{2+} permeable TRPV1 channels are present in the INS-1E cells, but not in the primary rat or human β-cells or the human insulinoma cells.

10 Future perspectives

It is important to identify which TRP channels are present and functional in the β-, α-, and δ-cells of the islets. Several TRP channels have already been identified in the β-cells and their role in the Ca^{2+} signaling and stimulus-secretion coupling needs to be studied in detail. The TRP channels might play an important role in mediating the depolarizing currents that lead to depolarization to the threshold for activation of the voltage-gated Ca^{2+} channels. Diverse physical second messengers like heat, swelling, stretch, and chemical factors like arachidonic acid, cAMP, PIP_2, and Ca^{2+} could act as links between insulin-secretagogues and activation of the TRP-channels. One of the challenges in the future will be to investigate the quantitative contribution of different second messengers and different TRP channels in stimulus-secretion coupling in the β-cells under different physiological and pathological conditions. The availability of more specific pharmacological tools and use of TRP channel knock-out mice models will hopefully give answers to many of the remaining questions. Eventually, some of these TRP channels may turn out to be molecular targets for the development of drugs for the treatment of impaired insulin secretion in diabetes.

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