Role of TRPV1 channel and P2Y1 receptor in Ca^{2+} signalling in β-cells: A study by single cell microfluorometry

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Författare
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Sammanfattning
Abstract:
Increase in the cytoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in the β-cells triggers insulin exocytosis. Among the Ca$^{2+}$ channels present in the plasma membrane, the transient receptor potential (TRP) channels receptors are currently of great interest. The mechanisms by which the extracellular adenosine diphosphate ribose (ADPr) increases the [Ca$^{2+}$]$_i$ is unknown. Our aims were to study the roles of the TRP channels in the tolbutamide induced [Ca$^{2+}$]$_i$ increase and to identify the surface receptor that is activated by ADPr.

We used S5 cells, a highly differentiated rat insulinoma cell line, as a model for β-cells. Single cell ratiometric microfluorometry was used to measure the [Ca$^{2+}$]$_i$, changes in the Fura-2 loaded cells.

Tolbutamide increased [Ca$^{2+}$]$_i$ in the form of oscillations. After tolbutamide increased [Ca$^{2+}$]$_i$, capsazepine, a potent blocker of the transient receptor potential vaniloid subtype 1 (TRPV1) channel was added to the β-cells, which reduced the tolbutamide-induced [Ca$^{2+}$]$_i$ increase. Capsazepine, N-(p-Amylccinnamoyl) anthranilic acid (ACA), TRPM2 channel blocker, and triphenyl phosphine oxide (TPPO), TRPM5 channel blocker were tested for their effect on potassium chloride (KCl) induced [Ca$^{2+}$]$_i$. response. These blockers did not inhibit the KCl induced [Ca$^{2+}$]$_i$, increase.

Adenosine diphosphate ribose (ADPr) increased [Ca$^{2+}$]$_i$ in the form of initial transient peak followed by an elevated plateau. Application of ADPr shortly after a prior application and washout of Adenosine diphosphate (ADP) elicited only small [Ca$^{2+}$]$_i$, increase indicating desensitization of the receptor involved. 2’deoxy-N$^6$-methyladenosine 3’5’bis-phosphate (MRS2179), and chloro N$^6$-methyl-(N)-methanoarca 2’deoxyadenosine 3’5’ bis-phosphate (MRS2279), two selective inhibitors of P2Y1 receptor, abolished the ADPr-induced [Ca$^{2+}$]$_i$, increase.

Tolbutamide closes ATP sensitive potassium (K$_{ATP}$) channels. Our results demonstrate that besides the closure of the K$_{ATP}$ channels, inward cation currents carried by Ca$^{2+}$ through the TRPV1 channel are necessary for depolarization to the threshold for the activation of the voltage gated calcium channels (VGCC) to increase the [Ca$^{2+}$]$_i$. Our results also show that ADPr increases [Ca$^{2+}$]$_i$, by activating the P2Y1 receptor.

Nyckelord
Keyword: Insulin exocytosis, P2Y1, tolbutamide, ADPr, microfluorometry, Islets of langerhans, β-cells, calcium signaling, signal transduction.
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1. Abstract

Increase in the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]\) in the β-cells triggers insulin exocytosis. Among the Ca\(^{2+}\) channels present in the plasma membrane, the transient receptor potential (TRP) channels receptors are currently of great interest. The mechanisms by which the extracellular adenosine diphosphate ribose (ADPr) increases the [Ca\(^{2+}\)\(_i\)] is unknown. Our aims were to study the roles of the TRP channels in the tolbutamide induced [Ca\(^{2+}\)\(_i\)] increase and to identify the surface receptor that is activated by ADPr.

We used S5 cells, a highly differentiated rat insulinoma cell line, as a model for β-cells. Single cell ratiometric microfluorometry was used to measure the [Ca\(^{2+}\)\(_i\)] changes in the Fura-2 loaded cells.

Tolbutamide increased [Ca\(^{2+}\)\(_i\)] in the form of oscillations. After tolbutamide increased [Ca\(^{2+}\)\(_i\)], capsazepine, a potent blocker of the transient receptor potential vanilloid subtype 1 (TRPV1) channel was added to the β-cells, which reduced the tolbutamide-induced [Ca\(^{2+}\)\(_i\)] increase. capsazepine, N-(p-Amylcinnamoyl) anthranilic acid (ACA), TRPM2 channel blocker, and triphenyl phosphine oxide (TPPO), TRPM5 channel blocker were tested for their effect on potassium chloride (KCl) induced [Ca\(^{2+}\)\(_i\)] response. These blockers did not inhibit the KCl induced [Ca\(^{2+}\)\(_i\)] increase.

Adenosine diphosphate ribose (ADPr) increased [Ca\(^{2+}\)\(_i\)] in the form of initial transient peak followed by an elevated plateau. Application of ADPr shortly after a prior application and washout of Adenosine diphosphate (ADP) elicited only small [Ca\(^{2+}\)\(_i\)] increase indicating desensitization of the receptor involved. 2’deoxy-N\(^6\)-methyladenosine 3’5’bis-phosphate (MRS2179), and chloro N\(^6\)-methyl-(N)-methanocarba 2’deoxyadenosine 3’5’ bis-phosphate (MRS2279), two selective inhibitors of P2Y1 receptor, abolished the ADPr-induced [Ca\(^{2+}\)\(_i\)] increase.

Tolbutamide closes ATP sensitive potassium (K\(_{ATP}\)) channels. Our results demonstrate that besides the closure of the K\(_{ATP}\) channels, inward cation currents carried by Ca\(^{2+}\) through the TRPV1 channel are necessary for depolarization to the threshold for the activation of the voltage gated calcium channels (VGCC) to increase the [Ca\(^{2+}\)\(_i\)]. Our results also show that ADPr increases [Ca\(^{2+}\)\(_i\)] by activating the P2Y1 receptor.

Key words:
Insulin exocytosis, P2Y1, tolbutamide, ADPr, microfluorometry, Islets of langerhans, β-cells, calcium signaling, signal transduction.
2. List of abbreviations

(K$_{\text{ATP}}$) - ATP sensitive potassium channels | MRS 2179 - 2’deoxy-N$^6$-methyladenosine 3’5’bis-phosphate  

[Ca$^{2+}$]$_i$ - cytoplasmic Ca$^{2+}$ concentration | MRS 2279’-chloro N$^6$-methyl- (N)-methanocarba 2’deoxyadenosine 3’5’ bis-phosphate  

ACA - N-(p-Amylcinnamoyl) anthranilic acid | P2Y1 – Purinergic receptor 2 subtype 1  

ADP - Adenosine diphosphate | SUR1 - Sulphonyl urea receptor subtype 1  

ADPr - Adenosine diphosphate ribose | TPPO - Triphenylphosphine oxide  

AM - acetoxymethyl ester | TRPM2 - Transient Receptor Potential Melastatin-like subtype 2  

EGTA - ethylene glycol tetraacetic acid | TRPM5 - Transient Receptor Potential Melastatin-like subtype 5  

ER - endoplasmic reticulum | TRPV1 - Transient Receptor Potential Vanilloid subtype 1  

KRBH – Krebs Ringer bicarbonate HEPES buffer | VGCC - Voltage gated calcium channels  

3. Introduction

3.1 Diabetes mellitus

Diabetes is a heterogeneous metabolic disorder associated with high blood glucose levels. Diabetes is classified into two types namely the type-1 diabetes mellitus, and the type-2 diabetes mellitus. Type-1 diabetes is an autoimmune disorder in which β-cells are killed by the immune system (Li et al., 2000). This type of diabetes is seen mostly in the children and the teenagers. Type-2 diabetes is associated with progressive failure of β-cells in secreting insulin due to aging or developing insulin resistance (Boden 1997, Martin et al., 1992). Changes in food habit, life style, lack of exercise, stress, obesity, aging, and sedentariness are some of the factors that lead to the type-2 diabetes mellitus (Astrup and Finer, 2000). There are a number of genes and pathways that are involved in insulin secretion as well as insulin action. Any defect in those genes or the pathways eventually result in the type-2 diabetes (Pillay et al., 1995; Kahn et al., 1996). Middle aged people are prone to be affected by type-2 diabetes. Sulphonylureas like tolbutamide have been widely used for treating Type-2 diabetes. The sulphonylureas inhibit the K$_{\text{ATP}}$ channels by binding to the
sulphonyl ureas receptor 1 (SUR1). They decrease the blood glucose levels by secreting insulin (Bryan et al., 1987, Ashcroft and Rorsman 1989).

3.2 The role of Ca\(^{2+}\) signaling in insulin exocytosis

The Islets of Langerhans are a crucial mini organs that contains five different cell types namely β-cells, α-cells, δ-cells, pancreatic polypeptide producing cells, and Epsilon cells. The Islets are aggregated more in the tail region compared to the head, and body region of the pancreas (Wittingen and Frey 1974). 70-80% of the adult human islets comprise of insulin secreting β-cells (Wierup et al., 2002). β-cells play crucial role in the maintenance of glucose homeostasis. They also act as sensors for fatty acids, amino acids, incretins other hormones, neurotransmitters and growth factors (Newsholme et al., 2010). Insulin exocytosis in β-cells is associated with an increase of free \([\text{Ca}^{2+}]_i\) (Lemmens et al., 2001, Fridlyand et al., 2003). Ca\(^{2+}\) homeostasis, and normal Ca\(^{2+}\) signalling are necessary for β-cells to function normally. Multiple mechanisms are involved in the increase of \([\text{Ca}^{2+}]_i\) in the β-cells which receive signals from the nutrient metabolism (Nadal and Soria, 1997). There are highly structured Ca\(^{2+}\) stores in β-cells that are part of the endoplasmic reticulum (ER) which contains Ca\(^{2+}\) pumps, and Ca\(^{2+}\) releasing channels (Zhang et al., 2011). These pumps and channels regulate Ca\(^{2+}\) signalling that are induced by plasma membrane depolarization, and activation of plasma membrane receptors that are coupled to the phospholipase C system (Islam, 1994).

3.3 K\(_{\text{ATP}}\) channels

The K\(_{\text{ATP}}\) channel is one of the extensively studied channels in pancreatic β-cells. These channels were first described by Cook and Hales (1984). K\(_{\text{ATP}}\) channels of β-cells are composed of four sulphonyl urea receptor subtype 1 (SUR1) and four inward rectifiers Kir6.2 arranged in a hetero-octameric complex (Clement et al., 1997). Kir6.2 senses the changes in ATP and ADP ratio in the cytoplasm in the β-cell and regulates the channel. The SUR1 has the binding sites for sulphonylureas like tolbutamide that acts as a blocker for the K\(_{\text{ATP}}\) channel (Babenko et al., 2000). Upon sensing the ATP or sulphonylureas, the K\(_{\text{ATP}}\) channels close and initiate membrane depolarization. The VGCC gets activated upon depolarization of the plasma membrane which increases \([\text{Ca}^{2+}]_i\) by allowing Ca\(^{2+}\) entry into the cytoplasm from entracellular region. The \([\text{Ca}^{2+}]_i\) increase triggers the insulin exocytosis (Hiriart and Bryan 2008).

3.3 Transient Receptor Potential (TRP) channels

TRP channels are tetrameric ion channels that are classified into seven families TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin) and TRPN (Venkatachalam and Montell 2007). Many TRP channels form heterotetramers that give rise to different kinds of ion channels involved in various cellular processes. The TRP channels are involved in hormone secretion, membrane depolarization by providing background current after K\(_{\text{ATP}}\) channel closure (Hiriart and Bryan 2008).
TRP channels are mostly involved in mediating many sensory functions like vision, olfaction, hearing, thermo-sensation, and taste (Benham et al., 2003). In β-cells, some of the TRP channels like TRPM2, TRPM5, and TRPV1 are involved in mediation of inward depolarizing currents that lead to activation of VGCC. One of the aims of our project was to explore the involvement of TRP channels in tolbutamide-induced Ca^{2+} increase in the S5 cells.

3.4 Purinergic receptor 2 (P2Y) and Adenosine dipshosphate ribose (ADPr)
P2Y receptors are metabotropic, G-protein coupled receptors. Eight subtypes of P2Y receptors have been discovered (Burnstock 2006). Intracellular ADPr is found to increase the [Ca^{2+}]_{i} through activation of the TRPM2 channels (Bari et al 2009). However the mechanism involved in [Ca^{2+}]_{i} increase by extracellular application of ADPr is not clear. We were interested in finding out the cell surface receptor involved in the interaction with ADPr to increase [Ca^{2+}]_{i}. ADP interacts with P2Y1 receptor and mobilizes [Ca^{2+}]_{i} for mediating platelet aggregation (Hollopeter et al., 2001). As ADP moiety is present in ADPr, there was a possibility that P2Y1 receptor may be involved in the ADPr-induced [Ca^{2+}]_{i} increase.

3. Aims of the thesis
- To study whether the TRP channels are involved in the tolbutamide-induced [Ca^{2+}]_{i} increase.
- To identify the surface receptor involved in the ADPr-induced [Ca^{2+}]_{i} increase.

4. Materials and methods

4.1 Cell lines
We used a highly differentiated rat insulinoma cell line (S5 cells) that was subcloned from the INS-1E cells. The cells were cultured in RPMI-1640 medium supplemented with fetal bovine serum (2.5% v/v), penicillin (50 IU/ml), 2-mercaptoethanol (500 µM), HEPES (10 mM), streptomycin (50 µg/ml) and sodium pyruvate (1 mM). The cells were cultured and maintained in humidified incubator in 5% CO_{2} at 37°C. The cells were provided with new medium every alternate day. They were trypsinised and passaged every week. On the day of passaging, the cells were plated on coverslips for the experiments. The cells were cultured on the coverslips for 3-4 days before measuring the [Ca^{2+}]_{i}.

4.2 Fura-2
The fluorescent probe used was Fura-2 acetoxymethyl ester (AM) purchased from Invitrogen. The AM group which is lipophilic enables the hydrophilic Fura-2 to get across the plasma membrane into the cytosol. The esterases in the cytosol cleave the AM group and set the Fura-2 free in the cytoplasm. The Fura-2 binds to the Ca^{2+} and changes its own fluorescent properties as they are excited by the two wavelengths (340nm and 380nm). The absorption maxima for Fura-2 are around 340nm, and 380nm and emission
maxima at 510 nm (fig 1). By the ratiometric method the amount of Ca$^{2+}$ bound to the Fura-2 can be measured (Grynkiewicz et al., 1985; Tsien., 1980). We used Fura-2 as a probe for measuring $[\text{Ca}^{2+}]_i$ in all our experiments.

![Excitation spectrum of Fura-2 dye measured at emission wavelength of 510nm. Two excitation peaks were seen around 340nm and 380nm wavelength. The excitation peak for 340nm increases with increase in $[\text{Ca}^{2+}]_i$ concentration, and the excitation peak for 380nm decreases with increase in the $[\text{Ca}^{2+}]_i$ concentration.](image)

Fig 1: Excitation spectrum of Fura-2 dye measured at emission wavelength of 510nm. Two excitation peaks were seen around 340nm and 380nm wavelength. The excitation peak for 340nm increases with increase in $[\text{Ca}^{2+}]_i$ concentration, and the excitation peak for 380nm decreases with increase in the $[\text{Ca}^{2+}]_i$ concentration. (The molecular probes handbook. chapter 19).

4.3 Loading cells with Fura-2

Fura-2-acetoxyethyl ester (AM) is a ratiometric lipophilic indicator that enters through the plasma membrane and binds Ca$^{2+}$ in the cytoplasm. Cells grown on coverslips were incubated for 35 minutes in 2ml loading buffer that contained RPMI, Fura-2AM (1µM), and 2% bovine serum albumin (BSA). It was then incubated for 10 minutes in modified Krebs–Ringer bicarbonate–HEPES buffer (KRBH) containing NaCl 140 mM, KCl 3.6 mM, NaH$_2$PO$_4$ 0.5 mM, MgSO$_4$.7H$_2$O 0.5 mM, CaCl$_2$ 1.5 mM, HEPES 10 mM, glucose 3mM and 0.1% bovine serum albumin (pH 7.4) to let the endogenous esterases in the cells hydrolyze the AM bond.

4.4 Measurement of $[\text{Ca}^{2+}]_i$ by microfluorometry

The microfluorometry is a sensitive method that makes it possible to measure $[\text{Ca}^{2+}]_i$ from single living cells. The system consisted of an inverted epifluorescence microscope (Olympus CK 40). The cell was studied using a 40×1.3 NA oil immersion objective (40× UV APO). Two lights of wavelengths 340 nm and 380 nm were produced by a monochromator from a white light source, and focused on the cells.

A coverslip was mounted on an open perfusion chamber that was designed for these types of experiments, and placed on the stage of the microscope. The physiological salt
solution was superfused by closed loop flow system by means of peristaltic pump. A water bath and a thermistor connected to the perfusion chamber were used to control the temperature of the flowing physiological solution. The \([\text{Ca}^{2+}]_i\) was measured by dual wavelength excitation fluorometry. The monochromator (PhotoMed DeltaRam) produces two excitation wavelengths 340 nm and 380 nm. The emitted light chosen by a 510 nm filter was detected by the photomultiplier tube detector and the signals were fed to a computer containing the Felix32 software. The cells were excited at two wavelengths of light (340 nm and 380 nm) alternately. 340 nm signal corresponds to \(\text{Ca}^{2+}\) bound Fura-2 molecules, and the 380 nm signal corresponds to free Fura-2 molecules. In order to consider a \([\text{Ca}^{2+}]_i\) increase, the 340 nm signal should increase, and the 380 nm signal should decrease. For each second, one 340/380 ratio was obtained. The ratio between emitted fluorescence intensities of 340 nm and 380 nm signals were calculated by Felix32 software. The corresponding \([\text{Ca}^{2+}]_i\) increase was elucidated from the calibration data.

The region of interest containing single cells was located with the help of diaphragm. The cell selected for experiment should be round, relatively big with sharp edge, and should posses a relatively high and stable fluorescent signal. The experiments were done in a dark room to avoid the interference of external light sources. The background fluorescence was measured by moving the focus away from the examined cell, and then subtracted from original fluorescence signal of 340 nm and 380 nm wavelengths, and a new ratio was calculated by using Felix32 software.

4.5 Calibration to calculate the \([\text{Ca}^{2+}]_i\)

Calibration is done to estimate the \([\text{Ca}^{2+}]_i\) from the fluorescence ratios. The protocol used was that described by Poenie et al. Two solutions namely \(F_{\text{max}}\) and \(F_{\text{min}}\) were used. \(F_{\text{max}}\) Solution contains Ethylene glycol tetraacetic acid (EGTA) 0.5mM, KCl 125mM, HEPES 10mM, MgCl\(_2\) 1mM and saturated with \(\text{Ca}^{2+}\) by adding CaCl\(_2\) 6mM and \(F_{\text{min}}\) solution contains Ethylene glycol tetraacetic acid (EGTA) 0.5mM, KCl 125mM, HEPES 10mM, MgCl\(_2\) 1mM were used. The \(F_{\text{min}}\) solution was devoid of CaCl\(_2\). Sucrose 2M was added to these solutions to mimic the viscosity of the cytoplasm. The pH of \(F_{\text{max}}\) and \(F_{\text{min}}\) solutions were adjusted to 7.2. The fluorescence from the \(F_{\text{max}}\) and \(F_{\text{min}}\) solutions upon excitation by 340 nm and 380 nm were recorded, and the corresponding fluorescence ratios, \(R_{\text{max}}\) and \(R_{\text{min}}\) were calculated. The dissociation constant (\(K_d\)) for \(\text{Ca}^{2+}\)-Fura-2 was taken as 225 nM. The \([\text{Ca}^{2+}]_i\) was calculated from the fluorescence ratio by using the formula described by Grynkiewicz et al.

4.6 Statistical Analysis

Results were expressed as means ± SEM. Student’s unpaired T test was used to compare data from two groups. \(P\)-value less that 0.05 was considered as significant. Sigma plot software was used to perform the T-test.
5 Results

5.1 capsazepine blocked [Ca\textsuperscript{2+}]\textsubscript{i} response to tolbutamide.

Simultaneous application of TPPO, ACA and capsazepine blocks the [Ca\textsuperscript{2+}]\textsubscript{i} increase by tolbutamide

Extracellular application of tolbutamide (100µM) in the presence of basal glucose (3mM) increased the [Ca\textsuperscript{2+}]\textsubscript{i} in the S5 cells (fig 2A). The [Ca\textsuperscript{2+}]\textsubscript{i} increase was in the form of numerous oscillations. The [Ca\textsuperscript{2+}]\textsubscript{i} returned to the baseline after washout of tolbutamide indicating that the effect of tolbutamide was reversible. The simultaneous application of TPPO (20µM), ACA (20µM), and capsazepine (10µM), which are blockers of TRPM5, TRPM2, and TRPV1 respectively, abolished the [Ca\textsuperscript{2+}]\textsubscript{i} increase by tolbutamide (100µM) (fig 2B), suggesting the participation of the TRPM5, TRPM2 or TRPV1 channels in [Ca\textsuperscript{2+}]\textsubscript{i} increase by tolbutamide.

Fig 2. Simultaneous application of TPPO, capsazepine and ACA inhibited the increase of [Ca\textsuperscript{2+}]\textsubscript{i} by Tolbutamide. S5 cells exposed to tolbutamide (100µM) increased [Ca\textsuperscript{2+}]\textsubscript{i} rapidly (A). Simultaneous application of TPPO (20µM), ACA (20µM) and capsazepine (10µM), which are antagonists of the TRPM5, TRPM2, and TRPV1 channels respectively inhibited the [Ca\textsuperscript{2+}]\textsubscript{i} increase by tolbutamide (100µM) (B). The traces are representative of three experiments each.
TRPM2 and TRPM5 are not involved in \([\text{Ca}^{2+}]_i\) increase by tolbutamide

We tested whether the TRPM2 channel could be involved in inhibiting the \([\text{Ca}^{2+}]_i\) increase by tolbutamide. The S5 cells were pre-treated with ACA (20µM) for 10 minutes and ACA was present in the perfusion throughout the experiment. ACA did not increase \([\text{Ca}^{2+}]_i\) by itself. When tolbutamide (100µM) was applied in the presence of ACA, \([\text{Ca}^{2+}]_i\) increase was seen (fig 3B). This ruled out the possibility of involvement of TRPM2 channel in tolbutamide induced \([\text{Ca}^{2+}]_i\) increase. Similar type of experiments were done by substituting ACA (20µM) with TPPO (20 µM) to test the involvement of TRPM5 in tolbutamide induced \([\text{Ca}^{2+}]_i\) increase. Tolbutamide (100µM) increased \([\text{Ca}^{2+}]_i\) even in the presence TPPO (20 µM) (fig 3A). Simultaneous application of both TPPO (20µM), ACA (20µM) also did not inhibit the \([\text{Ca}^{2+}]_i\) increase by tolbutamide (fig 3C).

capsazepine abolished the tolbutamide induced \([\text{Ca}^{2+}]_i\) increase.

capsazepine (10µM), a selective blocker of TRPV1 was applied after \([\text{Ca}^{2+}]_i\) increase by tolbutamide (100µM). The application of capsazepine reduced \([\text{Ca}^{2+}]_i\) to baseline (fig 3D). The \([\text{Ca}^{2+}]_i\) increased again when the capsazepine was removed from the perfusion.
Fig 3. *capsazepine inhibited the increase of [Ca$^{2+}$]$_i$ by tolbutamide.* Application of TPPO (20µM), a TRPM5 channel blocker (A), ACA (20µM) a TRPM2 channel blocker (B) did not inhibit the increase of [Ca$^{2+}$]$_i$ by TOL (100µM). The combined application of both antagonists TPPO (20µM), and ACA (20µM) also did not inhibit the [Ca$^{2+}$]$_i$ increase (C). However capsazepine (10µM) abolished the [Ca$^{2+}$]$_i$ increase by TOL (100µM) (D). When capsazepine (10µM) was washed out, [Ca$^{2+}$]$_i$ increased again indicating that the effect of capsazepine (10µM) was reversible. The traces are representative of at least three experiments each.
TPPO, ACA and capsazepine did not inhibit the VGCC.

KCl increases the \([\text{Ca}^{2+}]_i\) by depolarizing the plasma membrane which in turn opens the VGCC. To check whether the inhibitors used inhibit the VGCCs and thereby inhibit the \([\text{Ca}^{2+}]_i\) increase by tolbutamide, we tested the effect of these inhibitors on KCl response in S5 cells. We found that the inhibitors (TPPO 20µM, ACA 20µM and capsazepine 10µM) did not significantly inhibit the VGCC-mediated \([\text{Ca}^{2+}]_i\) increase caused by KCl (25mM) (fig 4A, 4B). In the presence of TPPO, ACA and capsazepine (10µM) the KCl-induced peak \([\text{Ca}^{2+}]_i\) increase was 1316±36 nM (n=4), and in the absence of the inhibitors KCl-induced peak \([\text{Ca}^{2+}]_i\) increase was 1133±172 nM (p=0.36, n=4) (fig 4C).

**Fig 4. TPPO, ACA and capsazepine do not inhibit the VGCC.** Application of TPPO (20µM), ACA (20µM), capsazepine (10µM) did not inhibit the KCl-induced \([\text{Ca}^{2+}]_i\) increase (A). Control experiment with KCl (25mM)(B). The bar graph shows that the peak \([\text{Ca}^{2+}]_i\) increase in the control and experiment groups did not differ significantly (p=0.36, n=4)(C). The traces are representatives of four experiments each.
5.2 ADPr increases \([\text{Ca}^{2+}]_i\) by activating P2Y1 receptors in the plasma membrane

Effect of repeated exposure of ADP and the effect of ADP on ADPr induced \([\text{Ca}^{2+}]_i\) increase

ADPr increased \([\text{Ca}^{2+}]_i\) in the S5 cells. The increase was biphasic. There was a rapid and transient \([\text{Ca}^{2+}]_i\) increase in the first phase and an elevated plateau in the second phase (fig 5A). ADP is the known agonist for the P2Y1 receptor, and it is known to desensitize the receptor. The effect of repetitive application of ADP and the effect of prior exposure of the ADP to the ADPr induced \([\text{Ca}^{2+}]_i\) increase were then studied. ADP (5µM) increased \([\text{Ca}^{2+}]_i\), and after a prolonged washout when the \([\text{Ca}^{2+}]_i\) remained in the baseline, ADP was added again. ADP increased \([\text{Ca}^{2+}]_i\), to the extent similar to that seen after the first exposure. This indicated that ADP did not desensitize the receptor (fig 5B). However, application of ADP for the second time shortly after the first exposure reduced the \([\text{Ca}^{2+}]_i\) response, implying desensitization of the receptor involved (fig 5C). Under identical conditions application of ADPr shortly after the application of ADP showed only a small increase in \([\text{Ca}^{2+}]_i\) (fig 5D).
Fig 5. Effect of extracellular application of ADP and ADPr on $[Ca^{2+}]_i$ in the S5 cells. ADPr (10µM) increased $[Ca^{2+}]_i$ in S5 cells. ADP (5µM) increased $[Ca^{2+}]_i$ (A). After prolonged washout and new application of ADP, there was a second, almost similar $[Ca^{2+}]_i$ increase by ADP (B). Another application of ADP shortly after the first application of ADP showed only a small $[Ca^{2+}]_i$ increase (C). Prior application of ADP decreased the ADPr-induced $[Ca^{2+}]_i$ increase (D). The figures are representative of at least three experiments each.
Activation of P2Y1 receptors was evident during ADPr induced \([\text{Ca}^{2+}]_i\) increase.

MRS 2279 and MRS 2179 are two selective inhibitors of the P2Y1 receptor (Boyer et al. 2002; Moro et al. 1998). MRS 2279 and MRS 2179 were tested to examine the involvement of P2Y1 receptors in ADPr-induced \([\text{Ca}^{2+}]_i\) increase. MRS 2179 (1-10 µM) completely blocked the ADPr-induced \([\text{Ca}^{2+}]_i\) increase. MRS 2279 (10 µM), which is an even more selective inhibitor of the P2Y1 receptor, also inhibited the ADPr-induced \([\text{Ca}^{2+}]_i\) increase completely (fig 6A, B and D). In control experiments without MRS 2179 or MRS 2279, ADPr increased \([\text{Ca}^{2+}]_i\). (fig 6C and E).
Fig 6. ADPr-induced $[Ca^{2+}]_i$ increase was due to the activation of P2Y1 receptors. The S5 cells were incubated for 10 min with either MRS 2179 (1 and 10 µM) (fig. 5A and 5B) or MRS 2279 (10 µM) (fig. 5D). The inhibitors were also present in the perfusion during the experiment. Both MRS 2179 and MRS 2279 completely inhibited the $[Ca^{2+}]_i$ increase by ADPr (10 µM). Control experiments shows that ADPr-induced $[Ca^{2+}]_i$ increase in the absence of the inhibitors (fig. 5C and 5E). MRS 2179 and MRS 2279 did not block the carbachol(Cch)-induced $[Ca^{2+}]_i$ increase (fig. 5A, B and D). The traces are representatives of at least three experiments each.
6. Discussion

6.1 Closure of the $K_{ATP}$ channels, and inward cation current through the TRPV1 channels are required for membrane depolarization

Defects in $[Ca^{2+}]_i$ oscillations in the β-cells are associated with non-insulin dependent diabetes mellitus (Roe et al., 1994). Sulphonylurea drugs like tolbutamide are widely used for treatment of non-insulin-dependent diabetes mellitus (Panten et al., 1996). The β-cells have $K_{ATP}$ channels on the plasma membrane. It is known that tolbutamide leads to the closure of the $K_{ATP}$ channels, and thereby depolarization of the plasma membrane (Trube et al., 1986). The depolarization activates the VGCCs (Safayhi et al., 1997), allows entry of extracellular $Ca^{2+}$ into the cytoplasm, and thereby triggers insulin exocytosis (Lang J., 1999).

Our results show that cation currents through TRPV1 channels along with the closure of the $K_{ATP}$ channels is required to depolarize to the threshold potential for the activation of the VGCCs. TRP channel like TRPM5, TRPM2 and TRPV1 are expressed in rat β-cells (Akiba et al., 2004, Bari et al., 2009, Palmer et al., 2010). The concentrations of pharmacological tools used in our experiments have been used in the past studies (Docherty et al., 1997, Bari et al., 2009, Schöfl et al., 2000, Palmer et al., 2010 and Roenfeldt et al., 1992). Initially it appeared that TRPM5, TRPM2, and TRPV1 are involved in the $[Ca^{2+}]_i$ increase by tolbutamide as the simultaneous application of the antagonists of these three TRP channels completely inhibited the tolbutamide induced $[Ca^{2+}]_i$ increase. However in separate experiments, the antagonists of TRPM2 and TRPM5 channels, ACA and TPPO respectively, (Bari et al., 2009, Palmer et al., 2010) did not inhibit the $Ca^{2+}$ response to tolbutamide in the S5 cells. This indicated that the TRPM2 and TRPM5 channels were not involved in the tolbutamide-induced $[Ca^{2+}]_i$ increase.

Capsazepine, a potent blocker of TRPV1 channel (Nguyen et al., 2010 and Akerman et al., 2003) inhibited the tolbutamide induced $[Ca^{2+}]_i$ increase in the S5 cells. TRPV1 is a $Ca^{2+}$ permeable channel located in the plasma membrane of the S5 cells. Docherty et al., reported that capsazepine non-specifically inhibits the VGCC in sensory neurons with moderate potency. To rule out the possible non-specific effect of capsazepine and the other two inhibitors, on the VGCCs, we studied the effect of capsazepine, ACA and TPPO on the KCl-induced $[Ca^{2+}]_i$ increase. KCl increases $[Ca^{2+}]_i$ by inducing $Ca^{2+}$ influx through the VGCCs (Roenfeldt et al., 1992). The peak calcium increase in the presence of capsazepine, ACA and TPPO did not vary significantly from that in the controls. Thus, it is unlikely that the effect of capsazepine on $Ca^{2+}$ current was due to the inhibition of VGCCs. Our speculation is that tolbutamide closes the $K_{ATP}$ channels, such closure of the $K_{ATP}$ channels in the presence of some inward cation currents through the TRPV1 channels leads to depolarization to the threshold for the activation of the VGCCs to increase the $[Ca^{2+}]_i$. 
6.2 ADPr is an endogenous ligand for purinergic receptor type P2Y1.

The second part of the study was aimed to find out the effect of extracellular ADPr on [Ca$^{2+}$]$_i$, and to identify the cell surface receptor that is responsible for the ADPr mediated [Ca$^{2+}$]$_i$ increase in the S5 cells. The concentration of ADPr used in our experiment to elicit [Ca$^{2+}$]$_i$ increase is high compared to that of ADP. Nevertheless, such high concentration of ADPr was used in various studies to show its biological effects in various tissues (Bortell et al., 2001; Broetto-Biazon et al., 2008; Hoyle and Edwards, 1992; Miller et al., 1999; Zhang et al., 2001).

ADP is a cognate agonist of P2Y1 receptor, and it is known to desensitize the receptor. In our experiments, we found that a first application of ADP increased [Ca$^{2+}$]$_i$, which reached baseline after washout of ADP. When ADP was applied again to the same cell, an identical [Ca$^{2+}$]$_i$ response was seen. This shows that the receptor was not desensitized provided that the intervening washout period was long. However, when ADP was applied repeatedly without an intervening prolonged washout period, there was a reduction in [Ca$^{2+}$]$_i$ increase suggesting that the receptor was desensitized when the washout period was short. The application of ADPr after ADP, without prolonged washout period also showed decreased [Ca$^{2+}$]$_i$ response. These results imply that ADP and ADPr activate the same receptor.

Previous studies had demonstrated that the intracellular application of ADPr activates the TRPM2 channel in the insulin secreting cells (Inamura et al., 2003; Togashi et al., 2006; Bari et al., 2009). However the ADPr induced [Ca$^{2+}$]$_i$ increase in our experiments was not due to the activation the TRPM2 channel. This is supported by the following facts. TRPM2 is located on the plasma membrane and allows entry of Ca$^{2+}$ into the cytoplasm (Togashi et al., 2006). Opening of TRPM2 channel requires binding of ADPr to its cytosolic C-terminal Nudix motif. Extracellularly applied ADPr is a polar substance which does not pass through the plasma membrane and thus is unlikely to bind to the cytosolic part of the channel (Kühn and Lückhoff, 2004).

The key finding in our study was that ADPr induced [Ca$^{2+}$]$_i$ increase was completely inhibited by two selective inhibitors of the P2Y1 receptors, MRS2179 and MRS2279 (Boyer et al., 2002; Moro et al., 1998). MRS2279 selectively inhibits the P2Y1 receptors; MRS2179 inhibits P2X1 and P2X3, in addition to the P2Y1 receptors (Brown et al., 2000). The inhibition of ADPr induced [Ca$^{2+}$]$_i$ increase by two structurally different selective inhibitors of P2Y1 receptor, MRS2179 and MRS2279 indicates the involvement of the P2Y1 receptor in the ADPr induced [Ca$^{2+}$]$_i$ increase. Previous studies had reported that many other purinergic receptors namely P2Y2, P2Y4, P2Y6, and P2Y12 are expressed in the pancreatic β-cells (Lugo-Garcia et al., 2007; Verspohl et al., 2002). Among them P2Y1, P2Y2, P2Y4, and P2Y6 are linked to the PLC-mediated Ca$^{2+}$ signaling system (Abbraccchio et al., 2006). As the ADPr induced [Ca$^{2+}$]$_i$ increase was blocked by the selective inhibitors of the P2Y1 receptors, it is likely that the P2Y1 receptor is the target for the ADPr-induced [Ca$^{2+}$]$_i$ increase. We conclude that ADPr is an endogenous ligand for P2Y1 receptor.
7. Conclusion
Ratiometric microfluorometry is an established technique to study the \([Ca^{2+}]_i\) changes in single \(\beta\)-cell. There are two main findings in our research work. Firstly, cation current through the TRPV1 channel is necessary for membrane depolarization during tolbutamide-induced \([Ca^{2+}]_i\) increase. Secondly, ADPr increases \([Ca^{2+}]_i\) by activating the P2Y1 receptor present on the plasma membrane. Understanding the function of ion channels and receptors involved in \(Ca^{2+}\) signalling helps better understanding of how failure of these ion channels or receptors might alter insulin secretion.

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